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Differences in enzyme activities between two species of *Hematodinium*, parasitic dinoflagellates of crustaceans

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Abstract

Parasitic dinoflagellates of the genus *Hematodinium* infect several commercially important decapod crustaceans. Different species of *Hematodinium* have different levels of virulence in their respective hosts. Enzyme activities were studied from two species of *Hematodinium*, one isolated from the Norway lobster (*Nephrops norvegicus*) and the other from the American blue crab (*Callinectes sapidus*). We report the identification of differences in secretion of acid phosphatase (AP) and leucine arylamidase from two parasite species. Leucine arylamidase was only contained and secreted by the species infecting the blue crab. Both parasite species contained AP, but only the species infecting the Norway lobster secreted this enzyme. In this species, AP activity was predominantly in the soluble fraction (69.5%). AP activity was localized to cytoplasmic granules and on the membranes surrounding the cell nucleus. In addition to providing information on the cellular metabolism of the parasite, the pattern of activities of these enzymes may also be useful in distinguishing among different species of *Hematodinium*.
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1. Introduction

Parasitic dinoflagellates of the genus *Hematodinium* infect several species of decapod crustaceans, including the harbour crab *Liocarcinus depurator* (Chatton and Poisson, 1931), the sand crab *Portunus pelagicus* (Hudson and Shields, 1994), the blue crab *Callinectes sapidus* (Newman and Johnson, 1975), the edible crab *Cancer pagurus* (Latrouite et al., 1988), several crabs of the genus *Chionoecetes* (Bower et al., 2003; Meyers et al., 1987; Taylor and Khan, 1995), and the Norway lobster *Nephrops norvegicus* from the West Coast of Scotland (Field et al., 1992) and the Irish Sea (Briggs and McAliskey, 2002).

In the Norway lobster, *Hematodinium* infections apparently progress from an unidentified mode of entry to a tissue-based infection, then to a fully developed systemic infection with the parasite multiplying to large numbers in the hemolymph (Field and Appleton, 1995; Stentiford et al., 2001). Similarly, the progression of *Hematodinium* infections in experimentally infected blue crabs has been shown to undergo a series of logarithmic increases resulting in densities of over 10^8 parasites ml^{-1} in the hemolymph (Shields and Squyars, 2000). In contrast to the above study in the blue crab, experimental infections of Norway lobsters with its associated *Hematodinium* sp. have never been successful (Appleton and Vickerman, 1998; Vickerman, 1994), which suggests a difference in virulence (defined within the discipline of invertebrate pathology as the degree of pathogenicity, Steinhaus and Martignoni, 1970) between these species.

Shields et al. (2003) demonstrated that the enzymatic profile of hemolymph from *Hematodinium* infected blue crabs differed from that of uninfected hemolymph, with acid phosphatase (AP), napthol AS-BI phosphohydrolase

and β-galactosidase all elevated in infected sera. The authors proposed that parasite-derived AP may function as an inhibitor of the innate host defences, thus aiding parasite survival, as has been suggested for several other protozoan parasites (Baca et al., 1993; Remaley et al., 1984; Talamás-Rohana et al., 1999; Volety and Chu, 1997). Little other work on the enzymes of *Hematodinium* spp. has been done.

In vitro culture of both species of *Hematodinium* has facilitated investigation of the enzymatic profiles of each species. We report on the identification of differences in intracellular and secreted amounts of AP and leucine aryl-amidase from *Hematodinium* spp. infecting *N. norvegicus* and *C. sapidus*. In particular, focus is placed upon the identification, characterization and localization of acid phosphatase activity in the species of *Hematodinium* infecting *N. norvegicus*.

2. Materials and methods

2.1. Collection and maintenance of experimental animals

Norway lobsters (*N. norvegicus*) were caught by otter bottom trawl (70 mm mesh size) at locations south of Little Cumbrae in the Clyde Sea Area, Scotland (55.41° N, 4.56° W). Lobsters were transported in a cool, damp environment after capture, then maintained in a closed seawater system at 10 °C and 33 psu salinity prior to use in experimental studies. Blue crabs (*C. sapidus*) were obtained from commercial crab pots at Wachapreague Creek, Virginia, USA. Crabs were transported to the Virginia Institute of Marine Science (VIMS) in ice-chilled coolers. Blue crabs were held in aquaria at 20–21 °C and 24 psu salinity.

