ELSEVIER

Journal of Experimental Marine Biology and Ecology, 207 (1996) 217–228

JOURNAL OF EXPERIMENTAL MARINE BIOLOGY AND ECOLOGY

The effects of *Hematodinium* sp.-infection on aspects of the respiratory physiology of the Norway lobster, *Nephrops norvegicus* (L.)

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Received 24 January 1996; revised 26 April 1996; accepted 22 May 1996

Abstract

Populations of the Norway lobster *Nephrops norvegicus* from the west coast of Scotland are infected with a dinoflagellate parasite belonging to the genus *Hematodinium*. The rates of oxygen consumption of infected *N. norvegicus* were significantly greater than those of uninfected lobsters. This may be attributed partly to the oxygen demands of the very large numbers of parasite cells present in the haemolymph and in the body tissues since the rates of oxygen consumption of the haemolymph of heavily-infected lobsters were approximately 5 times greater than those of uninfected or less heavily infected lobsters. The presence of such large numbers of cells in the haemolymph, which may block haemal sinuses in the gills, appears to compromise oxygen delivery to the tissue of the host since the PO_2 of the haemolymph of heavily-infected lobsters ($P_aO_2 = 2.99 \pm 0.91$ kPa) was significantly lower than that of uninfected lobsters ($P_aO_2 = 9.4 \pm 1.39$ kPa). The oxygen carrying capacity of the haemolymph of heavily-infected lobsters was reduced by approximately 50% compared with that of uninfected animals. In addition, the haemolymph pH was lower and the L-lactate concentration was significantly higher in infected lobsters indicating that these animals resort, in part, to anaerobic metabolism.

Keywords: Crustacea; Decapoda; Hemocyanin; *Hematodinium*; *Nephrops norvegicus*; Respiration

1. Introduction

Populations of the Norway lobster, *Nephrops norvegicus* from the west coast of Scotland are known to harbour an infection of a parasitic dinoflagellate belonging to the genus *Hematodinium* (Field et al., 1992). The prevalence of infected animals in the

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PII S0022-0981(96)02649-4

population shows a distinct seasonal pattern; throughout much of the year the prevalence of infection is low, but it increases markedly during the spring and early summer with peak infection levels reaching 70% at this time (Field et al., 1992). Heavily-infected animals (stages III and IV using the classification scheme of Field and Appleton, 1995) may appear moribund with the tail muscles having a watery consistency. A characteristic feature of such animals is the presence of extremely large numbers of parasite cells within the haemolymph giving it a milky white appearance. Heavy infection with the parasite is fatal in most if not all cases (Field et al., 1992, 1995).

The cause of death has yet to be clearly established but it has been suggested that a contributing factor may be the presence of very high concentrations of parasite cells in the haemolymph which, in histological studies, have been shown to occlude haemal spaces especially in the gills. The disruption of the circulation, together with the reduction in the haemocyanin concentration of the haemolymph that has been recorded previously (Field et al., 1992) and the potentially high oxygen demand of the cells within the haemolymph, may seriously compromise oxygen supply to the tissues of the host. The present study has investigated this possibility by comparing rates of oxygen consumption of infected and uninfected lobsters and by examining some aspects of oxygen transport by the haemolymph.

2. Materials and methods

2.1. Experimental animals

Adult *Nephrops norvegicus* (carapace length = 25–45 mm) were collected by trawling on grounds around the Isle of Cumbrae, Firth of Clyde, Scotland between April and June when the prevalence of the disease among the population is at its highest (Field and Appleton, 1995). Immediately after capture, the lobsters were returned to the University of Glasgow where they were maintained in aquaria containing circulating, aerated sea water (temperature 10 °C; salinity 33‰) until required. The lobsters were maintained under a 12:12 h light:dark cycle for at least a week before the experiments were carried out. During this time they were fed ad libitum on fish flesh. The moult stage of all lobsters was determined by the method of Aiken (1980). Dinoflagellate infection was diagnosed by the pleopod examination method reported by Field et al. (1992) and by Field and Appleton (1995) in which the degree of infection is classified into five stages based on the number of cells visible in the haemal spaces of the pleopod through the transparent pleopod cuticle (stage 0 = uninfected, stages 1–4 = increasing levels of infection). Diagnosis of infection with the dinoflagellate parasite was confirmed by microscopical examination of fixed or fresh haemolymph (as detailed in Field and Appleton, 1995).

