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Carbohydrate Dynamics and the Crustacean Hyperglycemic Hormone (CHH): Effects of Parasitic Infection in Norway Lobsters (*Nephrops norvegicus*)

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The effects of a dinoflagellate parasite (*Hematodinium* sp.) on carbohydrate metabolism were examined in the Norway lobster, *Nephrops norvegicus.* Five stages of infection were observed. These included uninfected (Stage 0), subpatently infected (SP), and patently infected (Stage 1–4) lobsters. During patent infection, the concentration of glucose in the hemolymph was reduced significantly from its value of 180 μg ml^{-1} in uninfected (Stage 0) lobsters to 25.3 μg ml^{-1} in Stage 3–4. These changes were accompanied by significantly lower levels of hepatopancreatic glycogen in lobsters at Stage 2 (2.01 mg g^{-1}) and Stage 3–4 (0.84 mg g^{-1}) of infection than in those at Stage 0 (16.19 mg g^{-1}) and Stage 1 (14.71 mg g^{-1}). Due to disruption of the normal feedback loops which control the release of crustacean hyperglycemic hormone (CHH), plasma concentrations increased with the severity of infection from 32.2 fmol ml^{-1} in Stage 0 to 106.6 fmol ml^{-1} in Stage 3–4. The increased CHH concentrations occurred concomitantly with reduced concentrations of plasma glucose and tissue glycogen. A significantly increased hemolymph CHH titer (107.7 fmol ml^{-1}) was also observed during SP infection. It is concluded that the parasite places a heavy metabolic load on the host lobster.

Key Words: Nephrops norvegicus; Hematodinium; CHH; hyperglycemia; hormonal disruption; parasitism.

INTRODUCTION

Stress responses occur in all animals when regulated physiological systems are extended beyond their normal range by external stressors. Failure of all or part of the integrated homeostatic response may lead to increasing physiological disturbance and ultimately death (Morris and Airriess, 1998). Indicators of such stress responses may therefore be useful in assessing the short-term well-being or long-term health status of an animal (Fossi *et al.*, 1997; Paterson and Spanoghe, 1997) and such indicators have received considerable attention in commercially important decapod crustacean species (Paterson and Spanoghe, 1997; Chang *et al.*, 1999a).

An important stressor is the infection of an animal by parasites (see Thompson, 1983 for review). The Norway lobster (*Nephrops norvegicus*) is seasonally infected by a dinoflagellate of the genus *Hematodinium*, with prevalence levels reaching 70% in populations of *N. norvegicus* from the inshore waters off the west coast of Scotland (Field *et al.*, 1992). A number of studies have established the basic characteristics of *Hematodinium* infection in *N. norvegicus* in terms of its progression, diagnosis and pathology (Field and Appleton, 1995, 1996), and its effect on host physiology (Taylor *et al.*, 1996), hemolymph and tissue biochemistry (Stentiford *et al.*, 1999a, 2000b), and locomotion (Stentiford *et al.*, 2000a). The ability to estimate the

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infection's severity by a simple morphological index (Field and Appleton, 1995) and by an immunoassay for subpatent (SP) infection (Field and Appleton, 1996) has greatly facilitated these epidemiological studies of *Hematodinium* infection in *N. norvegicus* (see Stentiford *et al.*, 1999a). It therefore offers a very useful model system in which to investigate the stresses imparted by parasitic infection in crustaceans.

A number of researchers have suggested different methods for quantifying the stress reactions in crustaceans; these include the measurement of different hemocyte types in the hemolymph (Jussila *et al.*, 1997), physiological, biochemical (Paterson and Spanoghe, 1997; Stentiford *et al.*, 1999a), and molecular changes in tissue and hemolymph (Fossi *et al.*, 1997), and the elevated transcription of heat shock proteins during periods of stress (Chang *et al.*, 1999a). A response that has received recent attention is that of the change in the plasma titer of the crustacean hyperglycemic hormone (CHH) in relation to imposed stressors (see Chang *et al.*, 1999b).