2.2. Diagnosis of Hematodinium infection and parasite isolation

Hemolymph samples were drawn from the base of the fifth pereiopod using a disposable 1-ml syringe and 25-G needle following sterilization of the cuticle with 70% (v/v) ethanol. Norway lobsters with advanced infections were identified by the gross body colour method of Field et al. (1992). To confirm infections, 100 μl samples of hemolymph from lobsters were subjected to ELISA, as described by Small et al. (2002). Serum samples from infected and uninfected Norway lobsters and blue crabs were prepared by immediately centrifuging hemolymph samples at 400g for 10 min at 4 °C. The cell-free serum was removed, filter-sterilized (0.2 μm) and frozen at −80 °C prior to analysis.

Infected blue crabs were identified by examining individual hemolymph smears for the uptake of neutral red solution by parasite cells (see Stentiford and Shields, 2005). Parasites take up the stain and host haemocytes do not. Briefly, one drop of hemolymph was mixed (1:1) with neutral red solution (0.25% w/v) in isolation buffer. The isolation buffer consisted of a physiological saline (MAM:

Modified Appleton Medium) adapted from Appleton and Vickerman (1998), containing NaCl 19.31 g l^{-1}; KCl 0.65 g l^{-1}; CaCl$_2$ 1.38 g l^{-1}; MgSO$_4$ 1.73 g l^{-1}; Na$_2$SO$_4$ 0.38 g l^{-1}; HEPES 0.82 g l^{-1}, adjusted to pH 7.8, and autoclaved. To this, glucose (1.0 mg ml^{-1}) was added, along with penicillin (80 U ml^{-1}) and streptomycin (50 μg ml^{-1}) to suppress bacterial contamination. The isolation medium was filter-sterilized (0.2 μm) prior to use. The stained cell suspension was then examined for *Hematodinium* cells as a wet smear at 200× magnification.

Parasites were isolated from host haemocytes of Norway lobsters and blue crabs using a similar technique. Previous attempts at *in vitro* culture using this method of parasite isolation and purification (below) indicated that during such initial incubations crab haemocytes adhered to the plastic surfaces of culture flasks, leaving only parasite cells in suspension in the medium (J. Shields, unpublished data). For the Norway lobster; 0.25 ml of infected hemolymph was added to 10 ml of isolation medium in sterile 25-cm^2 tissue culture flasks, which were then incubated for 30 min at 8 °C. For the initial isolation, a minimum of 4 flasks were prepared from each lobster. The isolation medium consisted of autoclaved balanced *Nephrops* saline (Appleton and Vickerman, 1998, containing NaCl, 27.99 g l^{-1}; KCl 0.95 g l^{-1}; CaCl$_2$ 2.014 g l^{-1}; MgSO$_4$ 2.465 g l^{-1}; Na$_2$SO$_4$ 0.554 g l^{-1}; HEPES 1.92 g l^{-1}) adjusted to pH 7.8, with penicillin G (10 U ml^{-1}) and streptomycin (10 μg ml^{-1}) added to inhibit bacterial contamination. The medium was then filter-sterilized (0.2 μm) prior to use. After initial incubation for 30 min, parasite suspensions were transferred into new sterile 25-cm^2 culture flasks and incubated at 8 °C for a further 30 min. Isolates were pooled and centrifuged at 400g for 10 min at 4 °C. The resulting parasite pellet was resuspended and washed three times by centrifugation with isolation medium to remove contaminating hemolymph factors. Parasites were isolated from the hemolymph of blue crabs by the addition of 1 ml infected hemolymph to 9 ml MAM buffer (detailed above) in sterile 25-cm^2 tissue culture flasks, and were incubated for 30 min at 23 °C. Suspensions of parasites were then transferred into new sterile culture flasks and incubated at 23 °C for a further 30 min. The remaining parasite suspensions in the culture flasks were pooled, sedimented at 400g for 10 min at 22 °C, then washed three times with MAM buffer. For the initial parasite isolation, a minimum of 3 flasks were prepared from each crab. Isolated parasite cell suspensions were assessed for any contaminating haemocytes by light microscopy.

2.3. Preparation of parasite lysates

After resuspension of purified parasites in isolation medium, cell density was assessed using a haemocytometer. Suspensions were adjusted to give a range of cell densities (1–10 × 10^6 cells ml^{-1}). Cell-free lysates were prepared by freezing and thawing the samples twice, followed by centrifugation (10,000g for 10 min at 4 °C). The resultant

supernatants were filtered (0.2 μm) and stored frozen at −80 °C and represented the cell-free parasite lysate.