2.2. Oxygen consumption of whole animals

The resting rates of oxygen consumption ($\dot{M}O_2$) of 95 adult *Nephrops norvegicus* of both sexes (fresh weight = 32–60 g) were determined using flow-through respirometry.

All animals used were in the intermoult stage. Data were obtained for both apparently uninfected *N. norvegicus* and for lobsters diagnosed as being at infection stages I to IV (as detailed above). Lobsters were introduced individually into clear Perspex tubes of approximately 0.5 l capacity, through which fully aerated sea water (temperature = 10°C; salinity = 33‰) flowed at a low rate (< 30 ml $\cdot$ min^{-1}), and were left undisturbed overnight to settle. The exact flow rate and the oxygen partial pressure (PO_2) of the sea water entering the respirometer and emerging from it were then measured. Samples of the water leaving the respirometer were collected at hourly intervals between 0800 h and 1800 h which allowed sufficient time between samples to ensure complete equilibration of the water within the tubes (Steffensen, 1989). Water samples (approximately 0.5 ml) were taken by slowly drawing water into a 1 ml syringe via a hypodermic needle inserted into respirometer outflows. The PO_2 of each water sample was determined by injecting the samples into a glass chamber (maintained at 10°C) containing a microcathode oxygen electrode, which was in turn connected to an oxygen meter (Strathkelvin Instruments, Glasgow) and to a pen recorder. This procedure allowed simultaneous measurements of $\dot{M}O_2$ to be carried out on nine lobsters.

The rates of oxygen consumption of 17 active, apparently uninfected, lobsters were measured using a closed respirometry system. The animals were stimulated to exercise by repeatedly touching them with a rod. Care was taken to ensure that the lobsters did not respond by performing the normal tail-flip escape response which may result in the production of L-lactate (Field et al., 1992). Following the period of induced activity, each lobster was placed immediately into a 2 l respirometer containing sea water at a temperature of 10 °C and salinity of 33‰. An oxygen electrode (Strathkelvin Instruments, Glasgow) was inserted through the lid of the respirometer and the respirometer sealed. A magnetic stirrer ensured constant mixing of the water. The respirometer was maintained in a water bath (at 10 °C) for the duration of the experiment. The changes in the PO_2 of respirometer water were then measured over a 15 min period. In both sets of experiments, the oxygen electrode was calibrated prior to use against aerated sea water at the experimental temperature (10 °C) and against a solution having a PO_2 of 0 kPa (sodium sulphite in 0.01 M sodium tetraborate). The sea water used in the experiments was sterilized by exposure to UV light for approximately 1 h prior to use to eliminate microbial activity. However, a blank run was carried out at the end of each set of experiments to measure the rates of any microbial respiration.

At the end of the experiments, the lobsters were removed from the respirometers, blotted dry and weighed, and their weight specific rates of oxygen consumption ($\dot{M}o_2$, μmol $\cdot$ g^{-1} $\cdot$ h^{-1}) calculated.

2.3. Oxygen consumption of the haemolymph

The rates of oxygen consumption of the haemolymph of both infected and apparently uninfected *Nephrops norvegicus* were also measured. Samples of haemolymph were taken from 22 adult *N. norvegicus*, by inserting the 25G needle of a 1 ml disposable syringe into the arthrodial membrane of a fifth pereiopod. Haemolymph samples (0.5 ml) were injected immediately into a microrespiration chamber (RC200, Strathkelvin Instruments, Glasgow) containing a small magnetic stirrer and maintained at 10 °C. The

change in the PO_2 of the haemolymph sample was measured continuously over periods of between 20 min and 1 h using the oxygen electrode inserted into the cell. Very few problems were experienced with haemolymph clotting during these recordings. On those rare occasions when this occurred the data were rejected.

A small quantity (150μl) of the remaining haemolymph from each sample was diluted in 2% formol saline (33‰) at a ratio of 1:10, and the total number of haemocytes and parasites counted using a haemocytometer. Haemocyte:parasite ratios were determined from fixed haemolymph smears, stained with Leishman's stain (see Field and Appleton, 1995). Knowledge of the numbers of haemocytes and parasites in the haemolymph enabled the rates of oxygen consumption to be expressed both as rates per unit volume of haemolymph and per cell.