The occurrence of some "diabetogenic" factor from the eyestalks of crustaceans has been known for many years (Abramowitz *et al.*, 1944), as has evidence for the elevation of plasma glucose levels in crustaceans undergoing stress (Telford, 1968). The CHHs from several species have now been well described and their amino acid profiles sequenced (see Keller *et al.*, 1985; Kegel *et al.*, 1989, 1991; Lacombe *et al.*, 1999 for review). These 8- to 9-kDa neuropeptides produced by perikarya in the medulla-terminalis X-organ can be selectively released from the sinus gland into the blood stream (Santos and Keller, 1993b), after which they are known to target the hepatopancreatic plasma membranes (Kummer and Keller, 1993), the abdominal musculature (Santos and Keller, 1993a) and the hemocytes (Santos and Stefanello, 1991), with the liberated glucose either moving to the extracellular fraction or being converted intracellularly to lactate via glycolysis (Santos and Keller, 1993a). The advantage of such a system is not entirely understood, but due to the predictable elevation of plasma CHH during certain stresses (notably hypoxia) in crustaceans (Santos and Keller, 1993a; Webster, 1996; Chang *et al.*, 1999a), it is envisaged that hyperglycemia may be involved in the so-called "fight or flight" response in these animals. An interesting feature of this process is that the lactate resulting from enhanced glycolysis, released to the extracellular medium, could serve as a positive feedback mechanism for CHH release, with the increased CHH in turn stimulating glycogenolysis which then increases glucose availability (Santos and Keller, 1993b).

Variations in blood glucose have been observed in crustaceans under several different environmental and physiological conditions (such as extremes of temperature, salinity, anoxia, starvation, and emersion—see Hall and van Ham, 1998). It has been shown that the plasma CHH titer is also consistently increased during emersion and hypoxia and that this leads to elevated plasma glucose concentration (Santos and Keller, 1993a; Webster, 1996; Chang *et al.*, 1999a). Since concentrations of plasma glucose change during parasitism by bacteria (Stewart, 1980), it is presumed that these changes may be at least partly mediated by alterations in plasma CHH. However, it has not yet been shown whether the plasma CHH titer is altered in relation to parasitism.

Therefore, we have investigated the effect of progressive infection of the Norway lobster by *Hematodinium* parasites on the plasma CHH titer, as a possible sensitive indicator of stress and assessed whether these changes may be implicated in the altered plasma glucose and tissue glycogen concentrations that often accompany these infestations.

MATERIALS AND METHODS

Collection and Treatment of Animals

Norway lobsters were collected with an otter trawl from a location south of Little Cumbrae in the Clyde Sea area, Scotland, United Kingdom. Lobsters were maintained in a closed aquarium (9°C, 33 ppt salinity) at the University of Glasgow. Animals were allowed to settle in the aquarium for 1 week and were fed *ad libitum* on squid (*Loligo* spp.) and mussel (*Mytilus* spp.) tissue. Animals were not fed for 3 days prior to the experiment to avoid any effects of differential feeding and all animals were in the intermolt state as defined by Aiken (1980). Lobsters were staged for patent *Hematodinium* infection using the pleopod staging method of Field and Appleton (1995). According to the pleopod staging method, Stage 1 denotes a lightly

infected animal, with infection progressing through to Stage 4 (late stage infection). Stages 3 and 4 (heavily infected) animals were grouped in all analyses, as animals in these groups show very similar disease characteristics (see Stentiford *et al.*, 1999a). For diagnosis of true Stage 0 (uninfected) and SP infection, samples of hepatopancreas from previously diagnosed Stage 0 animals underwent Western blot analysis using a polyclonal antibody against the *N. norvegicus* isolate of *Hematodinium* sp. (see Field and Appleton, 1996 for details of antibody production). All animals showing a positive reaction to the anti-*Hematodinium* antibody in hepatopancreas tissue were diagnosed as SP (i.e., *Hematodinium*-infected, but below the limit of detection using the pleopod staging method). Animals showing no reaction to the antibody were diagnosed as uninfected (Stage 0).