To investigate AP activity in cell membranes and soluble components of crude cell lysates from Norway lobster samples, aliquots of crude lysates (10×10^6 cells ml^{-1}) were centrifuged at 10,000g for 10 min at 4 °C. The resulting supernatant (soluble fraction of the crude lysate) was removed and filtered (0.2 μm) prior to use. The pellet was washed three times with isolation medium and resuspended in the original sample volume of isolation medium (as the "all membrane" fraction). All three samples (crude lysate, soluble fraction and cell membrane fraction) were assayed for AP activity.

2.4. Parasite culture

Isolated sporoblasts of the two *Hematodinium* spp. were resuspended in *Nephrops* saline (for parasites from lobsters) or MAM buffer (for parasites from blue crabs) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS: Sigma). Cell density was assessed using a haemocytometer and adjusted to give 1×10^6 or 2×10^6 cells ml^{-1}. Cell suspensions (2 ml) were incubated in triplicate in 12-well culture plates at 8 °C for 7 days (for *Hematodinium* cells derived from the Norway lobster), or 23 °C for 6 days (for *Hematodinium* cells derived from blue crabs). Cultures were examined daily. On day 6/7, the cell suspensions in each well were collected, and cell viability was assessed using trypan blue (0.25% w/v in culture medium) for *Hematodinium* cells derived from the Norway lobster, and both trypan blue and neutral red solution (0.25% w/v in culture medium) for *Hematodinium* cells derived from blue crabs. To examine exogenous enzyme release, parasites were separated from the cell culture medium by centrifugation at 800g for 10 min. The cell-free culture medium was removed, passed through a 0.2 μm filter and frozen at −80 °C. Culture medium in which no *Hematodinium* cells had been incubated was also frozen at −80 °C.

2.5. API ZYM enzyme analysis

Parasite cell lysates, the isolation medium and culture media from incubations were assayed in duplicate (from each of 3 cultures/media samples) following the manufacturer's (API Zym kit, BioMérieux) protocol for the following enzymes: alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, napthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase. Briefly, 65 μl of the sample was added to each cupule and the test strips were incubated for 4 h at 37 °C. Following incubation, 1 drop of ZYM A (API; tris-hydroxymethyl-aminomethane, hydrochloric acid, sodium lauryl sulphate, H$_2$O) and ZYM B (API; fast blue BB, 2-methoxyethanol) was added to each cupule and the reaction was allowed to proceed for 5 min. The test strips were then read and the results scored using the following scale: −, negative reaction; ±, weak positive; +, positive reaction; ++, strong positive reaction.

2.6. Acid phosphatase (AP) assay

AP activity in parasite cell lysates, the culture medium, and serum samples from both Norway lobster and blue crab *Hematodinium* species was measured using the colorimetric method of Bodley et al. (1995), with minor modifications. The assay is based on the release of *p*-nitrophenol (PNP) and inorganic phosphate (IP) from *p*-nitrophenylphosphate (PNPP) by the AP enzyme. The substrate, *p*-nitrophenylphosphate, was prepared at a concentration of 40 mg ml^{-1} in 1 M sodium acetate at pH 5.5. The assay was initiated by the addition of 100 μl cell lysate/cell culture medium or 50 μl lobster serum to 10 μl of substrate solution. Samples were incubated for 2–8 h at 37 °C. The resulting yellow-coloured reaction product was measured at 405 nm on a microtiter plate reader (Titertek Multiscan MCC/340).

Controls comprised isolation and cell culture media without the addition of *Hematodinium* cells, and uninfected crab and lobster serum. The activity of AP enzyme was determined using an extinction coefficient of 1.83×10^4 M^{-1} cm^{-1} and a path length of 0.4 cm on the plate reader. Activities were expressed as the amount (mol) of PNP formed per hour by the volume of sample assayed. Statistical analysis of the enzymatic activities in the culture media with different densities of *Hematodinium* cells and the lobster serum samples was performed using the Mann-Whitney *U* test. Significance was considered to be $p < 0.05$.

2.7. AP histochemistry

Hemolymph smears from a *Hematodinium*-infected Norway lobster were air-dried and stained using the simultaneous azo dye coupling method for AP (Burnstone, 1958) as detailed in Bancroft (1967). The recommended substrate, naphthol AS-BI phosphate, was used along with hexazonium pararosanilin as the diazonium salt. The combination of hexazonium pararosanilin and naphthol AS-BI phosphate allows the accurate localization of AP as a bright red reaction product at the sites of enzyme activity. Following the localization of AP, the smears were lightly stained with haematoxylin (Carazzi, 1911) as a nuclear counterstain.

