THE ROLE OF EYESTALK FACTORS IN CARBOHYDRATE REGULATION IN THE CRAB *CANCER MAGISTER*

by

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Abstract

Crustacean hyperglycemic hormone (CHH), located in the sinus gland of the decapod crustacean eyestalk, is proposed to be the primary carbohydrate regulatory hormone in these animals. However, its physiological role is poorly characterized. In this study, the role of eyestalk factors in regulating carbohydrates under control and severely hypoxic (emersion) conditions was investigated in the crab *Cancer magister*. Previously, dramatic increases in hemolymph flow to the sinus gland, *via* the anterior aorta, had been proposed to be essential for increases in circulating concentrations of CHH observed during emersion stress. Therefore, ligation of this artery was hypothesized to be an effective means of manipulating circulating hormone titers.

The data presented here indicates that, under control conditions, eyestalk extracts (ESE) from *C. magister* contain an active factor(s) which, when injected into whole animals, cause hyperglycemia in a dose dependent manner. Eliminating flow through the anterior aorta does not annul ESE injection induced hyperglycemia. Carbohydrate stores in heart, hepatopancreas and muscle tissue were affected by anterior aorta ligation and, may therefore be target tissues. Injection of eyestalk extract had no effect on heart, gill, hepatopancreas or muscle carbohydrate content. Treatment of isolated muscle tissue with eyestalk extract had no effect on glucose release and, therefore, did not support the proposed mode of action of CHH. Under emersion stress conditions, hyperglycemia occurs but is prevented if eyestalk factors cannot circulate (anterior aorta ligated animals). Changes in glycogen content of muscle and hepatopancreas identified these tissues as potential targets of eyestalk factor(s) during emersion. Again, *in vitro* studies of isolated muscle tissue did not support the proposed mode of action of CHH. Finally, the presence of CHH like peptides in eyestalk extracts prepared from *C. magister* tissue was demonstrated and provides support for the hypothesis that this hormone is responsible for the results observed. The results are discussed in relation to hormonal involvement in metabolic depression displayed by emersed aquatic decapod crustaceans.

Table of Contents

List of Tables

List of Figures

Abbreviations

AP	adapter primer
AUAP	abridged universal anchor primer
cDNA	complementary DNA
CHH	crustacean hyperglycemic hormone
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CPRP	CHH-Precursor-Related-Peptide
dH_2O	distilled water
EDTA	ethylenediamineteraacetic acid
ESE	eyestalk extract
EtBr	ethidium bromide
GIH	gonad inhibiting hormone
HPLC	high performance liquid chromatography
$InsP_3$	inositol 1,4,5-triphosphate
Ligated	anterior aorta ligated
MIH	moult inhibiting hormone
MTXO	medulla terminalis X-organ
P_{crit} O_2	critical ambient oxygen tension
PCR	polymerase chain reaction
RACE	rapid amplification of cDNA ends
RP-HPLC	reverse phase high performance liquid chromatography
RT-PCR	reverse transcribed polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean

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Chapter One: General Introduction

In decapod crustaceans, the sinus gland comprises an endocrine structure of primary importance and is located in the eyestalk. It is a classical neurohemal organ, composed of axon terminals from cell bodies located within the medulla terminal X-organ (MTXO) which is supported by muscle and connective tissue (Chaigneau, 1983; Fingerman, 1987; Stuenkel and Cooke, 1988). Peptide hormone precursors are synthesized in perikarya in the MTOX and transported axonally to the sinus gland for storage in secretory granules (Andrew, 1983; Castany et al., 1997). Among the synthesized neurohormones are chromatophorotropins and members of the crustacean hyperglycemic hormone/moult-inhibiting hormone/gonad-inhibiting hormone (CHH/MIH/GIH) family (Stuenkel and Cooke, 1988; Keller, 1992; De Kleijn and Van Herp, 1995; Van Herp, 1998). Of this latter hormone family, CHH is the best characterized. It is present in more than 40% of axon terminals in the sinus gland and accounts for up to 10% of the total protein present in this structure (Kallen et al., 1990; Keller et al., 1994; Glowik et al., 1997). Significantly smaller stores, estimated at less than 1% of the total (Keller et al., 1985), are found in the pericardial organs, ventral nerve cord and thoracic ganglion (Chang et al., 1999; Dircksen et al., 2001). As a consequence of the elevated concentration of this peptide in the sinus gland, the primary amino acid, cDNA and DNA nucleotide sequences have been characterized (Weidemann et al., 1989; De Kleijn et al., 1994; De Kleijn et al., 1995; Ohira et al., 1997; Gu and Chan, 1998). However, studies investigating the physiological role(s) of this hormone have proven less successful and point to a variety of potential functions as well as modes of action.

1.1. Peptide characterization

Thus far, eleven primary amino acid sequences are known for CHHs from species of five decapod infraorders and from one isopod species. These peptides share a high degree of amino acid homology and have conserved features including: 6 cysteine residues, three disulfide bridges, blocked C-termini and, in astacideans (lobsters and crayfish) and brachyurans (true crabs), blocked N-termini (Soyez et al., 1990; Kegel et al., 1991; Huberman et al., 1993; Martin et al., 1993; Aguilar et al., 1995; Chung et al., 1998; Lin et al., 1998; Marco et al., 1998). All characterized peptides are 71 to 73 amino acids in length and between 8.4 and 8.7 kDa in molecular weight. Sequencing of cDNA coding for CHH has revealed that these peptides are encoded and translated as pre-prophormones (Weideman et al. 1989; De Kleijn et al., 1994, 1995). This structure consists of (from the N-terminus to the C-terminus) a short signal peptide (24 to 26 amino acids), a cleavage site (2 to 3 basic amino acids), CHH-related-

precursor-peptide (CPRP, 17-38 amino acids), a second cleavage site, and the CHH coding region (Weideman et al., 1989; De Kleijn et al., 1994; De Kleijn et al., 1995).

Interestingly, in all species studied to date, CHH is present in more than one isoform (Chung et al., 1998; Van Herp, 1998). Generally, two isoforms are found in brachyuran species. The source of variation between these isoforms appear to be the substitution of pyroglutamate for glutamine in the N-terminal position (Chung and Webster, 1996). Species from other decapod infraorders tend to have more than 2 hyperglycemic hormones. Post-translational epimerization of L-phenyalanine to D-phenylalanine at position 3 of the mature peptide and gene duplication and divergence are sources of isoform diversity in these species (De Kleijn et al., 1994; Soyez et al., 1994; Yasuda et al., 1994; Aguilar et al., 1995; De Kleijn et al., 1995; Ohira et al., 1997; Marco et al., 2000).

1.2 CHH and the hyperglycemic response

The classical endocrinology techniques of extirpation and re-implantation are the most frequently employed tools in investigating the physiological roles of CHH. For decapod crustaceans the methods used to remove and replace circulating CHH are eyestalk ablation and eyestalk extract (ESE) injection, respectively. However, more recently, purified hormone and immunological techniques have been adopted. The initial study by Abramowitz et al. (1944), in which eyestalk ablation and crude ESE injection were employed, identified a "diabetogenic factor" capable of elevating hemolymph glucose levels within the crustacean eyestalk. Recently, other roles for CHH have been identified and include: amylase secretion (Sedlmeier, 1988), osmoregulation (Charmantier-Daures et al, 1994; Spanings-Pierrot et al., 2000), lipid metabolism (Santos et al., 1997), moult regulation (Liu et al., 1997; Chung et al., 1999), immune response (Lorenzon et al., 1997) and vitellogenesis (De Kleijn et al., 1998; Khayat et al., 1998) have all been proposed. Despite the plethora of potential roles for this hormone, the best characterized remains the regulation of hemolymph glucose concentrations.

The proposed mode of action of CHH in eliciting a hyperglycemic response is binding of G-protein-coupled receptors at target tissues and subsequent stimulation of glycogenolysis and inhibition of glycogenesis resulting in glucose release to the hemolymph (Huggins and Munday, 1968; Sedlmeier, 1982; Keller and Sedlmeier, 1988). Evidence that CHH interacts with G-protein-coupled receptors comes from investigation of secondary messengers in target tissues. Injection of sinus gland extract and purified CHH has been shown to increase cyclic adenosine monophoshphate (cAMP) and cyclic guanosine monophosphate (cGMP) concentrations in heart, hepatopancreas and muscle tissue (Sedlmeier and Keller, 1981; Goy, 1990). Membrane permeable analogues of both cAMP and cGMP have been shown to be effective at mimicking the effect of CHH on glycogen synthase activity

(Sedlmeier, 1982). However, Smullen et al. (1996) presented data indicating that the secondary messenger may be inositol 1,4,5-triphosphate ($InsP_3$). Therefore, while support for the interaction of CHH with G-proteins-coupled receptors is convincing, the type of receptor(s) and second messenger(s) involved are currently not well characterized.