Chemicals

For glycogen analyses, potassium hydroxide, ethanol, and the anthrone and Folin-Ciocalteu's phenol reagents were obtained from Sigma–Aldrich Co. (Poole, UK). For glucose analysis, perchloric acid (PCA), sodium phosphate (dibasic), glucose, and orthophosphoric acid (85%) were obtained from Sigma–Aldrich Co., and the glucose oxidase assay kit was obtained from Boehringer Mannheim (Kit No. 124028). For CHH analysis, bovine serum albumin (fraction V), EDTA, glycine ethyl ester, Tween 20, and 2,2′-azino-bis-3-ethylbenz-thiazoline-6-sulfonic acid were obtained from Sigma–Aldrich Co., and the streptavidin–peroxidase solution was obtained from Boehringer Mannheim.

Hemolymph and Tissue Preparation

A total of 100 hemolymph samples from uninfected and *Hematodinium*-infected lobsters were drawn from the base of the fifth pereiopod into sterile syringes and centrifuged immediately at 17,000*g* for 10 min at 10° to remove cellular material and suspended debris. For the CHH assay, aliquots of plasma were frozen immediately in liquid nitrogen before freeze-drying for 24 h. Dried samples were stored at −20° until transport to, and analysis at, the Bodega Marine Laboratory. For the glucose assay, the clear plasma was deproteinized using an equal volume of 0.6 M PCA for 10 min at 4°. Protein precipitate was removed by centrifugation and the supernatant was stored briefly at −20° until analysis at the University of Glasgow.

The hepatopancreas from 40 lobsters at different stages of *Hematodinium* infection was dissected out in physiological saline. Excised samples were blotted to remove excess moisture and weighed (wet weight) before rapid freezing in liquid nitrogen and freeze-drying for 36 h. Tissue was then reweighed (dry weight) and samples were individually ground using a standard mortar and pestle. Ground samples were stored at −20° until analysis of total glycogen content at the University of Glasgow.

Measurement of Plasma Glucose Concentration

Glucose concentration in the hemolymph of uninfected and infected lobsters was measured using the glucose oxidase method (Boehringer Mannheim) in a microplate method as described by Webster (1996). Briefly, 50-μl samples of deproteinized plasma were added to 450 μl of 0.2 M phosphate buffer (pH 7.4) and 100-μl samples of this solution were used in the assay with 200 μl of the enzyme chromogen reagent. Concentrations of plasma glucose were read from a standard curve constructed for glucose.

Measurement of Plasma CHH Titer

The production of an antibody against purified CHH-A from *H. americanus* has been described by Chang *et al.* (1998) and previous studies have shown that *N. norvegicus* CHH can be detected using this antibody (G. D. Stentiford and E. S. Chang, unpublished data). In the current study, freeze-dried plasma samples were resuspended to their original volume with ddH_2O and assayed for CHH-A using the ELISA method of Chang *et al.* (1998).

HPLC-purified CHH from the crayfish *Orconectes limosus* (Kegel *et al.*, 1991) was used as the standard. Since purified *N. norvegicus* CHH was not available, it was not possible to quantify absolute concentrations of *N. norvegicus* CHH. The data were therefore presented as *O. limosus* equivalents, which allow relative quantification.