3. Results

3.1. Cell culture of Hematodinium spp.

After 6/7 day incubation, cultures of *Hematodinium* sporoblasts from the Norway lobster and blue crab aggre-

gated into clump colonies in the bottom of cell culture wells (Small, 2004), thus preventing the estimation of cell densities. However, cell viability was consistently over 99% based on trypan blue and neutral red staining.

3.2. API ZYM enzyme profiles of Hematodinium cell lysates and cell culture media

Cell lysates of *Hematodinium* sp. from the Norway lobster and blue crab showed strong positive activity for several enzymes (Table 1). For lysates of *Hematodinium* from the Norway lobster, strong positive activity was noted for acid phosphatase and positive activity was observed for napthol-AS-BI-phosphohydrolase and α-fucosidase. Weak positive reactions were observed for esterase (C4), leucine arylamidase, β-glucuronidase, α-glucosidase, β-glucosidase and *N*-acetyl-β-glucosaminidase. Negative reactions were obtained for all other enzymes of the API ZYM system. Cell lysates of *Hematodinium* from the blue crab had a similar profile, with strong positive activity observed for acid phosphatase, and weak positive activity for esterase (C4), napthol-AS-BI-phosphohydrolase and *N*-acetyl-β-glucosaminidase. In contrast to the lysates of parasites from the Norway lobster, strong positive reactions were observed for leucine arylamidase. Negative reactions were observed for all other enzymes tested. Negative reactions for all enzyme reactions were obtained from both of the cell isolation media used in obtaining the parasites analysed in this part of the study.

Table 1
Enzymatic activities in cell free lysates of *Hematodinium* sp. from the Norway lobster and blue crab (1×10^6 cells ml^{-1}) detected by the API ZYM system

Enzyme	Hematodinium ex N. norvegicus	Hematodinium ex C. sapidus
Alkaline phosphatase	−	−
Esterase (C4)	±	±
Esterase lipase (C8)	−	−
Lipase (C14)	−	−
Leucine arylamidase	±	++
Valine arylamidase	−	−
Cystine arylamidase	−	−
Trypsin	−	−
α-Chymotrypsin	−	−
Acid phosphatase	++	++
Napthol-AS-BI-phosphohydrolase	+	±
α-Galactosidase	−	−
β-Galactosidase	−	−
β-Glucuronidase	±	−
α-Glucosidase	±	−
β-Glucosidase	±	−
N-Acetyl-β-glucosaminidase	±	±
α-Mannosidase	−	−
α-Fucosidase	+	−

++, Strong positive; +, positive; ±, weak positive; −, negative. Both isolation medium controls had negative results for all enzymes tested.

In culture media, enzymatic activities differed between *Hematodinium* from the Norway lobster versus the blue crab (Table 2). Strong positive reactions for several enzymes were observed for the control *Hematodinium*-free culture medium which prevented them from being assayed in the *Hematodinium* cell culture media. Marked increases in enzyme activity between control culture media and *Hematodinium* cell culture media were observed with AP (weak positive to strong positive) and napthol-AS-BI-phosphohydrolase (weak positive to positive). Cell culture media containing *Hematodinium* cells from the blue crab were observed to have only one enzyme, leucine arylamidase, with a measurable difference in activity between control and cell culture media (positive to strong positive).

3.3. AP activity in cell lysates of Hematodinium

No AP activity was detected in the isolation medium that was used to make *Hematodinium* cell suspensions for lysates. AP activity was observed from crude cell lysates for both species of *Hematodinium* (Fig. 1). The species from the Norway lobster had significantly higher enzyme activities ($p < 0.05$) at both cell densities tested.

3.4. AP activity in cell culture medium containing Hematodinium

A low level of AP activity was detected in the control, *Hematodinium*-free, cell culture media for both species (0.11 ± 0.01 and 0.17 ± 0.02 nmol h^{-1} 100 μl sample^{-1}). An increase in enzyme activity was observed in Norway lobster *Hematodinium* culture media after 7 days incubation (Fig. 2). Levels of AP activity in culture media seeded with 1×10^6 cells ml^{-1} (0.34 ± 0.02 nmol h^{-1} 100 μl sample^{-1}) and 2×10^6 cells ml^{-1} (0.50 ± 0.03 nmol h^{-1} 100 μl sample^{-1}) were both significantly higher ($p < 0.05$) than in the control medium (Mann-Whitney *U* test: $p = 0.01$). The culture media from *Hematodinium* from the blue crab showed no statistically significant increase in AP activity at either cell density tested (0.11 ± 0.01 and 0.14 ± 0.009 nmol h^{-1} 100 μl sample^{-1}).