The hepatopancreas has long been considered a prime target of CHH based on the proposal that its function is analogous to that of the vertebrate liver (O'Connor and Gilbert, 1968; Keller and Andrew, 1973; van Weel, 1974). The only conclusive evidence that this organ is a target of CHH in the hyperglycemic response it elicits comes from *in vitro* results investigating the incorporation of C^{14}-glucose into glycogen. Net incorporation was observed to decrease in the presence of purified CHH (Sedlmeier, 1985, 1987). However, results from both *in vivo* and *in vitro* studies do not support a role for hepatopancreas tissue in supplying circulating glucose (Ramamurthi et al., 1968; Rangneker and Madhyastha, 1971; Keller and Andrew, 1973; Telford, 1975; Santos et al., 1988). Muscle tissue has also been postulated to be a primary target of CHH (Keller and Andrew, 1973; Keller and Sedlmeier, 1988). Results of *in vivo* experiments investigating changes in glycogen content in response to eyestalk ablation and ESE or purified CHH injection support a role for this tissue in CHH elicited hyperglycemia (Schwabe, 1952; Rangneker and Madhyastha, 1971; Keller and Andrew, 1973). Results of *in vitro* studies provide further support for this hypothesis (Telford, 1975). Nevertheless, conflicting data, in which eyestalk ablation and ESE injection were ineffective at altering glycogen content in muscle tissue has been presented (Ramamurthi et al., 1968). Also, treatment of isolated muscle tissue with purified CHH resulted, in one species, not in increases in glucose release, but decreases in glucose uptake (Nery et al., 1993). Other proposed target tissues include: hypodermis (Schwabe, 1952; Keller and Andrew, 1973), gills (Keller and Andrew, 1973) and hemocytes (Johnston et al., 1971; Santos and Stefanello, 1991; Nery et al., 1993).

Support for a stimulatory effect of CHH on glycogenolysis is provided by data on glycogen phosphorylase activity, the enzyme responsible for the breakdown of glycogen. This enzyme cleaves glucose-1-phosphate molecules from glycogen and is present in two forms; the more active phosphorylated form, *a,* and the dephosphorylated form, *b,* (Huggins and Munday, 1968; Ramamurthi et al., 1968; Bauchau et al., 1968). Eyestalk ablation results in an increase in phosphorylase *b,* supporting the presence of an activating factor within the eyestalk. ESE injection increases the proportion found in the *a* form (Bauchau et al., 1968; Ramamurthi et al., 1968). The injection results have been confirmed with purified CHH (Sedlmeier, 1985). Despite support for this mode of action, some results indicate that eyestalk ablation alters the ratio of active to inactive glycogen phosphorylase in favour of the active form, suggesting that removal of eyestalks constitutes the removal of an inhibitory factor and not an activating factor as proposed (Sedlmeier, 1985). The data

for inhibition of glycogenesis (synthesis of glycogen) by CHH comes from investigations of glycogen synthase activity. This enzyme, much like glycogen phosphorylase, is present in two forms; the active dephosphorylated glucose-6-phosphate (G-6-P) independent, I, form and the less active phosphorylated G-6-P dependent, D, form (Hers, 1976; Sedlmeier, 1985). Eyestalk ablation results in increases in total glycogen synthase activity and ESE/purified CHH injection returns enzyme activity levels to normal (Wang and Scheer, 1963; Ramamurthi et al., 1968; Sedlmeier, 1982), suggesting that CHH has a role in decreasing glycogenesis *via* its effects on that ratio of I to D glycogen synthase.

Despite support for the proposed mode of action of CHH in eliciting hyperglycemia, it is apparent from reviewing the literature that conclusive and unequivocal evidence is lacking. In fact, a great deal of contradictory and species-specific data has been presented (Scheer and Scheer, 1951; Santos and Colares, 1986; Santos et al., 1988; Rao et al., 1991). Some of the data that is most inconsistent with the proposed mode of action has led investigators to speculate that the role of CHH is not to increase glucose mobilization to the hemolymph but to reduce glucose uptake and utilization by tissues (Scheer and Scheer, 1951; Santos et al., 1988). Even researchers supporting a mobilizing role of CHH on carbohydrate stores suggest that the name hyperglycemic hormone may not accurately reflect hormonal activity. Instead, a more appropriate description of the physiological function of CHH is proposed to be general carbohydrate regulation (Honhke and Scheer, 1970; Santos and Keller, 1993b).

1.3 Emersion

During normoxic conditions, carbohydrates as well as lipids and proteins are used as fuel sources (Randall et al., 1997). The degree to which each fuel is utilized is organ and tissue specific (McMahon, 1988). However, during severe hypoxia, lipid and protein utilization is superceded by carbohydrate utilization (Hochachka, 1980). Consequently, severe hypoxia represents a stressful condition in which carbohydrate utilization must be well regulated. Therefore, if CHH is in fact, as suggested, a general carbohydrate regulatory hormone, its activity should be evident under conditions of severe hypoxia. For some aquatic decapods, air exposure (emersion) represents a natural occurrence of severe hypoxia.

Typically, terrestrial brachyurans have anatomical adaptations facilitating air breathing and, are therefore, able to benefit from increased oxygen supply, decreased energy demand for ventilation and improved oxygen diffusion across respiratory surfaces as a result of the increased solubility of oxygen in air (Rahn, 1966). These brachyuran species tend to exhibit vascularization and enlargement of the branchial chambers to form air cavities which act as lungs (Maitland, 1990). Primarily aquatic

species that are active in air exhibit both behavioural and anatomical adaptations for extracting oxygen from air. Several intertidal crabs have been observed to re-circulate branchial water over the carapace (Santos et al., 1987; Maitland, 1990), thereby increasing oxygen content for extraction *via* the gills. The European shore crab, *Carcinus maenas,* accomplishes a similar increase in branchial water oxygen content by reversing ventilatory flow, causing air to bubble through branchial water (Taylor et al., 1973; Wheatly and Taylor, 1979). Anatomical adaptations in these species include wider spacing and thicker chitin coating of the gill lamellae, presumably to prevent collapse in air (Taylor and Butler, 1978). However, aquatic species that do not display behavioural or anatomical adaptations to air breathing suffer from severely reduced oxygen intake during aerial exposure. Oxygen present in water retained in the gill chambers is rapidly depleted (deFur and McMahon, 1984) and, more importantly, the gill lamellae collapse, drastically reducing the surface available for gas exchange (deFur et al., 1988; Taylor and Whiteley, 1989). These animals must, therefore, rely on physiological mechanisms to compensate or circumvent the deleterious effects of insufficient oxygen supply. Upon exposure to hypoxia, some decapod species are capable of mobilizing glycogen to facilitate increased rates of anaerobic respiration and reducing their overall metabolic rate, much like other facultative anaerobes (Teal and Carey, 1967; Hochachka and Somero, 1984; Hill et al., 1991b). The involvement of CHH in the response of aquatic decapods to emersion stress was initially proposed based on observation of eyestalk dependent hyperglycemia. Primarily aquatic decapods are frequently observed to respond to air emersion with a pronounced elevation in hemolymph glucose concentrations, a response which is absent from eyestalk ablated animals (Kleinholz and Little, 1949; Telford, 1975; Spicer et al., 1990; Santos and Keller, 1993a; Webster, 1996; Chang et al., 1998). More recently, rapid and large increases in circulating CHH concentrations have been recorded for emersed lobster, *Homarus americanus*, and crab, *Cancer pagurus* (Webster, 1996; Chang et al., 1998).

1.4 Experimental animals

Cancer magister Dana, a brachyuran crab native to the Pacific coast of North America, is a fully marine species that is found from Alaska to California in the near shore region and to depths of 196 m (Butler, 1961). This species inhabits sandy bays and estuaries (Butler, 1984) and can occasionally be stranded in air by large tidal fluctuations (Airriess and McMahon, 1994). Evidence indicates that this species is unable to extract sufficient oxygen from air and must rely on anaerobic metabolism and metabolic depression to survive emersion (deFur and McMahon, 1984; Airriess and McMahon, 1996).

1.5 Goals

The goals of the current investigation were to characterize the role of eyestalk factor(s) in carbohydrate metabolism in the crab, *Cancer magister*, during control and emersion stress conditions. Specifically, this research addresses the questions: i) is there a hyperglycemic hormone in crude eyestalk extracts of *C. magister* ii) what tissues are affected by eyestalk factor(s) and iii) what is the mode of action? This preliminary study relies, primarily, on the investigative tools of removal and re-implantation. In this case, anterior aorta ligation (see chapter two section 2.2.2) and injection of crude eyestalk extract were employed to remove and replace circulating eyestalk factors, respectively.