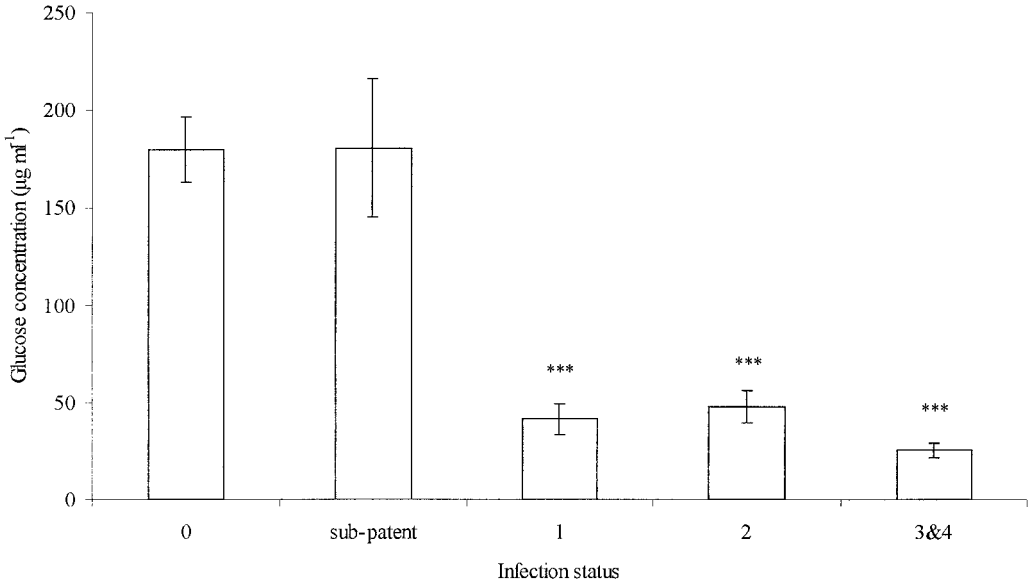

FIG. 1. Concentration of glucose (μg ml^{-1}) in the plasma of uninfected (Stage 0) and *Hematodinium*-infected (subpatent and Stages 1, 2, 3–4) *N. norvegicus.* Statistically significant difference from Stage 0 value given as ***($P < 0.01$). Stage 0, $n = 22$; subpatent, $n = 8$; Stage 1, $n = 22$; Stage 2, $n = 33$; Stage 3–4, $n = 15$.

Measurement of Hepatopancreatic Glycogen Content

For determination of muscle and hepatopancreatic glycogen, 400 μl of 30% KOH was added to 20 mg of the freeze-dried samples from lobsters at different stages of infection; then the samples were boiled for 20 min in a water bath maintained at 95–100°. Samples were cooled and added to 700 μl absolute ethanol before being placed on ice for 2 h. Following precipitation, samples were spun at 17,000*g* for 10 min and the supernatant was discarded. One milliliter of ddH_2O was added to each sample before sonication, and 50 μl of each sonicated sample was incubated at 95–100° in 1 ml of anthrone reagent before measurement of total glycogen (see Carroll *et al.,* 1956).

Data Analysis

Comparisons of plasma glucose concentration, plasma CHH titer, and hepatopancreatic glycogen content of uninfected and infected *N. norvegicus* were performed either by one-way analysis of variance (ANOVA) for normally distributed data or by a Kruskal–Wallace test for nonnormal distributions. Comparisons between stages were made with a Tukey's pairwise analysis (normal distributions) and a Mann–Whitney test (nonnormal distributions). Significance was considered to be at $P < 0.05$.

RESULTS

Plasma Glucose Concentrations

The mean concentration of glucose in the plasma of uninfected *N. norvegicus* was 180.0 μg ml^{-1} plasma. The glucose concentration in SP-infected lobsters (180.1 μg ml^{-1} plasma) was not significantly different from that of Stage 0 animals ($P = 0.979$), but patently infected animals of all stages showed significantly lower concentrations of plasma glucose than Stage 0 [Stage 1 (41.4 μg ml^{-1} plasma; $P < 0.001$), Stage 2 (47.8 μg ml^{-1} plasma; $P < 0.001$), and Stage 3–4 (25.3 μg ml^{-1} plasma; $P < 0.001$)]. All patently infected animals also showed significantly lower concentrations of plasma glucose than SP-infected lobsters (all $P < 0.001$), but different stages of patently infected lobsters showed no significant difference in glucose concentration (all $P > 0.05$) (Fig. 1).