3.5. Localization of AP activity in Hematodinium cells

Fractionation of crude cell lysates from *Hematodinium* from the Norway lobster into soluble and membrane components allowed the distribution of intracellular AP activity to be examined (Fig. 3). The soluble and membrane components accounted for $69.5 \pm 1.4\%$ and $33.0 \pm 1.7\%$, respectively, compared to the total crude lysate activity. Light microscopic examination of smears of *Hematodinium* from the Norway lobster, stained for AP activity using the described procedure, indicated that the enzyme was localized in cytoplasmic granules and on the membranes surrounding the cell nucleus (Fig. 4). No deposition of reaction product was detected in the nucleus, or associated with the outer membrane or any other cellular structures.

Table 2
Enzymatic activities present in media from cultures of *Hematodinium* sp. from the Norway lobster and blue crab (1×10^6 cells ml^{-1}) after a six- or seven-day incubation, detected by the API ZYM system

Enzyme	*Hematodinium* ex *N. norvegicus* control medium	*Hematodinium* ex *N. norvegicus* cell culture medium (1×10^6 ml^{-1})	*Hematodinium* ex *C. sapidus* control medium	*Hematodinium* ex *C. sapidus* cell culture medium (1×10^6 ml^{-1})
Alkaline phosphatase	++	++	±	±
Esterase (C4)	+	+	+	+
Esterase lipase (C8)	+	+	+	+
Lipase (C14)	−	−	−	−
Leucine arylamidase	++	++	+	++
Valine arylamidase	−	−	−	−
Cystine arylamidase	−	−	−	−
Trypsin	−	−	−	−
α-Chymotrypsin	−	−	−	−
Acid phosphatase	±	++	±	±
Napthol-AS-BI-phosphohydrolase	±	+	±	±
α-Galactosidase	−	−	−	−
β-Galactosidase	±	±	−	−
β-Glucuronidase	−	−	−	−
α-Glucosidase	±	±	−	−
β-Glucosidase	±	±	−	−
N-Acetyl-β-glucosaminidase	±	±	±	±
α-Mannosidase	±	±	−	−
α-Fucosidase	−	−	−	−

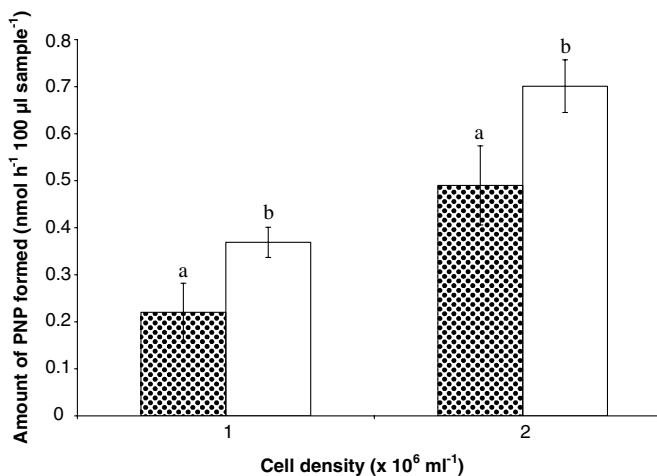++, Strong positive; +, positive; ±, weak positive; −, negative.

Fig. 1. AP activity of crude *Hematodinium* cell lysates from *Callinectes sapidus* (filled columns) and *Nephrops norvegicus* (open columns). Mean ± SD, $N = 5$. Assay incubation period: 6 h. The presence of different lowercase letters indicates statistical significance ($p < 0.05$) between the different species.
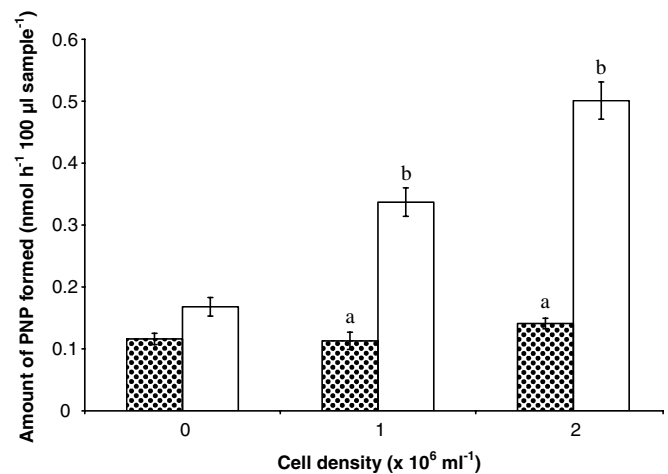