The data presented here indicate that factor(s) with hyperglycemic activity are present in the eyestalk of *C. magister*. Immunological investigation identified the presence of a CHH-like peptide and physiological experiments confirmed the occurrence of hyperglycemia resulting from ESE injection. Putative target tissues were identified based on changes in carbohydrate stores and the mode of action of hyperglycemic eyestalk factor(s) was investigated in muscle tissue by quantifying changes in glucose release/uptake in the presence of ESE. Carbohydrate regulation during emersion stress was investigated with the goal of clarifying the role of hyperglycemic eyestalk factor(s) *in vivo*. The occurrence and involvement of eyestalk factor(s) in emersion hyperglycemia were confirmed. Identity of target tissues and mode action of eyestalk factor(s) were also investigated. The results of these studies support the presence of a carbohydrate regulating substance in the eyestalk of *C. magister* and putatively identify it as CHH. Additionally, a role for this hormone in metabolic reduction during emersion stress was suggested.

Chapter Two: General Methods

2.1 Animals

Adult male intermoult *Cancer magister*, mean weight 665 ±16 g (range 570 g to 861 g) were purchased from a local commercial fisherman and allowed a minimum of one week to acclimate to laboratory conditions. Animals were fed a diet of frozen smelt twice weekly but were starved for 48 hours prior to experiments.

Animals were held in indoor holding tanks in a medium of artificial seawater made up of a solution of commercially available seasalt (Instant Ocean^{TM}) in distilled water or natural seawater from Burrard inlet (English Bay). Natural day night cycles were maintained in the animal holding room but incident light levels were reduced. Seawater was re-circulated through two holding tanks and a biological filtering tank containing crushed coral, bacterial supplement (Cycle^{TM}) and bio-balls to regulated pH and nitrite levels. Salinity, pH and nitrite levels were checked daily with a refractometer, pH meter (corning pH/ion meter 450) and nitrite test kit (TetraTest ^{TM} NO_2 test kit). Salinity was relatively constant between 27-30, pH values were between 7.18 –7.98 and nitrite levels were maintained at < 0.1 mg/L. Half the total seawater volume was changed twice weekly. Water temperature was maintained at 9 ± 1° C by two cooling units. Adequate aeration was achieved with airstones connected to standard fish tank air pumps drawing on room air placed in each of the holding tanks.

2.2 Animal Preparation

2.2.1 Hemolymph sampling

A small hole was drilled through the carapace (not piercing the hypodermis) on the right lateral margin of the pericardial sinus. The area was sealed with dental dam to prevent any excessive bleeding that may occur during hemolymph sampling.

2.2.2 Anterior aorta ligation

Ligation of the anterior aorta, one of the 7 arteries leaving the decapod heart, has been proposed as a means of manipulation circulating CHH concentrations. Flow from this artery supplies the eyestalks, specifically, the sinus glands, antennules and supraoesophageal ganglion (Sandeman, 1967). At rest, flow through this artery is minimal, 0.5% of total cardiac output, and eyestalk structures may receive supplementary hemolymph from branches of the anterolateral arteries. During emersion, flow through the major arteries decreases except for the anterior aorta, where flow increases to 7% of the total cardiac output (Airriess and McMahon, 1996). Airriess and McMahon (1996) proposed that flow through this artery is necessary for secretion and/or distribution of CHH during emersion stress.

By eliminating flow, the observed increase in circulating CHH concentration during emersion (Webster, 1996; Chang et al., 1998) could be annulled, and the role of CHH in carbohydrate regulation investigated by virtue of its absence.

Ligation surgery was performed a minimum of 24 hours prior to experimentation. Acclimated animals were cold anaesthetized at - 20° C for 20 minutes prior to commencing the procedure. Anesthetized animals were restrained with plastic ties (affixed to solid surface) placed over the left and right sides of the body including the chelae. A 2 x 1 cm portion of the carapace above the posterior margin of the pericardial sinus was removed leaving the hypodermis exposed. Position and flow through the anterior aorta was confirmed with a pulsed-Doppler flowmeter. A piezoelectric crystal electrode was placed at a 45° angle to the artery with the range initially set to 3 mm. An ultrasonic burst generated by the piezoelectric crystal (Titronics Medical Instruments, Iowa city, IA) was directed into the lumen of the artery. Large hemolymph-borne proteins and hemocytes reflect the signal back to the crystal where the echo is amplified and emitted through a speaker on the pulsed-Doppler flowmeter (University of Iowa Bioengineering).

Once the artery had been located a sterile needle and thread were drawn under artery and tied off superior to the hypodermis. Successful ligation was confirmed by repositioning the electrode. If, after careful placement and minor adjustment, no pulsing noise was heard the ligation was determined to be successful. Often the process needed to be repeated a second time.

The hole in the carapace was sealed with dental dam glued in place using a fast drying adhesive (Krazy Glue ®). The animals were returned to individual holding chambers and returned to the main holding tank within 40 minutes of completing the surgery. All animals survived for more than 2 weeks.

2.3 *Cancer magister* saline

Cancer magister saline was prepared according to Morris and McMahon (1989). The composition of saline was 468 mM NaCl, 11mM $CaCl_2 \cdot 2H_2O$, 9 mM KCl, 13 $MgSO_4 \cdot 7H_2O$, 9mM $MgCl_2 \cdot 6H_2O$ and 2 mM $NaHCO_3$ and pH adjusted to 7.6. For experimental procedures requiring saline containing glucose, D-Glucose was added to achieve a final concentration of 0.4 mM immediately prior to use. Saline solutions were not kept longer than 3 days and were stored at 4° C when not being used.

2.4 Eyestalk extract preparation

Eyestalks were isolated from crabs by first cold anaesthetizing them for 30 min at -20° C. Eyestalks were removed by cutting through the arthrodial membrane at the base of each eyestalk using clean dissecting scissors. The isolated eyestalks, exoskeleton intact, were frozen in liquid nitrogen and

stored at - 70° C. Frozen eyestalk were thawed briefly on ice and the exoskeleton removed with dissecting instruments. Soft tissue was gently teased away from the shell and kept as intact as possible. The photoreceptive portion of the eyestalk (most distal) was removed and the remaining tissue placed in a chilled microcentrifuge tube containing 50 µl of *C. magister* saline per eyestalk. Tissue was homogenized using a mini-pestle and centrifuged at 15 000 rpm (Hettich Zentrifugen) for 10 minutes at 2° C. The supernatant was collected in a pre-chilled microcentrifuge tube and the homogenate re-extracted as before. The combined supernatants were then centrifuged for 10 minutes at 15 000 rpm at 2° C. The final supernatant containing the eyestalk extract (ESE) was aliquoted into cold microcentrifuge tubes and stored at -20° C.

2.5 Glucose quantification

Hemolymph samples for glucose determination were taken via the arthrodial membrane at the base of one of the pereiopods or from the pericardial sinus using chilled 1 ml syringes and 27 gauge needles (Becton Dickinson). Samples, between 100 and 200 µl in volume, were stored in pre-chilled 1.7 ml microcentrifuge tubes on ice prior to processing, generally less than 15 minutes. Samples were centrifuged for 10 minutes at 13 000 rpm (VSMC-13 mini-centrifuge, Shelton) to precipitate blood clots, cells and other heavy material.

Glucose was assayed according to a glucose test kit (Sigma). A 50 µl sample of centrifuged hemolymph was added to 950 µl of assay reagent (oxidase/peroxidase/dye) and incubated at 37° C for 30 minutes in a dry bath incubator. The formation of end product, oxidized o-Dianisidine (brown), was measured at 450 nm using a spectrophotometer (Ultrospec ® 3000, Pharmacia Biotech). The coupled enzymatic reaction is shown below.

$$\text{Glucose} + 2H_2O + O_2 \xrightarrow{\text{glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{o-Dianisidine} \xrightarrow{\text{peroxidase}} \text{oxidized o-Dianisidine}$$

(colourless) (brown)

Absorbance at 450 nm is directly proportional to the glucose concentration in the sample. Glucose standards spanning the expected range were prepared by serial dilution of a stock glucose solution (5.56 mmol/L) and used to generate a standard curve.

2.6 Tissue samples

Animals to be used for tissue sampling were sacrificed by chilling at -20° C for 1.5-2 hours. Subjects were removed from the freezer and a portion of the carapace removed with a drill (Foredom Electric Co. Inc). Using sterile dissecting instruments tissue samples of heart, gill, hepatopancreas and muscle tissue were taken. For sampling; the entire heart, approximately 5 g of hepatopancreas tissue from the antero-lateral lobe, sections of the 5^{th} to 7^{th} gills, and muscle tissue from the meropodite of the 4^{th} or 5^{th} pereiopod were removed and blotted dry of hemolymph on filter paper. Samples were transferred to 15 ml polypropylene centrifuge tubes and placed in a dewar containing liquid nitrogen. Once fully frozen, samples were placed in a -70° C freezer for storage.