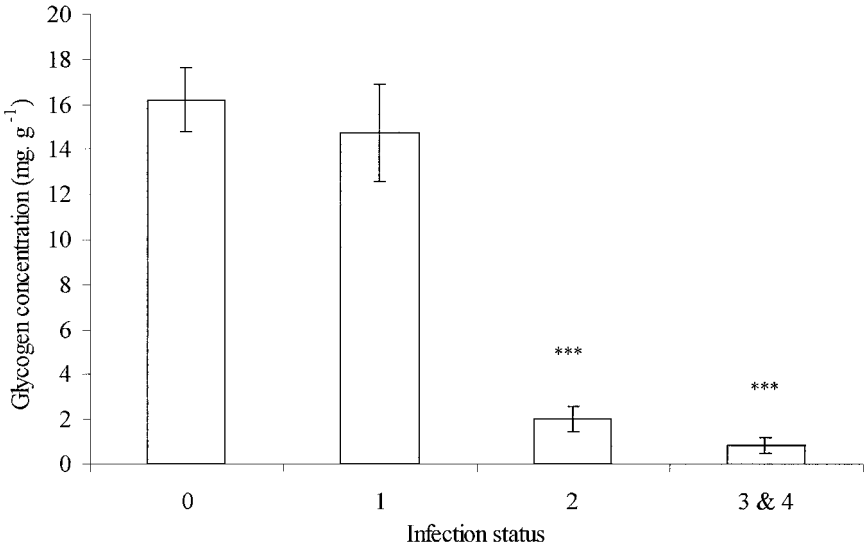

FIG. 2. Concentration of glycogen (mg g^{-1} dry weight) in the hepatopancreas of uninfected (Stage 0) and *Hematodinium*-infected (Stages 1, 2, 3–4) *N. norvegicus.* Statistically significant difference from Stage 0 value given as *** ($P < 0.001$). Sample sizes were the same as those described in the legend to Fig. 1.

Hepatopancreatic Glycogen Concentrations

The mean glycogen concentration of the hepatopancreas of uninfected *N. norvegicus* was 16.19 mg g^{-1} dry weight. Hepatopancreatic glycogen concentration was reduced during patent *Hematodinium* infection. The reduction was not significant in Stage 1 lobsters (14.71 mg g^{-1} dry weight; $P = 0.574$), but was highly significant in both Stage 2 (2.01 mg g^{-1} dry weight; $P < 0.001$) and Stage 3–4 (0.84 mg g^{-1} dry weight; $P < 0.001$) *Hematodinium* infection. When different stages of infection were compared, significant reductions in hepatopancreatic glycogen were found between Stage 1 and Stage 2 ($P < 0.001$) and between Stage 1 and Stage 3–4 ($P < 0.01$), but not between Stage 2 and Stage 3–4 ($P = 0.098$) (Fig. 2).

Plasma CHH Concentrations

The mean concentration of CHH in the plasma of uninfected *N. norvegicus* was 32.2 fmol ml^{-1}. The mean concentration of CHH in the plasma of SP-infected lobsters (107.65 fmol ml^{-1}) was significantly higher than that of Stage 0 animals ($P < 0.05$), and at Stage 1 (light patent infection), the mean concentration was higher (though not significantly, $P = 0.057$) than that of Stage 0 and lower (though not significantly, $P = 0.070$) than that of subpatently infected lobsters. In later stages of patent *Hematodinium* infection, the plasma CHH concentration was significantly higher than that of uninfected animals [Stage 2 (77.2 fmol ml^{-1}, $P < 0.001$) and Stage 3–4 (106.6 fmol ml^{-1}, $P < 0.001$). The significant increase in plasma CHH concentration between Stage 1 and Stage 3–4 animals ($P < 0.05$) and the almost significant increase between Stage 1 and Stage 2 animals ($P = 0.080$) suggest that the titer of CHH increases with the severity of patent infection, but also that initiation of SP infection may involve a temporary rise in plasma CHH titer. It is interesting to note, however, that the mean plasma CHH titer of SP-infected lobsters is not significantly different from that of Stage 3–4 lobsters ($P = 0.997$) (Fig. 3).

DISCUSSION

Carbohydrate Dynamics in Hematodinium-Infected N. norvegicus

This study has identified large alterations in the carbohydrate profile in the plasma and tissues and in the crustacean hyperglycemic hormone titer in the