Fig. 2. AP activity in media from cultures of *Hematodinium* from *Callinectes sapidus* (filled columns) and *Nephrops norvegicus* (open columns). Initial densities were 0, 1 and 2×10^6 cells ml^{-1} and incubation was for 7-days. Mean ± SD, $N = 5$. Assay incubation period: 8 h. Data are representative of three separate experiments. The presence of different lowercase letters indicates statistical significance ($p < 0.05$) between the different species.

3.6. AP activity in infected N. norvegicus and C. sapidus serum

Cell-free sera from infected Norway lobsters had significantly higher ($p < 0.05$) AP activity (5.53 ± 1.67 nmol h^{-1} 50 μl sample^{-1}) than was present in cell-free hemolymph from uninfected lobsters (0.42 ± 0.06 nmol h^{-1} 50 μl sample^{-1}) (Mann-Whitney *U* test: $p = 0.001$) (Fig. 5). Cell-free sera from infected and uninfected blue crabs had no signif-

icant difference in AP activity (1.3 ± 0.13 and 1.17 ± 0.16 nmol h^{-1} 50 μl sample^{-1}).

4. Discussion

Differences were observed in the enzyme profiles (parasite lysates and cell culture supernatants) from two

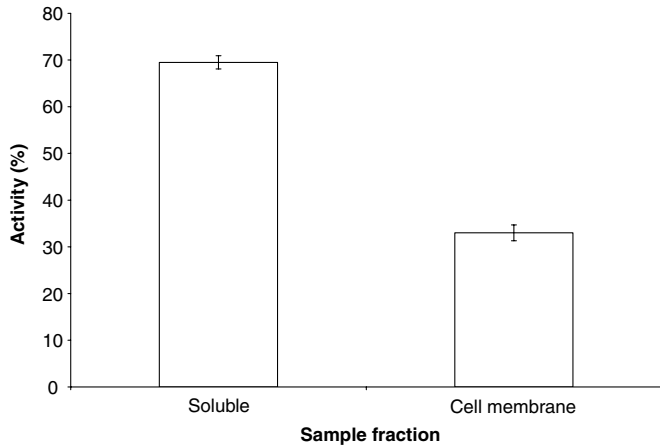Fig. 3. Determination of AP activity in *Hematodinium* cell lysates from *Nephrops norvegicus* (10×10^6 cells ml^{-1}). Data are presented as percentage of activity associated with each fraction, where activity of 100% (crude lysate) is equivalent to 3.02 ± 0.023 nmol PNP formed h^{-1} 100 µl^{-1} sample during the 6 h assay incubation period. Mean $\pm$ SD, $N = 5$.
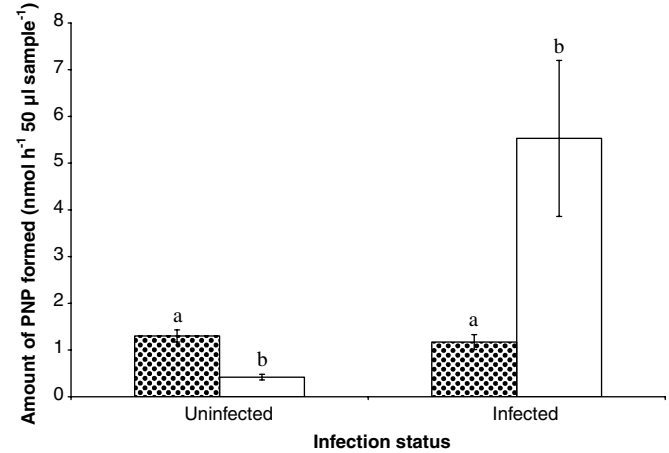