2.7 Glycogen and free glucose quantification

The protocol for glycogen determination was modified from that of Keppler and Decker (1974) and Schmitt and Santos (1993). Frozen tissues were thawed, 400 mg samples weighed and placed in a 8.5 ml centrifugation tube containing 2 ml of a perchloric acid solution (0.6 M). Samples were homogenized using a tissue homogenizer (Tissue Tearor™, Biospec Products), placed in a boiling water (85° C) bath for 5 minutes and cooled on ice. A 200 µl sample of the homogenate was removed to new 8.5 ml tube and neutralized with 100 – 150 µl of a 1 M $KHCO_3$ solution. The neutrality of random samples was tested with pH paper (color pHast® Indicator Strips, EM Science).
Glycogen in the samples was digested to D-glucose by the addition of 2 ml of amyloglucosidase solution -1mg/ml *Asperigillus niger* amyloglucosidase (SIGMA) and acetate buffer (0.2 M, pH 4.8). Samples were incubated at 55° C for 2.5 hours in a water bath (Immersion Circulator, VWR). After digestion was completed the reaction was stopped by the addition of 500 µl perchloric acid solution (0.6 M) and stored on ice. Chilled samples were centrifugation for 10 min at 13 000 rpm at 5° C (RC5C, Sorvall) to precipitated remaining tissue particles. 50 µl of the supernatant was assayed for glucose content according to section 2.5. Duplicate samples for each tissue were taken.
A second set of tissue samples were incubated with acetate buffer without amyloglucosidase to calculate free glucose. A set of 6 glycogen standards, spanning the range from 0 to 0.25 mg, was prepared from type III rabbit liver glycogen (Sigma) dissolved in distilled water and processed as described above. Calculations for glycogen content are given below.

$$G= ((A-N)*(S/O)*V)/(100*W)$$

G = glycogen (mg/g wet weight tissue)
A = glucose in tissue (mg/100ml) corrected by dilution factor
N = free glucose in tissue (mg/100ml) corrected by dilution factor
S = theoretical glucose concentration of glycogen standard (mg/100ml)

O = observed glucose concentration of glycogen standard (mg/100ml)
V = volume of homogenate (ml)

W = weight of tissue (g)
Note: free glucose was calculated as above but omitting A, S and O

free glucose=N*V/100*W

2.8 Data analysis

Because of heterogeneous covariance matrices, it was not appropriate to analyze hemolymph glucose data using between/within repeated measures ANOVA. These data were analyzed with t-tests, using a Bonferroni adjustment to control the type I error rate. Hemolymph glucose data at individual time points were analyzed with one-way ANOVAs or t-tests, as suitable. Glycogen data were compared by one way ANOVA with Dunett's controlled comparisons or t-tests. *In vitro* data was analyzed by t-test or one way ANOVA at specified time points. Significant difference was taken as a p value less than 0.05. Data are shown as mean ± 1 standard error of the mean (SEM) unless otherwise stated. n refers to the sample size.

Chapter Three: Characterization of the Carbohydrate Regulatory Role of Eyestalk Factors Under Control Conditions

3.1 Introduction

To date, investigations of the carbohydrate regulatory role of CHH have focused primarily on hemolymph glucose regulation. These studies have identified a "classic" hyperglycemic response to ESE and/or purified CHH injection which is dose dependent, has a rapid onset and decline and generally peaks after 2 hours with maximum increases in glucose titers 2- to 4-fold above resting levels (Abramowitz et al., 1944; Keller and Andrew, 1973; Keller et al., 1985; Santos and Keller, 1993b). Data on target tissues of CHH in its role in eliciting hyperglycemia comes from studies of glycogen mobilization and changes in enzyme activity levels. Several target tissues have been identified but much of the data is inconsistent and, in some cases, equivocal (Loret, 1993). Results pertaining to the effects of CHH on enzyme activity are more cohesive and support an inhibitory role for the hormone on glycogen synthase and a stimulatory role on glycogen phosphorylase activity (Ramamurthi et al., 1968; Sedlmeier and Keller, 1981; Keller and Sedlmeier, 1988).

Data from investigations of the carbohydrate regulatory role of CHH in *Cancer magister* are limited to the effects of ESE injection and eyestalk ablation on glycogen synthase and glycogen phosphorylase activity and changes in carbohydrate stores of hepatopancreas and muscle tissues (Wang and Scheer, 1961; Ramamurthi et al., 1968). The current investigation reports on the occurrence of eyestalk factor(s) with hyperglycemic activity in this species and specifically, characteristics of the hyperglycemic response, the identity of potential target tissues, and the mode of action of the hyperglycemic factor(s).

3.2 Experimental Protocol

3.2.1 Hyperglycemic response

Subjects were removed from the main holding tank 1 ½ hours prior to the commencement of experimental procedures and placed in individual holding chamber supplied with flowing seawater (1L/min, 9 ± 1° C, aerated with individual airstones connected to an aquarium pump). Chambers were shielded on three sides with black plastic to minimize incident light and visual disturbances. Trials were conducted during daylight hours to minimize the effect of diurnal rhythms in hemolymph glucose titers.

Crabs (mean 662 ± 12 g) were injected with saline, 0.1, 0.5 or 1.0 ESE equivalents (diluted in *C. magister* saline to a final volume of 100 µl). Solutions were injections via the arthrodial membrane at the base of the 5th walking leg were using a cold 1 ml syringe and 27 gauge needle (Becton Dickinson). Immediately prior to injection the first hemolymph sample (ca. 200 µl) was taken from the pericardial sinus. Hemolymph samples were taken from each subject hourly over the next 5 hours and a final sample taken 7.75 hours post-injection. Animals injected with 0.1 ESE equivalents were sampled for the first 4 hours following injection. Hemolymph glucose samples were kept on ice for less than 15 minutes prior to processing. Glucose quantification was completed as described in Chapter 2 section 2.5.

3.2.2 Glycogen quantification

Glycogen content for heart, gill, hepatopancreas and muscle tissue of non-ligated, anterior aorta ligated and eyestalk extract injected crabs (mean weight 699 ± 10 g) was determined according to a procedure modified from Keppler and Decker (1974) and Schmitt and Santos (1993) as described in Chapter 2 section 2.7. Control crabs and 1-week post-operative anterior aorta ligated crabs were removed from the holding tank and placed in the a -20° C freezer. Eyestalk extract injected crabs were placed in holding chambers and the procedure used for hyperglycemia assays followed. Three hours after receiving injections of 0.5 ESE equivalents subjects were transferred to a -20° C freezer. The procedure for tissue sampling and anterior aorta ligation are outlined in Chapter 2 section 2.6 and 2.2.2 respectively.

3.2.3 Muscle tissue bioassay

Muscle tissue for *in vitro* trials was obtained from autotomized legs. Crabs (non-ligated) were encouraged to autotomize the 3rd or 4th walking legs by applying pressure to the meropodite. This procedure was completed in less than 30 seconds. Autotomized legs were placed on ice until dissection at which time the exoskeleton, hypodermis and tendons were removed. Two samples of muscle tissue, 1 g each, were taken from each leg. Tissue was held in aerated *C.magister* saline (0.4 mM D-glucose) at room temperature prior to the start of *in vitro* trials (not more than 20 minutes). Muscle fibers were gently teased apart to increase the exposed surface area.

At the beginning of a trial, tissue samples were blotted dry of excess fluid on filter paper and transferred to 7 ml flat bottomed test tubes containing 4 ml of *C. magister* saline (0.4 mM D-glucose). Immediately prior to the addition of muscle tissue, either 10 µl of saline of 10 µl of an ESE solution (0.1 ESE/100 µl, roughly the same concentration per ml as 0.5 ESE injection into whole animals) and 10 µl anti-foam (Sigma) were added to each test tube. Paired samples were distributed between saline

controls and ESE treatments. Test solutions were aerated with room air via Intramedic® PE-50 tubing connected to an aquarium air pump drawing on room air. Micro-stir bars were placed in all test tubes to ensure thorough mixing.

At 20 minute intervals, starting at time 0 and continued over 240 minutes, 60 µl samples of the incubation solution were taken. Samples were centrifuged for 5 minutes at 13 000 rpm and 50 µl of the supernatant was used for glucose determination as described in Chapter 2 section 2.5.

3.2.4 Data analysis

Due to covariance homogeneity violations, a repeated measures analysis of variance was not suitable for analyzing the hemolymph glucose concentration data. Data were therefore analyzed by Bonferroni modified t-tests. Glycogen data was analyzed by t-tests. *In vitro* data was analyzed by t-test at the final (240 minute) time point and rate of glucose release was determined for the time period between 120 and 160 minutes. The significance level was set at 0.05. Data are presented as means ± 1 SEM unless otherwise indicated.