2.4. Measurements of PO_2, pH, oxygen carrying capacity, copper and L-lactate concentrations of the haemolymph

The PO_2, pH and L-lactate concentration of pre- and post-branchial haemolymph of both apparently uninfected and infected lobsters were determined. Lobsters of known disease and moult stage were left undisturbed in small individual tanks at 10 °C for 24 h prior to experimentation. Thereafter, a haemolymph sample (0.5–1.0 ml) was taken from each individual. Haemolymph samples were taken from either the pericardium, via the dorsal posterior margin of the carapace (post-branchial) or from the ventral thoracic sinus, via the arthrodial membrane of a fifth pereiopod (pre-branchial). Care was taken to ensure that the samples were taken as quickly as possible and with minimal disturbance. Part of each haemolymph sample was then immediately transferred to the chamber containing an oxygen electrode (see above) and the PO_2 determined. The remainder of each sample was retained for determination of the pH and the concentrations of copper and L-lactate. The pH of the sample was measured (at 10 °C) using the micro-capillary pH electrode of a BMS2 (Radiometer, Denmark). L-lactate concentrations were determined using the method of Gutmann and Wahlefeld (1974) incorporating the modifications of Engel and Jones (1978) and Hill (1989). The oxygen carrying capacity of the haemolymph was determined on 10μl samples using the method of Tucker (1967) as modified by Bridges et al. (1979). The oxygen carrying capacity of the haemocyanin was then calculated by subtraction of the physically dissolved fraction. The copper concentration of the haemolymph was determined using atomic absorption spectrophotometry (Philips PU9200) following dilution with dilute nitric acid (Aristar, Merck, U.K.). The spectrophotometer was calibrated using standard copper solutions (Spectrosol, Merck).

2.5. Statistical analysis

Data were log transformed and tested for normality using the Anderson–Darling test, and for homogeneity of variances using Bartlett's test. Analyses of parametric data were made by one-way analysis of variance (ANOVA) coupled with Tukey's pairwise comparison ($P < 0.05$). Due to the low number of lobsters at infection stages III and IV that were available, the two groups were combined for statistical analysis.

3. Results

3.1. Oxygen consumption of whole animals

The mean values for the $\dot{M}O_2$ of quiescent, *Hematodinium*-infected *Nephrops norvegicus* of all infection stages were significantly higher than those of apparently uninfected lobsters (ANOVA, $P < 0.05$ Tukey pairwise comparison) (Fig. 1). Tukey pairwise comparisons also indicated that the mean values for the $\dot{M}O_2$ of infected animals increased with infection stage; the $\dot{M}O_2$ values of lobsters at infection stages III and IV were significantly higher than those of stage I lobsters ($P < 0.05$). Furthermore, the mean values for the $\dot{M}O_2$ of active, uninfected *N. norvegicus* were significantly higher than those of stage I and II lobsters but not those at infection stages III and IV (ANOVA, Tukey pairwise comparison $P < 0.05$). The differences in $\dot{M}O_2$ between the different groups of lobsters can not be attributed to differences in activity during the recording period since, in nearly all cases, the animals remained quiescent in the respirometer tubes throughout this time. Unfortunately, no data were obtained for active lobsters showing high levels of infection with the parasite. This was because heavily-infected lobsters were very lethargic and could not be induced to become active. On two occasions, such animals died when attempts were made to stimulate them to become active.

3.2. Oxygen consumption of the haemolymph

Measurements of the rates of oxygen consumption of haemolymph samples taken from individual *Nephrops norvegicus* at different infection stages showed that there were no significant differences in the $\dot{M}O_2$ of the haemolymph from lobsters showing stage I and stage II infections (Fig. 2). In contrast, the $\dot{M}O_2$ of the haemolymph from lobsters at

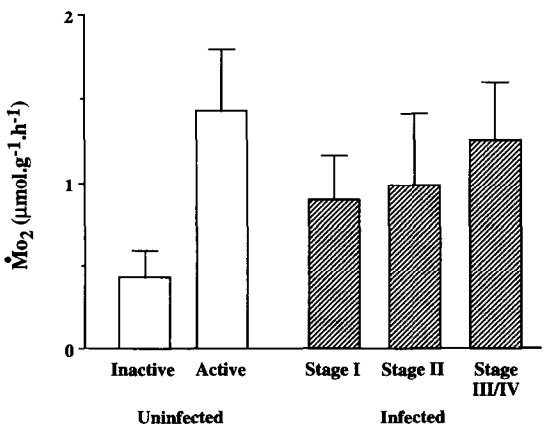

Fig. 1. Rates of oxygen consumption ($\dot{M}O_2$) of active and inactive, uninfected *Nephrops norvegicus* and of lobsters showing different stages of infection with the *Hematodinium* sp. parasite (shaded). Error bars are standard deviations. All recordings were carried out at 10 °C.