Fig. 5. AP activity of serum samples (3) from *Hematodinium*-infected and uninfected *Callinectes sapidus* (filled columns) and *Nephrops norvegicus* (open columns). Mean $\pm$ SD, $N = 5$. Assay incubation period: 3 h. The presence of different lowercase letters indicates statistical significance ($p < 0.05$) between host serum samples.
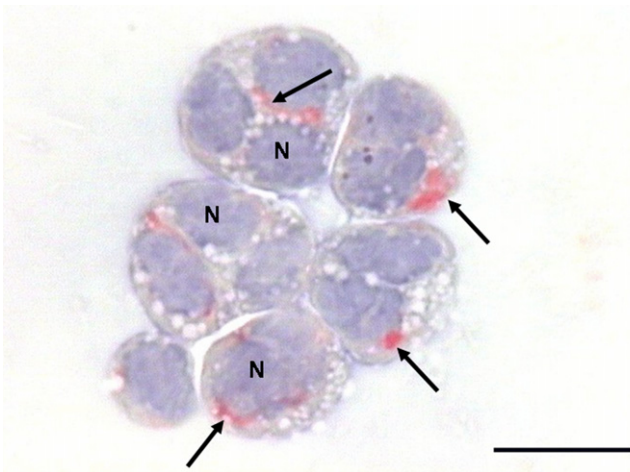

Fig. 4. Light micrograph of *Hematodinium* cells from *Nephrops norvegicus* showing AP activity as a red reaction product (arrows) localized in single, bi- and tri-nucleated parasites. N = nucleus. Scale bar = 10 µm.

species of *Hematodinium* isolated from the blue crab, *C. sapidus*, and the Norway lobster, *N. norvegicus*. Elevated levels of leucine arylamidase were found in the parasite lysates and cell culture supernatants from the *Hematodinium* species from blue crabs, but not from the species infecting Norway lobsters. Both species were shown to possess intracellular acid phosphatase (AP) activity, but only one species, that from the Norway lobster, secretes AP during *in vitro* culture. In this *Hematodinium* species, AP activity was predominantly in the soluble fraction and was localized to cytoplasmic granules and on the nuclear membrane of the parasite. Elevated levels of AP were also measured in cell-free sera from infected lobsters.

The APIZYM system (BioMérieux) has previously been used to identify and type several medically important bac-

terial and fungal pathogens (García-Martos et al., 2000; Hermosa de Mendoza et al., 1993; Heroldová et al., 2001; Malinowski et al., 2001; Poh and Loh, 1985; Poh and Loh, 1988; Youngchim et al., 1999) Using this system, we have shown that cell lysates of *Hematodinium* sp. from the Norway lobster and blue crab have different enzyme profiles. High levels of AP were present in the lysates of parasites from the blue crab and from the lobster, but high levels of leucine arylamidase were only present in the parasite from the blue crab. Differences also existed in the secretion of enzymes as reflected in the content of the conditioned culture media. Medium from cultures of *Hematodinium* from the blue crab had no AP activity, whereas that from cultures of *Hematodinium* from the Norway lobster had high AP activity. This finding indicates that AP is located intracellularly and is not secreted by the *Hematodinium* sp. from blue crabs. This is consistent with Shields et al. (2003) who reported the enzyme from whole hemolymph but not in the cell-free serum from an infected blue crab. Shields et al. (2003) also suggested that the AP found within *Hematodinium* cells from the blue crab may inhibit innate host defences such as superoxide-mediated cell death or act as a marker of phagocytic or pinocytic activity. We speculate that the differences in the pattern of enzyme secretion between the two *Hematodinium* species may also account for their different levels of virulence in their respective hosts. That is, *Hematodinium* infections in blue crabs tend to be of relatively short duration (Shields and Squyars, 2000), whereas those in the Norway lobster tend to be of longer duration (Field et al., 1992). Differences in enzyme secretions may be reflected in the duration and virulence of these infections.

In many microorganisms, AP activity is associated with pathogenic mechanisms and virulence. Studies on AP from *Leishmania donovani* (Remaley et al., 1984), *Entamoeba histolytica* (Talamás-Rohana et al., 1999), *Coxiella burnetii*

(Baca et al., 1993), and *Legionella micdadei* (Saha et al., 1985) suggest that this parasite-derived enzyme plays an important role as a virulence factor by modulating the host's immune response. Similarly, in their molluscan hosts, the parasites *Bonamia ostreae* (Hervio et al., 1988), *Perkinsus marinus* (Volety and Chu, 1997), and rickettsi-ales-like organisms (Le Gall et al., 1991), all contain or secrete AP, which is thought to interfere with the production of superoxide radicals (O_2^-) in host haemocytes. Parasite-derived acid phosphatases can also act as virulence markers among isolates (Katakura and Kobayashi, 1988; Lovelace and Gottlieb, 1986; Singla et al., 1992). The reasons for the lack of AP secretion from the *Hematodinium* from the blue crab vs. that from the lobster are unclear at present. However, the results suggest that the former species may have different mechanisms for evading the host's immune reaction, as indicated by secretion of leucine arylamidase (a protease, see below). Studies involving the measurement of possible inhibition of reactive oxygen species by *Hematodinium* AP may clarify this potential pathogenic role for the enzyme.