3.3 Results

Resting hemolymph glucose of anterior aorta ligated crabs (0.37 ± 0.03 mmol/L, n=31) was not significantly different from values for control animals (0.32 ± 0.03 mmol/L, n=41) by t-test (p=0.084).

3.3.1 Hyperglycemic response to ESE injection

ESE injection results are shown in figure 3.1. Injection of 0.1 ESE equivalents (A) did not result in a significant increase in hemolymph glucose. Injection of 0.5 ESE equivalents resulted in a profound and prolonged hyperglycemia. Elevated hemolymph glucose levels were observed two hours post injection as shown in panel B. Control values (0.05 ± 0.01 mmol/L) and ESE injected values (0.15 ± 0.03 mmol/L) were significantly different by Bonferroni modified t-test (p=0.0005). Hemolymph glucose levels remained elevated over the next 3 hours. Maximal hyperglycemia occurred 3 hours post-injection (0.48 ± 0.09 mmol/L) and represented an 8-fold increase from levels of saline injected control animals. After 8 hours, glucose levels had returned to normal. Injections of 1.0 ESE equivalents, shown in panel C, resulted in a hyperglycemia similar to that observed with 0.5 ESE equivalents. Significant hyperglycemia occurred 2 hours post injection (p=0.0009) and levels remained elevated for the following 2 hours. Glucose titers did not decrease significantly, even 8 hours post-injection. As with 0.5 ESE equivalents, maximum circulating glucose concentrations were observed three hours post-injection (0.61 ± 0.11 mmol/L), and represented a 10-fold increase over

controls. Panel D of figure 3.1 illustrates the dose dependency of the hyperglycemia elicited by ESE injections in *C. magister*. The mean value for the maximum increase in hemolymph glucose are plotted for each injection level (0 ESE equivalents =saline).

Removal of circulating eyestalk factors *via* anterior aorta ligation did not dramatically alter the hyperglycemic response of *C. magister*. The results of 0.5 ESE equivalents injection into anterior aorta ligated animals is shown in figure 3.2. As for non-ligated 0.5 ESE equivalents injected crabs, significant elevation of hemolymph glucose levels was observed 2 hours post-injection ($p=0.0059$). Maximum hyperglycemia occurred 4 hours post-injection (0.51 ± 0.09 mmol/L) and represented a 5.5-fold increase from saline injected control values. Hemolymph glucose concentrations of ESE injected anterior aorta ligated animals had decreased significantly 8 hours post-injection. Peak hyperglycemia of non-ligated and ligated animals were not significantly different.

3.3.2 Glycogen and free glucose

The effects of eyestalk extract injection and anterior aorta ligation on carbohydrate reserves in *C. magister* are shown in Table 3.1 and 3.2. Glycogen content of heart, gill, hepatopancreas and muscle tissue are given in Table 3.1. Control animals were non-ligated, submersed and resting. Anterior aorta ligated crabs, operated one week prior, were submersed and resting. 0.5 ESE animals were held, submersed and unrestrained for three hours prior to tissues sampling. It is presumed that peak hyperglycemia represents the time of maximal carbohydrate mobilization, therefore changes in carbohydrate reserve content should be most evident at this time. As determined by the hyperglycemia results, the time of maximum hyperglycemia with injections of 0.5 ESE equivalents occurs 3 hours post-injection (see figure 3.1 panel B). The data are summarized in Table 3.2. Anterior aorta ligation resulted in increases in glycogen content of heart, hepatopancreas and muscle tissue. Injection of 0.5 ESE equivalents did not alter glycogen content of any of the four tissues examined.

3.3.3 Muscle tissue bioassay

Treatment of isolated muscle tissue with eyestalk extract had no effect on glucose release. Tissues were incubated in *Cancer magister* saline under room air conditions with or without eyestalk extract. The results are shown in figure 3.3. The observed increases in glucose content of the incubation medium were not significantly different for ESE treated tissue, 0.38 ± 0.06 mmol/L, compared to

Table 3.1. Glycogen content of heart, gill, hepatopancreas and muscle tissue of non-ligated resting (control), anterior aorta ligated (ligated) and 0.5 ESE equivalents injected (0.5 ESE) *C. magister* (see text for details). Data are presented as means ± one SEM. Control n=11, ligated n=6 and n=9 for 0.5 ESE.

Glycogen content (mg/g wet weight)				
	Heart	**Gill**	**Hepato-pancreas**	**Muscle**
Control	8.0 ± 1.5	4.2 ± 1.2	3.0 ± 0.7	2.0 ± 0.5
Ligated	13.5 ± 0.8	3.4 ± 1.2	7.0 ± 2.1	4.4 ± 0.6
0.5 ESE	12 ± 2.0	3.0 ± 0.7	4.6 ± 0.9	2.7 ± 0.4

Table 3.2. Change in glycogen content of heart, gill, hepatopancreas and muscle tissue of anterior aorta ligated (ligated) and 0.5 ESE equivalents injected (0.5 ESE) *C. magister* compared to control values. Arrows indicate significant difference and the direction of change (up=increase, down=decrease). Dashes indicate no significant difference.

Change in glycogen content compared to control values				
	Heart	**Gill**	**Hepato-pancreas**	**Muscle**
Ligated	↑	–	↑	↑
0.5 ESE	–	–	–	–

controls, 0.24 ± mmol/L, after 240 minutes (p=0.14). The rates of glucose release, calculated for 120 to 160 minutes, did not differ between controls and ESE treated tissue.

3.4 Discussion

3.4.1 Hemolymph glucose

Although a reduction in resting hemolymph glucose concentration has been reported for the lobster, *Panulirus japonicus* (Scheer and Scheer, 1951), and the prawn, *Metapenaeus monoceros* (Rangneker and Madhyastha, 1971), in response to eyestalk ablation, this does not appear to be a generalized response of decapods. The brachyurans *Callinectes sapidus*, *Libinia emarginata* and *Chasmagnathus granulata* do not exhibit ablation hypoglycemia (Abramowitz et al., 1944; Kleinholz and Little, 1949; Santos and Colares, 1986). The results for anterior aorta ligated *Cancer magister* reported here are in agreement with those of eyestalk ablated brachyurans. These animals do not exhibit a significant reduction in circulating glucose concentrations.

Despite differences in time course, amount of ESE required and extent of increases in circulating glucose as compared with the "classic" hyperglycemia described by Keller et al. (1985), the response of *Cancer magister* to crude eyestalk extract injection was not dissimilar from that reported for other decapods. Much like the lobsters *Homarus americanus* (Soyez et al., 1990) and *Nephrops norvegicus* (Smullen and Bentley, 1994), the onset of significance and time of peak hyperglycemia were delayed for *C. magister*. Peak hyperglycemia did not occur until 3 and 5 hours post-injection for *H. americanus* and *N. norvegicus*, respectively. The initial onset of hyperglycemia was also delayed in these species (Soyez et al., 1990; Smullen and Bentley, 1994). Although, the literature indicates that CHH is a highly potent hormone, and able to elicit hyperglycemia by injection of as little as 0.001 ESE equivalents (Abramowitz et al., 1944), this was not observed for *C. magister*. Injection of 0.01 ESE equivalents were insufficient to elevate hemolymph glucose levels. Additionally, the extent of hyperglycemia observed, 8- to 10-fold, for *C. magister* was greater than described in the "classic" response. Increases of similar, or greater magnitude, were observed for the crayfishes, *Procambarus Clarkii* (Huberman et al., 1995) and *Orconectes limosus* (Sedlmeier, 1985) injected with purified CHH.

Lin et al. (1998) proposed that some of the observed variability in the hyperglycemic response may, in part, be due to the different temperatures at which experiments are conducted. Another potential contributing factor is the size of experimental subjects. Many studies are conducted with very small, less than 50 g, subjects while others, such as the results presented here, employ much larger, more than 500 g, animals. Both of these parameters are important in determining metabolic rate and, therefore, rates of carbohydrate mobilization and consumption.

Results for anterior aorta ligated *Cancer magister* indicate that ligation surgery does not impede the hyperglycemic response to crude eyestalk extract injection and that operated animals are otherwise healthy. Ligated animals respond to ESE injection with a prolonged and pronounced hyperglycemia similar to that observed for non-ligated animals.

3.4.2 Glycogen and free glucose

Glycogen content of gill, hepatopancreas and muscle tissues obtained for intact resting *Cancer magister* are in agreement with values reported in the literature. Data for glycogen content of heart tissue reported by Keller and Andrew (1973), the only available measurements, are inconsistent with the litarue and were not used for comparative purposes. Glycogen content of the hepatopancreas from several decapods, including *C. magister,* have been reported and were in the range of 3 to 18 mg/g wet weight (Meenakshi and Scheer, 1961; Winget et al., 1977; Rao et al., 1991; Schmitt and Santos, 1993). Glycogen content of this tissue is more commonly in the lower portion of this range. Gill and muscle glycogen measurements indicate content of between 1 to 5 and 1 to 1.5 mg/g wet weight, respectively (Rao et al., 1991; Schmitt and Santos, 1993).