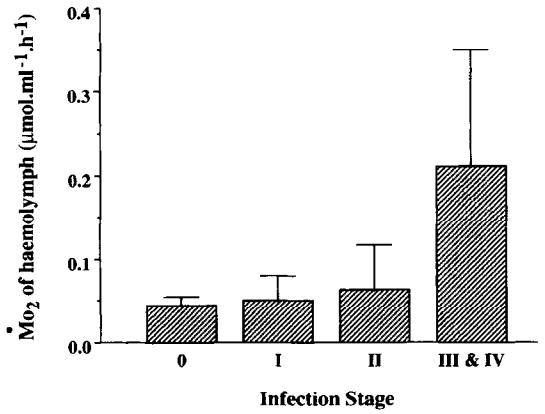

Fig. 2. Rates of oxygen consumption ($\dot{M}O_2$) of haemolymph from uninfected (Stage 0) *Nephrops norvegicus* and from lobsters showing different stages of infection with the *Hematodinium* sp. parasite (Stages I–IV). Error bars are standard deviations. All recordings were carried out at 10 °C.

stages III and IV of the infection was significantly greater (ANOVA $P < 0.05$). The possibility that this pronounced increase in the $\dot{M}O_2$ of haemolymph from lobsters showing extreme levels of infection was due primarily to the increased number of parasite cells in the haemolymph was tested by plotting the rates of oxygen consumption of haemolymph from individual *N. norvegicus* against the number of cells (both parasite cells and the haemocytes) present in the haemolymph (Fig. 3). Although few haemolymph samples containing extremely high concentrations of cells were available, a highly significant correlation ($r = 0.782$, $P < 0.001$) between log $\dot{M}O_2$ and log cell concentration was obtained.

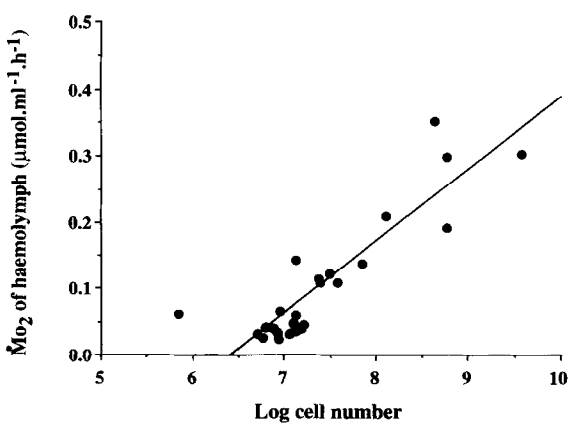

Fig. 3. The relationship between the rate of oxygen consumption ($\dot{M}O_2$) of the haemolymph and the concentration of cells (both haemocytes and parasite cells as $\log_{10}$ cell number) within the haemolymph of infected and uninfected *Nephrops norvegicus*. The regression line fitted to the data is shown.

3.3. Oxygen carrying capacity of the haemolymph

The copper concentration in the haemolymph of infected *Nephrops norvegicus* was significantly lower than that of uninfected lobsters with the greatest reduction shown in lobsters at infection stages III and IV (ANOVA $P < 0.05$) (Fig. 4). This reduction in the copper concentration of the haemolymph of infected *N. norvegicus* confirms the results of an earlier study of this species (Field et al., 1992) which was interpreted as representing a reduction in the haemocyanin concentration of the haemolymph of infected animals. The present data support this conclusion since measurements of the oxygen carrying capacity of the haemolymph made during the present study indicate that there is a significant reduction ($P < 0.01$), by approximately 50%, in the oxygen carrying capacity of the haemolymph of infected animals (Fig. 4).

3.4. PO_2, pH and lactate concentration of the haemolymph

Comparisons of the PO_2 and pH of the pre- and post-branchial haemolymph from infected and uninfected *Nephrops norvegicus* showed that, although there was no significant difference ($P > 0.05$) in the mean values for PO_2 and pH between uninfected animals and animals at infection stages I and II, there was a significant reduction in both the PO_2 and pH of the haemolymph of animals at infection stages III and IV (ANOVA)(Table 1). The mean L-lactate concentration of the haemolymph of uninfected *Nephrops norvegicus* was low (0.34 ± 0.09 mmol $\cdot l^{-1}$). The values for the lactate concentration of the haemolymph of animals at stages I and II did not differ significantly from those of uninfected animals (Table 1). There was, however, a highly significant