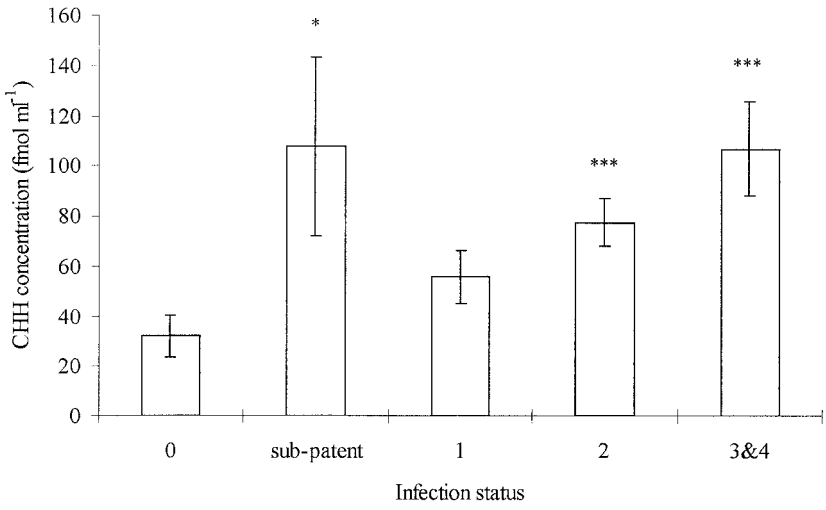

FIG. 3. Concentration of the crustacean hyperglycemic hormone (CHH) (fmol ml^{-1}) in the plasma of uninfected (Stage 0) and *Hematodinium*-infected (subpatent and Stages 1, 2, 3–4) *N. norvegicus.* Statistically significant difference from Stage 0 value given as * ($P < 0.05$), and *** ($P < 0.001$). Sample sizes were the same as those described in the legend to Fig. 1.

plasma of *N. norvegicus* infected by the parasitic dinoflagellate *Hematodinium* sp. The concentration of glucose in the plasma remains relatively unchanged in SP-infected lobsters, but is significantly reduced during all stages of patent infection. The 30% reduction in the concentration of plasma glucose in Stage 1 infection suggests that glucose provides a readily usable substrate for the growth of the *Hematodinium* parasite in the hemolymph of *N. norvegicus.* Similar reductions in the concentrations of simple carbohydrates in the host tissues have been reported for other parasitic infestations in crustaceans (Stewart, 1980; Cawthorn, 1997) and insects (Schmidt and Platzer, 1980). In these cases, the parasites may be acting as a "carbohydrate sink," absorbing hemolymph glucose and thereby forcing the host to resupply glucose to the hemolymph to maintain carbohydrate homeostasis. This resupply of glucose to the plasma occurs mainly via tissue-based glycogenolysis, and the main storage tissues for such polysaccharides in crustaceans are the hepatopancreas (Dall and Moriarty, 1983), the muscle (Schwoch, 1972), and the hemocytes (Johnson *et al.*, 1971). The dramatic decrease in the concentration of glycogen in the hepatopancreas that occurs between Stage 1 and Stage 2 of infection (Fig. 2) and the similar depletion found to occur in the deep abdominal flexor muscle of *Hematodinium*-infected *N. norvegicus* (Stentiford *et al.*, 2000b) are consistent with a response to an elevated burden of circulating parasites (Field *et al.*, 1992).

The control of carbohydrate dynamics in the hemolymph and tissues of crustaceans is known to be exerted mainly by circulating levels of CHH (Sedlmeier, 1985). The cellular reception of CHH neuropeptide molecules elicits the mobilization of glucose from intracellular glycogen stores via glycogenolysis (Santos and Keller, 1993b), with the liberated glucose either moving to the extracellular fraction or being converted intracellularly to lactate via glycolysis (Santos and Keller, 1993a).

In patently infected lobsters, the plasma CHH concentration shows a steady and significant increase in relation to infection severity (Fig. 3), which is mirrored by a progressive decrease in the concentration of plasma glucose (Fig. 1). The coexistence of a high plasma CHH concentration and a low plasma glucose concentration is the opposite of what may be expected in a normally operating feedback system and is probably a reflection of the fact that carbohydrate stores are exhausted by late infection. As infection progresses, the developing parasites will consume the glucose being liberated from the tissue glycogen reserves, lowering circulating glucose concentrations and thereby reducing the negative feedback on CHH release (see step 1 in Fig. 4 and Santos and Keller, 1993a). This, in turn, will initiate an increased release of CHH (step 2