Since anterior aorta ligation may be functionally analogous to eyestalk ablation in terms of removing circulating eyestalk factors, changes in carbohydrate stores should be similar under these two conditions. In fact, results obtained here for *C. magister* do not agree with those previously reported for eyestalk ablated animals of this species. Ramamurthi et al. (1968) reported no change in glycogen content of hepatopancreas or muscle tissue. The data obtained here indicates that ligation of the anterior aorta causes an increase in carbohydrate stores in both tissues. Nevertheless, increases in these tissues have been reported for other eyestalk ablated decapods including astacideans and brachyurans (Schwabe, 1952; Rangneker and Madhyastha, 1971; Reddy, 1990). Interestingly, the opposite, a reduction in glycogen content, has also been reported for hepatopancreas tissue (Rangneker and Madhyastha, 1971). Comparative data for glycogen content of heart and gill tissues of eyestalk ablated decapods is unavailable.

The results obtained for changes in glycogen content of *C. magister* tissues indicate that carbohydrate stores are not altered in response to ESE injection. While this result is in agreement with those of Ramamurthi et al. (1968) for ESE injection of the same species, results for other decapod crustaceans differ. Decreases in glycogen content of gill and muscle tissue in response to ESE injection has been reported for brachyurans (Keller and Andrew, 1973; Nery et al., 1993). However, in muscle tissue, the reduction is dependent on the source of the injected hormone. Purified CHH from a species of the same infraorder decrease glycogen content whereas purified CHH from a species of a different

infraorder increases glycogen content (Nery et al., 1993). Hepatopancreas glycogen content has not been observed to change in response to ESE injection (Keller and Andrew, 1973). Comparative data is unavailable for glycogen content of heart tissue.

Potential reasons for these differences in changes to carbohydrate stores are many. Eyestalk ablation has been cited as representing a severe stress to animals (Keller et al., 1985), therefore, the resulting changes, or absence thereof, could be unrelated to the removal of eyestalk factors. Anterior aorta ligation may represent a less severe stress, as it does not interfere with non-sinus gland circulation including visual reception (Sandeman, 1967). Consequently, changes in response to ligation may more accurately reflect the absence of circulating eyestalk factors, specifically CHH. More importantly, a variety of factors affect tissue glycogen. These include: diet, season, molt stage; temperature and experimental conditions (Reddy et al., 1981; Winget et al., 1977; Kucharski and Da Silva, 1991a; Kucharski and Da Silva, 1991b; Vinagre and Da Silva, 1992). Therefore, differences in these variables may account for some degree of the observed differences in glycogen content As well, some of the species specificity may also be attributable to the different habitats (terrestrial vs. aquatic) occupied by the diverse group of decapods used as experimental animals.

3.4.3 Muscle Tissue Bioassay

Telford (1975) reported that *in vitro* incubated muscle tissue from *Cambarus robustus* treated with ESE released significantly more glucose than control tissues. Santos et al. (1988) reported a decrease in glucose uptake by isolated muscle tissue of *Chasmagnathus granulata* in the presence of eyestalk extract. The results reported here for *in vitro* incubated muscle tissue of *C. magister* indicate that crude eyestalk extract is not effective at altering glucose release/uptake. Both prior studies report that inclusion of glucose in the incubation medium is important to the affect of ESE on glucose uptake/release. The experimental procedure used in this study included glucose in the incubation medium and this inclusion could account for the lack of significant effect of eyestalk extract. Nevertheless, it is also possible that, in *C. magister*, ESE is not effective at altering glucose release/uptake of muscle tissue.

3.4.4 Summary

Cancer magister responded to crude eyestalk extract injection with a hyperglycemic response similar to that described for other decapods. Anterior aorta ligation does not affect resting hemolymph glucose levels or the ability of crabs to respond to ESE injection with hyperglycemia. Anterior aorta ligation affects glycogen reserves in heart, hepatopancreas and muscle tissues. These tissues may, therefore, represent targets of eyestalk factors under control conditions. Injection of eyestalk extract

does not alter glycogen content of heart, gills, hepatopancreas or muscle tissues. Treatment of isolated muscle tissue with ESE does not affect glucose release/uptake and indicates that eyestalk factors are unlikely to function in the manner proposed for CHH in eliciting hyperglycemia.

Chapter Four: Characterization of the Carbohydrate Regulatory Role of Eyestalk Factors Under Emersion Stress Conditions

4.1 Introduction

During exposure to ambient oxygen tension below P_{crit} O_2, energy production by anaerobic metabolism must be initiated to compensate for the shortfall of energy supply from decreasing rates of aerobic respiration (Forgue et al., 1992; Pörtner and Greishaber, 1993). P_{crit} O_2 is defined as the ambient oxygen tension above which animals of a particular species are able to regulate oxygen uptake by physiological mechanisms. These mechanisms include: changes in ventilation, perfusion, cardiac output and oxygen binding by carrier molecules (McMahon, 1988). Below this oxygen tension, these mechanism are insufficient to compensate for reduced ambient oxygen.
During anaerobic metabolism resulting from either environmental hypoxia or functional hypoxia, animals, including crustaceans, utilize glycogen as their primary fuel source (Teal and Carey, 1967; Hochachka, 1980; Zebe, 1982; Gäde, 1983). In contrast to most invertebrates but in common with vertebrates, L-lactate is the only end-product of consequence of anaerobic metabolism in decapod crustaceans (Hochachka and Somero, 1984; Spicer et al., 1990; Hill et al., 1991a). Consequently, accumulation of lactate in the blood or hemolymph is indicative of exposure to ambient oxygen tension below P_{crit} O_2. Because severe hypoxia represents a condition in which carbohydrates are the only fuel of consequence, an investigation of the physiological role of CHH in carbohydrate regulation during emersion stress in aquatic decapod species not exhibiting behavioural or anatomical adaptations to aerial gas exchange could yield new insights into the physiological role of this hormone.

Crabs of the species *Cancer magister* may occasionally be stranded on land by the receding tide (Airriess and McMahon, 1994). Consequently, the longest air exposure these animals are likely to experience is 6 hours, in accord with the tidal cycle. Observations of *C. magister* indicate that emersion represents exposure to ambient oxygen tension below the P_{crit} O_2 of this species. Not only have circulating lactate levels been observed to increase (McDonald, 1977 cited in Airriess and McMahon, 1996), but also, measurements of cardiac function (Airriess and McMahon, 1996), branchial pressure and oxygen uptake (deFur and McMahon, 1984) indicate that physiological function cannot be maintained by theses animals when exposed to air.

The data reported here outlines the changes in hemolymph glucose concentrations of non-ligated and anterior aorta ligated *C. magister* in response to 6 hours of air exposure. Mobilization of

carbohydrate stores in heart, gill, hepatopancreas and muscle tissue of non-ligated and anterior aorta ligated crabs in response to emersion was investigated by measuring changes in glycogen content with the goal of identifying target tissues of hyperglycemic eyestalk factor(s). Finally, the mode of action of this factor(s) was investigated by recording changes in glucose release/uptake by isolated muscle tissue held under anoxic conditions in the presence or absence of ESE.

4.2 Methods

4.2.1 Emersion trials

Anterior aorta ligated and non-ligated animals were transferred to individual holding chambers supplied with flowing seawater (27-30, 9 ± 1° C, 1L/min). Crabs were allowed to acclimatize to the new chambers for 1½ hours. At the start of the experiment hemolymph samples (100-200 µl) were taken from each subject via a hole drilled through the carapace directly above the pericardial sinus. After sampling, seawater inflow for ½ of the 6 holding chambers was disconnected. Seawater supply was maintained for the other tanks which held the control submersed animals. Ambient humidity in the drained chambers remained high as the experiment took place on a flow table and small quantities of water were present in the seams of the plastic holding containers.

Hemolymph glucose samples were taken every hour for 6 hours and processed immediately as described in chapter 2 section 2.5. The 6 hour hemolymph glucose sample was tested for pH with litmus paper (color pHast® Indicator, EM Science). After the final sample was taken animals were either returned to the main holding tank or placed in a - 20° C freezer in preparation for tissue sampling.

4.2.2 Glycogen and free glucose quantification

In order to determine if glycogen content in the heart, gills, hepatopancreas or muscle tissue changed during emersion tissue samples were taken at the end of a 6 hour emersion/submersion trial period. Animals were placed in a - 20° C freezer for preparation for tissue sampling and for glycogen content quantification as described in chapter 2 section 2.6 and 2.7, respectively.