Although AP is classically considered to be a lysosomal marker in eukaryotic cells, it has an extra-lysosomal distribution in several protozoan parasites. The enzyme has been found on the surface membrane of *Leishmania donovani* promastigotes (Gottlieb and Dwyer, 1981), in the dense granules and rhoptries of *Toxoplasma gondii* (Metsis et al., 1995), in the dense bodies of *Bonamia ostrea* (Hervio et al., 1991), and in the vacuoles and at the amoeba-host cell interface of *E. histolytica* (Ventura-Juárez et al., 2000). Localization studies on the *Hematodinium* sp. from lobsters indicate that AP is associated with cytoplasmic vesicles and granules. Fractionation studies support this, and indicate that enzyme activity is predominantly within the soluble fraction, and approximately 30% associated with membranes. These vesicles might be lysosomes as *Hematodinium* cells have previously been shown to possess micropores (Appleton and Vickerman, 1996), which may permit endocytosis. Alternatively, the vesicles could contain soluble enzyme destined for exocytosis. Characterization of secreted, soluble, and membrane associated AP forms is needed to clarify its role in *Hematodinium*.

Parasite lysates from cultures of the *Hematodinium* sp. from the blue crab had high leucine arylamidase activity which is thought to be due to active secretion of this enzyme. The observation that AP activity was high in the cell lysates but absent in culture medium indicates that there was no significant cell death in the medium. Cell death, if it had occurred, would have caused false positive measures of AP secretion thereby strongly suggesting that the extra-cellular leucine arylamidase activity detected in the medium is the result of secretion, and not cell death. Proteolytic enzymes have been well documented from many parasites and play various roles in the establishment and progression of many diseases (Klemba and Goldberg, 2002; McKerrow, 1989). Many marine pathogens secrete proteases (Hussain et al., 2000; La Peyre et al., 1995; Small

et al., 2005). In particular, extracellular leucine arylamidase activity directed towards the N-terminus of proteins has been observed in several other pathogens (Dettori et al., 1995; Grehn et al., 1991) and has also been implicated as a virulence factor in a strain of *Vibrio* infecting turbot (Farto et al., 1998). Such may be the case for the *Hematodinium* species from the blue crab, which is highly virulent (Shields and Squyars, 2000). In contrast, infections in Norway lobsters appear to be chronic, lasting long periods (Field et al., 1992). Experimental infections in tanner and snow crabs also have extremely long durations (Eaton et al., 1991; Shields et al., 2005). Thus, the secretion of this aminopeptidase, by the *Hematodinium* species from blue crabs may explain its greater virulence compared with the *Hematodinium* sp. from *N. norvegicus*.

Finally, the enzymatic profiles of *Hematodinium* species may be influenced by life cycle stages and culture conditions. Although the *in vitro* cultures of both parasites consisted of morphologically similar sporoblast cells, the *in vitro* life cycle of the *Hematodinium* from blue crabs has not been fully documented and may differ from that of *Hematodinium* sp. from the lobster (Appleton and Vickerman, 1998; Stentiford and Shields, 2005). Cells of the crab parasite were cultured at 23 °C, whereas those of *Hematodinium* sp. from the lobster were cultured at 8 °C. Although the two culture media had the same basic constituents, some were at different concentrations, due to differences in blood osmolality of the hosts. For example, the medium for *Hematodinium* from the blue crab contained glucose, different salt concentrations, and a different source of FBS. Perhaps these differences significantly influenced the parasites.

In conclusion, we have identified differences in the secretion of acid phosphatase and leucine arylamidase from two different species of *Hematodinium* during *in vitro* culture. These differences may be important for parasite evasion of the host immune response, and may also account for the differences in virulence in their respective hosts. The profile of activities of these enzymes may also be useful in distinguishing among different species of *Hematodinium*.

Acknowledgments

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