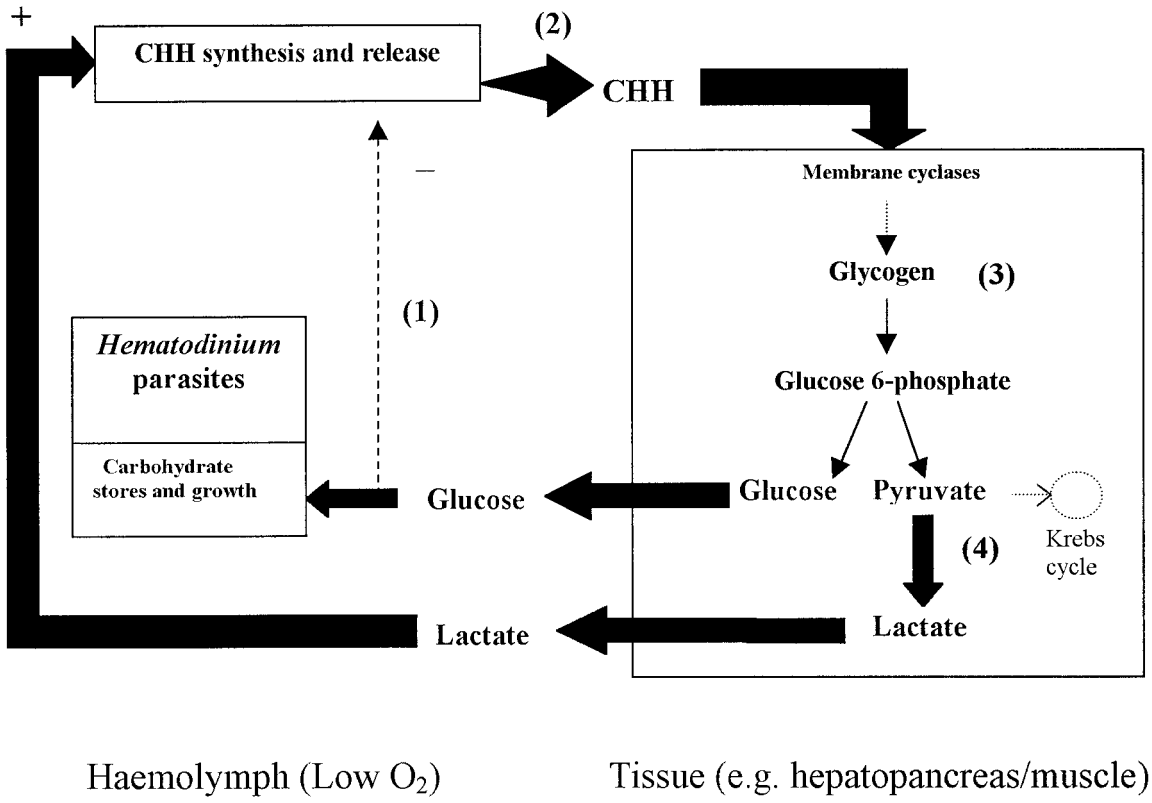

FIG. 4. Schematic diagram of the changing relationship among tissue glycogen, plasma glucose, and CHH release in the presence of *Hematodinium* parasites in the plasma of *N. norvegicus.* Key: + and − (positive and negative feedback loops for CHH release); numbers in parentheses refer to descriptions in the Discussion.

in Fig. 4), inducing progressive glycogen depletion of the tissues (step 3 in Fig. 4). As the parasite burden increases, a steadily reducing plasma glucose concentration, a progressive depletion of tissue glycogen stores, and an increasingly elevated plasma CHH titer are all to be expected. Although there is evidence for the synthesis and release of CHH from sites other than the X-Organ / sinus gland complex (Chang *et al.*, 1999b; Chung *et al.*, 1999), the eyestalk is likely to be the primary source of the CHH that is involved in the regulation of systemic carbohydrate metabolism.