4.2.3 Muscle tissue bioassay

Muscle tissue for *in vitro* trials was obtained from autotomized legs. Crabs (non-ligated) were encouraging to autotomize the 3rd or 4th walking legs by applying pressure to the meropodite. This procedure was completed in less than 30 seconds. Autotomized legs were placed on ice until dissection at which time the exoskeleton, hypodermis and tendons were removed. Two samples of muscle tissue, 1 g each, were taken from each leg. Tissue was held in aerated *C.magister* saline (0.4

mM D-glucose) at room temperature prior to the start of *in vitro* trials, not more than 20 minutes. Muscle fibers were gently teased apart to increase the exposed surface area.

At the beginning of a trial tissue samples were blotted dry of excess fluid on filter paper and transferred to 7 ml flat bottomed test tubes containing 4 ml of *C. magister* saline (0.4 mM D-glucose). Immediately prior to the addition of muscle tissue, either 10 µl of saline of 10 µl of an ESE solution (0.1 ESE/100 µl, for a final concentration roughly the same per ml as 0.5 ESE injection into whole animals) and 10 µl anti-foam (Sigma) were added to each test tube. Paired samples were distributed between saline controls and ESE treatments. Test solutions were bubbled with a CO_2/N_2 mixture (0.2% CO_2 balance N_2, Praxair) via Intramedic® PE-50 tubing. Micro-stir bars were placed in all test tubes to ensure thorough mixing.

At 20 minute intervals, starting at time 0 and continued over 240 minutes, 60 µl samples of the incubation solution were taken. Samples were centrifuged for 5 minutes at 13 000 rpm and 50 µl of the supernatant was used for glucose determination as described in chapter 2 section 2.5.

4.2.4 Data analysis

Hemolymph glucose measurements for non-ligated animals were analyzed with a between-within repeated measures analysis of variance with simple effects post-hoc analysis. Data for ligated animals violated the assumption of homogeneity of covariance and were analyzed with Bonferroni modified t-tests. Glycogen data was analyzed with t-tests or, if group variances where not homogeneous, with Welch's F-test. *In vitro* data were analyzed by t-test at the final, 240 minute, time point. The significance level was set at 0.05. Data are presented as means ± 1 SEM unless otherwise indicated.

4.3 Results

4.3.1 Non-ligated animals

The hyperglycemic response of non-ligated *C. magister* during 6 hours of emersion is shown in figure 4.1. Hemolymph glucose concentrations of emersed crabs had increased significantly, 0.68 ± 0.12 mmol/L, compared to submersed controls, 0.08 ± 0.14 mmol/L, 2 hours after the onset of emersion. Hemolymph glucose concentrations of emersed crabs continued to increase over the remaining four hours of the trial and were more than 7-fold higher than values for submersed animals at this time. Additionally, emersed crabs exhibited a decrease in hemolymph pH at the end of the trial period, from resting values, estimated at 8.0, to 6 hour emersed values, estimated at 7.6.

Glycogen content of heart, gill, hepatopancreas and muscle tissue of 6 hour submersed (control) and 6 hour emersed (stressed) non-ligated *C. magister* are given in table 4.1. A significant decrease in glycogen content of heart tissue occurred in emersed animals compared to values for submersed control animals (see table 4.5). Changes in glycogen content of gill, hepatopancreas and muscle tissue were non-significant. Free glucose content of these four tissues under the same conditions are given in table 4.2. An increase in hepatopancreas free glucose content was the only significant change recorded between submersed and emersed crabs (see table 4.6).

The results of *in vitro* trials designed to mimic *in vivo* submersed and emersed conditions for non-ligated *C. magister* are shown in figure 4.2. Isolated muscle tissue was incubated under normoxic (room air) conditions without ESE in the saline incubation medium (normoxic) or, under anoxic (0.2% CO_2, 99.8% N_2) conditions with ESE included in the incubation medium (anoxic ESE), representative of submersed and emersed conditions, respectively. Sufficient eyestalk extract was added to the incubation medium to give a per ml concentration equivalent to the per ml hemolymph titer resulting from injections of 0.5 ESE. After 240 minutes of incubation "submersed" muscle tissue had released 0.24 ±0.06 mmol/L glucose into the incubation medium, whereas "emersed" muscle tissue released 0.33 ± 0.12 mmol/L. These values were not significantly different by t-test, p=0.46. T-test analysis of the rates of glucose release, calculated for the time between 120 and 160 minutes, indicates that the rates do not differ between normoxic and anoxic ESE groups.

4.3.2 Anterior aorta ligated animals

Hemolymph glucose concentrations of emersed anterior aorta ligated *C. magister* were not significantly different from values of submersed control animals at any time during the 6 hour trial. Results are shown in figure 4.3. At the end of 6 hours, hemolymph glucose concentrations of emersed animals had increased by 0.86 ± 0.26 mmol/L from resting values and were not significantly different from values for 6 hour submersed animals, 0.18 ±0.13 mmol/L, p=0.08. As was the case for non-ligated *C. magister*, emersed ligated crabs experienced hemolymph acidosis. Submersed values were estimated at 8.0 whereas values after 6 hours of emersion were estimated at 7.7.

Glycogen content of heart, gill, hepatopancreas and muscle tissue of submersed (control) and emersed (stressed) anterior aorta ligated *C. magister* is given in table 4.3. Carbohydrate stores of all tissues, except gill, decreased in 6 hour emersed ligated crab compared to tissues of submersed animals (see table 4.5). Free glucose content of these four tissues under the same conditions for anterior aorta ligated crabs is given in table 4.4. Glucose content was not significantly different for any of the

Table 4.1. Glycogen content of heart, gill, hepatopancreas and muscle tissue of non-ligated *C. magister* after 6 hours submersion (control) or emersion (stressed). Data are presented as means ± one SEM. Control values n=11, stressed values n=6.

Glycogen content (mg/g tissue wet weight)				
	Heart	**Gill**	**Hepato-pancreas**	**Muscle**
Control	8.0 ± 1.5	4.2 ± 1.2	3.0 ± 0.7	2.0 ± 0.5
Stressed	2.0 ± 0.4	7.5 ± 2.1	6.1 ± 1.5	1.8 ± 0.5

Table 4.2. Free glucose content of heart, gill, hepatopancreas and muscle tissue of non-ligated *C. magister* after 6 hours of submersion (control) or emersion (stressed). Data are presented as means ± one SEM. Control values n=11, stressed values n=6.

Free glucose (mg/g tissue wet weight)				
	Heart	**Gill**	**Hepato-pancreas**	**Muscle**
Control	0.4 ± 0.2	0.5 ± 0.2	0.3 ± 0.1	0.4 ± 0.1
Stressed	0.2 ± 0.1	1.0 ± 0.5	0.7 ± 0.1	0.3 ± 0.1

Table 4.3. Glycogen content of heart, gill, hepatopancreas and muscle tissue of anterior aorta ligated *C. magister* after 6 hours of submersion (control) or emersion (stressed). Data are presented as means ± one SEM. Control values n=11, stressed values n=6.

Glycogen content (mg/g tissue wet weight)				
	Heart	**Gill**	**Hepato-pancreas**	**Muscle**
Control	13.5 ± 0.8	3.4 ± 1.2	7.0 ± 2.1	4.4 ± 0.6
Stressed	3.6 ± 1.2	0.8 ± 0.4	1.8 ± 0.8	2.1 ± 0.6

Table 4.4. Free glucose content of heart, gill, hepatopancreas and muscle tissue of anterior aorta ligated *C. magister* after 6 hours of submersion (control) or emersion (stressed). Data are presented as means ± one SEM. Control values n=11, stressed values n=6.

Free glucose (mg/g tissue wet weight)				
	Heart	**Gill**	**Hepato-pancreas**	**Muscle**
Control	0.2 ± 0.1	0.8 ± 0.6	0.3 ± 0.1	0.2 ± 0.1
Stressed	0.6 ± 0.3	0.7 ± 0.4	0.6 ± 0.2	0.3 ± 0.1

tissues examined between submersed and emersed animals.

Results of *in vitro* experiments designed to mimic submersed and emersed conditions for anterior aorta ligated *C. magister* are shown in figure 4.4. "Submersed" isolated muscle tissue was incubated in normoxic saline without eyestalk extract (normoxic). "Emersed" muscle tissue was incubated in anoxic (0.2% CO_2, 99.8% N_2 bubbled) saline without eyestalk extract (anoxic). After 240 minutes of incubation "submersed" tissue had released 0.33 ± 0.12 mmol/L glucose into the medium, whereas "emersed" tissue had released 0.48 ±0.16 mmol/L glucose into the medium. The amount of glucose released and rate of release between 120 and 160 minutes, were not significantly different between the two groups.