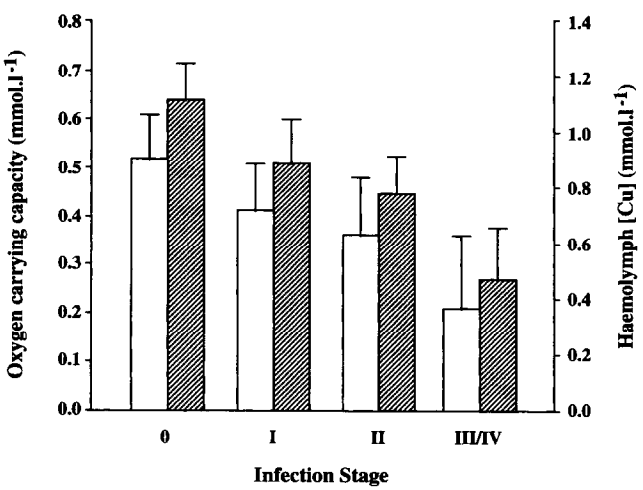

Fig. 4. Values for the oxygen carrying capacity of the haemolymph (unshaded) and of the copper concentration of the haemolymph (shaded) of uninfected (Stage 0) *Nephrops norvegicus* and lobsters showing different stages (Stages I–IV) of infection with *Hematodinium* sp. Values are means ± S.D.

Table 1
Values for the PO_2 and pH of the pre- and post-branchial haemolymph of *Nephrops norvegicus* showing differing degrees of infection with the *Hematodinium* sp. parasite

Infection stage	Pre-branchial PO_2 (kPa)	Post-branchial PO_2 (kPa)	Pre-branchial pH	Post-branchial pH	L-lactate ($mmol \cdot l^{-1}$)
0	2.45±0.43 (17)	9.44±1.39 (19)	7.878±0.075 (21)	7.920±0.076 (18)	0.34±0.08 (12)
I	2.59±0.49 (12)	8.81±1.59 (14)	7.812±0.152 (18)	7.846±0.049 (14)	0.49±0.11 (10)
II	2.32±0.63 (10)	9.13±1.63 (11)	7.747±0.092 (14)	7.779±0.143 (13)	0.52±0.12 (7)
III and IV	0.48±0.91 (8)	2.99±0.91 (10)	7.608±0.105 (10)	7.663±0.063 (8)	3.84±1.13 (7)

Values for the L-lactate concentration of the haemolymph are also given. The values presented are means±S.D. Numbers in brackets are the values of *n*.

increase ($P < 0.01$) in the lactate concentration of the haemolymph of animals at infection stages III and IV (Table 1).

4. Discussion

The present study has shown that there is a significant increase in the rate of oxygen consumption of *Nephrops norvegicus* infected with *Hematodinium* sp. with the highest rates being shown by animals having the heaviest parasite burdens. These differences cannot be attributed simply to changes in activity of infected animals since care was taken to ensure that the comparisons were made only on quiescent lobsters. In fact, infected *N. norvegicus* are generally very lethargic compared with uninfected individuals (Field et al., 1992).

The high $\dot{M}O_2$ of infected lobsters may be due to an increase in the metabolic rate of the host in response to the infection or to the additional demand of high numbers of parasites cells in the haemolymph. The $\dot{M}O_2$ values of heavily-infected, but quiescent, lobsters (stages III and IV) were not significantly different from those of apparently uninfected animals that had been induced to become very active. This suggests that the presence of *Hematodinium* cells within infected hosts results in a significant additional oxygen demand. This is further emphasised by the results of determinations of the oxygen consumption of the haemolymph. Although the $\dot{M}O_2$ per cell was approximately the same for haemocytes and parasites, a significant relationship was observed between the numbers of parasites and haemocytes and the $\dot{M}O_2$ of the haemolymph. Since total cell number tends to increase with infection stage (Field et al., 1992; Field and Appleton, 1995), the presence of parasites is likely to impose an additional burden on the respiratory system. Evidence to support this suggestion is provided by the fact that the values for the PO_2 of the pre- and post-branchial haemolymph of heavily-infected *N. norvegicus* were significantly lower than those of uninfected lobsters. The low values for the PO_2 of the pre-branchial haemolymph were not unexpected given the huge numbers of parasite cells in the haemolymph. The fact that the PO_2 of the post-branchial haemolymph was also very much lower than in uninfected animals suggests either that the haemolymph may be incapable of taking up sufficient oxygen during its passage through the gills (see below) or that the respiration of the high number of cells in the haemolymph has an immediate effect on the PO_2.