In addition to this change in negative-feedback control of CHH release by plasma glucose, the increased plasma lactate levels that are known to occur during *Hematodinium* infection (Taylor *et al.*, 1996) may also stimulate CHH release, with consequent effects on glycogenolysis (Santos and Keller, 1993b). The most likely cause of these elevated plasma lactate levels is a switch to anaerobic metabolism, which Taylor *et al.* (1996) have recorded in terms of increased lactate and a lowered hemolymph pH, a situation which is exacerbated by a reduction in the oxygen carrying capacity of the hemolymph. At the cellular level, pyruvate, the end product of glycolysis, is converted under anaerobic conditions to lactate, which either accumulates within the cell or is moved to the extracellular medium (step 4 in Fig. 4).

Potentially, tissue glycogen reserves could be replenished from the lactate by the process of gluconeogenesis, but this is unlikely to be significant, since in invertebrates these pathways are thought to be relatively inefficient compared with the Cori cycle in mammals (Schulman and Landau, 1992) and probably operate under aerobic conditions (Ellington, 1983). Moreover, gluconeogenesis is antagonized by CHH itself, which is known to activate cyclic nucleotide-dependent protein kinases, leading to a phosphorylation and therefore inhibition of glycogen synthase (Sedlmeier, 1985; Santos and Keller, 1993a). It is also inhibited by hyperosmolarity (Li *et al.*, 1992), which is

known to occur in *Hematodinium* infection (Stentiford *et al.*, 1999a). It is most likely, therefore, that the elevated plasma CHH concentration in patent infection is due primarily to a "functional hypoxia" in the infected lobster, which elicits a cascade response similar to that seen during the "environmental hypoxia" caused by emersion (Santos and Keller, 1993a; Webster, 1996; Chang *et al.*, 1999a).

Implications of Altered Carbohydrate Dynamics during Hematodinium Infection

Recently, it has been shown that the concentration of CHH in the plasma is increased by up to 100-fold in the hours leading up to molting, with levels returning to normal following ecdysis (Chung *et al.*, 1999). Due to the coincidence of the spring peak of *Hematodinium* infection in Scottish *N. norvegicus* with the onset of the main molting period for this species (Field *et al.*, 1998), it is tempting to suggest that an elevated concentration of plasma CHH at this time may create hemolymph conditions that are suitable for rapid growth of the parasite population, possibly explaining why heavy infections are often observed in recently molted lobsters (Stentiford *et al.*, 1999b). An increase in plasma CHH titer due to other stressors (such as seasonal hypoxia, temperature changes, or pollution) could also be implicated in infection progression. Thus, the elevated plasma CHH titer in SP-infected lobsters shown here may either be responsible for or be caused by the appearance of *Hematodinium* parasites in the hemolymph following latent infection. Further studies on the effect of elevated CHH concentration on latently infected lobsters are necessary to investigate the role of this hormone in initiation of hemolymph infection in *N. norvegicus.*

The severe depletion of plasma and tissue carbohydrate reserves during late infection probably represents a form of physiological starvation from which the host cannot recover. Following this, the postparasitic forms emerge and the host will die (see Field and Appleton, 1995; Appleton and Vickerman, 1998). During these terminal stages of *Hematodinium* infection, the arthrodial membranes appear swollen and the hemolymph volume is apparently increased (Appleton and Vickerman, 1998). Increased water uptake is associated with the high plasma CHH titer preceding ecdysis (Chung *et al.*, 1999), and it is possible that elevated levels of plasma CHH within during the final stages of *Hematodinium* infection in *N. norvegicus* could cause a similar water uptake, leading to the observed swelling of arthrodial membranes and thereby facilitating the exit of motile dinospores from the host lobster.

This study has, for the first time, identified a link between the disruption of carbohydrate handling during parasitic infection of crustaceans and the alterations in the expression of the crustacean hyperglycemic hormone. Whether the increase in the plasma CHH titer is caused directly by parasitic disruption of the lobster's endocrine system or indirectly by interfering with the positive and negative feedback loops in the plasma requires further investigation. Future studies which consider plasma and tissue carbohydrate dynamics in crustaceans should take into account the fact that the relatively large variations often seen in the hyperglycemic response (Hall and van Ham, 1998), while being related to the molt stage of the test animal, may also be due to its overall health status.

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