In an earlier study, Field et al. (1992) showed that the haemocyanin concentration (measured as the concentration of copper) and the oxygen carrying capacity of the haemolymph of heavily-infected *N. norvegicus* were significantly lower than in uninfected lobsters. The present study has confirmed this observation and has shown that there was a progressive reduction in the carrying capacity of the haemolymph with increasing levels of infection. Although the reason for this reduction in the haemocyanin concentration of the haemolymph has yet to be clearly established, it may be due to direct effects of the parasite on the copper metabolism of the host, or to indirect effects on their respiratory physiology. A similar reduction in haemocyanin concentration also occurs in *N. norvegicus* subjected to prolonged exposure to severe hypoxia (Hagerman and Baden, 1988; Baden et al., 1990). Internal hypoxia due to *Hematodinium* sp.

infection may cause a similar response in this species. In a recent study, however, it was shown that, although the haemocyanin concentration of the haemolymph decreased under these conditions, the concentration of copper in the haemolymph did not change significantly at this time (Baden et al., 1994).

An alternative explanation for the reduction in the haemocyanin concentration in infected lobsters could be that this results from a reduction in their feeding activity. Several previous studies have shown that starvation in decapods may lead to a significant reduction in the protein and/or haemocyanin concentration of the haemolymph (Uglow, 1969; Djangmah, 1970; Spoek, 1974; Hagerman, 1983). Similarly, a recent study of the effects of starvation in uninfected *Nephrops norvegicus* showed that haemolymph protein concentrations could be reduced by as much as 50% over an 8 week period of starvation (Coutts, Parslow-Williams and Taylor, unpubl. obs.). At present, however, starvation would not appear to be the likely cause of the reduction in haemocyanin concentration in infected lobsters since preliminary studies of feeding behaviour under laboratory conditions have shown that these animals do not show any loss of appetite. It has yet to be determined, however, if infection with the parasite affects the ability of lobsters to forage for food in the field.

There is evidence that the presence of such large numbers of parasite cells in the haemolymph can result in the occlusion of haemal spaces, especially those in the gills (Field et al., 1992; Field and Appleton, 1995). Clearly, this is likely to impair gas exchange at the respiratory surfaces and the distribution of oxygen throughout the body. This, together with the reduction in the oxygen carrying capacity of the haemolymph of infected lobsters, may compromise the ability of the haemolymph to supply sufficient oxygen to the host's tissues. Evidence that this does indeed occur is provided by the increased L-lactate concentration and the associated reduction in the pH of the haemolymph of heavily-infected lobsters. The increased L-lactate concentration indicates that at least some of the host's tissues have resorted to anaerobic metabolism in response to internal hypoxia. Vivares and Cuq (1981) observed increased concentrations of L-lactate in the haemolymph and muscle of *Carcinus mediterraneus* infected with *Thelohania maenadis,* which they attributed either to internal hypoxia induced by the parasites or to some action of the parasite. Furthermore, another microsporidian, *Ameson michaelis*, was observed to cause hyperlactosis and a concomitant reduction in haemolymph pH in the blue crab, *Callinectes sapidus* (Findley et al., 1981).

The presence of L-lactate in the haemolymph is now known to increase the oxygen affinity of the haemocyanin of many species of decapod and partly counteracts the effect of a reduction in pH on the oxygen affinity in vivo (Truchot, 1980; Booth et al., 1982; Morris, 1990). In the present study, however, this effect was unlikely to have offset the reduction in haemocyanin oxygen affinity caused by the large reduction in the pH of the haemolymph in heavily-infected lobsters. The resulting reduction in haemocyanin oxygen affinity will also reduce the ability of these lobsters to take up oxygen at the gills which will further compromise their ability to maintain the supply of oxygen to the tissues.

The cause of death of *Nephrops norvegicus* infected with *Hematodinium* sp. requires further investigation but it may be attributable to tissue hypoxia, as a result of the massive systemic infection, or perhaps to the acidosis resulting from the host switching to anaerobic metabolism in an attempt to combat this hypoxia.

Acknowledgments

The authors would like to thank the following for their assistance with this work; the skipper and crew of the *RV Aora* for collection of experimental animals and Mrs J. Freel and Dr A. Munroe for assistance with haemolymph PO_2 measurements. This study was financed by the Chief Scientist's Group, Ministry of Agriculture Fisheries and Food (Project Ref. CSA 2139). One of us (P.P.W) was supported by an N.E.R.C. Research Studentship.

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