4.3.3 Comparison of results for non-ligated and anterior aorta ligated animals

Ligation of the anterior aorta may eliminate circulating eyestalk factors, therefore, a hyperglycemic response to emersion stress is expected for non-ligated animals but not for ligated animals. The results for *C. magister* are shown in figure 4.5. After 6 hours of emersion, hemolymph glucose concentrations had increased significantly in non-ligated *C. magister* compared to submersed animals. Changes in hemolymph glucose content of ligated crabs were not significant. After 6 hours of emersion, circulating glucose concentration of non-ligated crabs had increase by 1.84 ± 0.28 mmol/L, whereas a significantly smaller increase, 0.86 ±0.26 mmol/L, was observed for ligated animals, $p=0.03$.

Table 4.5 summarizes the changes in glycogen content of the four tissues analyzed during 6 hours of emersion, for both non-ligated and ligated *C. magister,* compared to submersed control values. Animals with circulating eyestalk factors (non-ligated) exhibited a reduction in heart carbohydrate stores. Animals without circulating eyestalk factors (ligated), exhibited significant reductions in glycogen stores of heart, hepatopancreas and muscle tissue.

A summary comparing changes in free glucose content of heart, gill, hepatopancreas and muscle tissue of non-ligated and ligated crabs during 6 hours of submersion and 6 hours of emersion is given in table 4.6. The only significant change was an increase in hepatopancreas free glucose in emersed non-ligated animals compared to submersed control values.

Table 4.5. Change in glycogen content of heart, gill, hepatopancreas and muscle tissue of control (Non-ligated) and anterior aorta ligated (Ligated) *C. magister* during 6 hours of emersion compared to 6 hour submersed (resting) values. Arrows indicate significant difference and the direction of change. Dashes indicate no significant difference.

Change in glycogen content				
	Heart	**Gill**	**Hepato-pancreas**	**Muscle**
Non-ligated	↓	–	–	–
Ligated	↓	–	↓	↓

Table 4.6. Change in free glucose content of heart, gill, hepatopancreas and muscle tissue of control (Non-ligated) and anterior aorta ligated (Ligated) *C. magister* during 6 hours of emersion compared to 6 hour submersed (resting) values. Arrows indicate significant difference and the direction of change. Dashes indicate no significant difference.

Change in free glucose content compared to control values				
	Heart	**Gill**	**Hepato-pancreas**	**Muscle**
Non-ligated	–	–	↑	–
Ligated	–	–	–	–

4.4 Discussion

4.4.1 Responses of Cancer magister to emersion stress

Based on the number of species in which emersion hyperglycemia has been observed, Keller (1983) proposed that this represents a generalized response of decapod crustaceans to emersion stress. The results obtained here for *C. magister* indicate that this brachyuran species responds to air exposure with the expected hyperglycemia, but only if flow through the anterior aorta is not impeded. Similarly, removal of eyestalks eliminates the hyperglycemic response to emersion in other decapod species (Kleinholz and Little, 1949; Webster, 1996; Chang et al., 1998).

Data addressing changes in carbohydrate stores in response to emersion stress are scarce but available results indicate that, over 6 hours of emersion, glycogen content of gill tissue increases, muscle tissue glycogen decreases and hepatopancreas carbohydrate stores are unaffected in the supra-littoral crab *Chasmagnathus granulata* (Schmitt and Santos, 1993). The results obtained here for gill and muscle tissue of *C. magister* were not in agreement with these results. One reason for the differing results may be due to behavioural adaptations of *C. granulata* facilitating oxygenation of branchial water during air exposure (Santos et al., 1987). Consequently, emersed *C. granulata* may experience less severe hypoxic exposure compared to that experienced by *C. magister*, a species that does not possess behavioural or anatomical adaptations to aerial respiration. Other aspects of emersion stress may be more important in regulating carbohydrate utilization in *C. granulata.* Comparable data for an emersed eyestalk ablated decapod is not available.

Although changes in tissue free glucose content were not expected, the glycogen determination protocol used in the current investigation required measurement of this sugar. Cellular concentrations of glucose are known to be low in crustaceans (Schatzlein et al., 1973; Hill et al., 1991b) as a results of transport *via* facilitative diffusion (Rodnik et al., 1997) which allows for rapid equilibration between cells and the hemolymph. Also, intracellular glucose is rapidly phosphorylated to glucose-6-phosphate (Schatzlein et al., 1973; Santos and Keller, 1993b). The reported glucose content of gill, muscle and hepatopancreas were considerably higher in *C. granulata* (Schmitt and Santos, 1993) but, whole animal measurements from *Carcinus maenas* (Hill et al., 1991b) were in agreement with the values reported here for *C. magister.* Significant changes in free glucose were recorded for hepatopancreas tissue of emersed non-ligated crabs.

Although Telford (1975) had previously reported an increase in glucose release from muscle tissue incubated under anoxic conditions compared to normoxic condition in the crayfish, *Cambarus robustus*, this was not observed in the current investigation of *C. magister* muscle tissue.

4.4.2 Conclusions

The different responses of non-ligated and anterior aorta ligated animals suggest that eyestalk factor(s) may effect carbohydrate regulation by reducing mobilization. Only carbohydrate stores of heart tissue, a highly aerobic organ (Brooks and Storey, 1989; Lallier and Walsh, 1991), were observed to decrease in non-ligated crabs. However, significant reductions in glycogen content of heart, hepatopancreas and muscle tissue were observed in ligated animals without a concomitant increase in hemolymph glucose. Muscle tissue represents the largest percent of body mass in brachyurans (Telford, 1975; Schmitt and Santos, 1993). Therefore, changes in glycogen stores in this tissue constitute notable changes in whole animal carbohydrate content. The absence of hyperglycemia suggests that the mobilized sugar was metabolized and that these animals were lacking a restraining factor present in the eyestalk. Whether or not the presence of this restraining eyestalk factor is characterized by a reduction in overall metabolic rate or the absence of an increase in metabolic rate is not clear, but data for emersed decapods (discussed later) argues for the former situation.

4.4.3 Summary

The results obtained here indicate that *C. magister* respond to emersion with a pronounced hyperglycemia dependent on flow through the anterior aorta. Both non-ligated and ligated animals experience hemolymph acidosis during 6 hours of emersion. Flow through the anterior aorta is also important for regulation of carbohydrate stores of hepatopancreas and muscle tissue. Consequently, glycogen stores in these tissues may represent targets of eyestalk factors during emersion stress. Finally, the release of glucose by isolated muscle tissue is unaffected by anoxic conditions.

Chapter Five: Identification of CHH-like peptides in Eyestalk Extract

5.1 Introduction

Early evidence from disc electrophoresis, gel filtration and ion-exchange chromatography indicated the presence of more than one hyperglycemic peptide per decapod species (Kleinholz and Keller; 1973; Tensen et al., 1991a). Results from reverse phase high performance liquid chromatography (RP-HPLC), amino acid sequencing and molecular cloning have confirmed this finding for a number of species. Although preliminary results for the land crab *Cardisoma carnifex* indicated that 3 CHHs may be present for this species (Newcomb, 1983), generally two forms are observed for brachyuran crabs (Chung and Webster, 1996).

The 11 characterized CHHs have molecular masses between 8.4 and 8.7 kDa as determined by mass spectrometry and nucleotide transcribed peptide molecular weight calculation (Soyez et al., 1990; Kegel et al., 1991; Martin et al., 1993; Huberman et al., 1993; Aguilar et al., 1995; Lin et al., 1998; Marco et al., 1998; Chung et al., 1998).

5.2 Methods

Proteins from a crude eyestalk extract were first separated using a discontinuous Tris-Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system described by Shägger and von Jagow (1987). This form of SDS-PAGE is specialized for separation of small (1-100 kDa) proteins. The separated peptides were transferred electrophoretically to a nitrocellulose membrane (0.2 μm, Biorad) in a mini trans-blot apparatus (Biorad) for 1 hour at 100 V at 4° C. The gel and nitrocellulose membrane along with Whatman papers and fiber pads were soaked in Towbin transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) and assembled as a gel-membrane sandwich. With the gel on the cathode side of the nitrocellulose membrane the gel-membrane sandwich was placed in a tank with cold Towbin buffer and an icebox.

Once the blot transfer was complete the nitrocellulose membrane was removed from the transfer tank and the position of the protein standards marked in preparation for probing with anti-CHH antibodies from *Carcinus maenas* (prepared by R. Keller). After transfer was complete the membrane was blocked for 12-16 hours with 5% (w/v) milk powder in TBS-T (0.1% Tween 20 in Tris buffered saline, pH 7.5) at 4° C. After blocking, the membrane was washed twice in TBS-T at which point it was ready for probing with the first antibody. The blocked membrane was probed for 2 hours at room temperature in a 0.5% milk powder TBS-T solution containing anti-CHH antibodies diluted 1:2000.

The membrane was washed for 15 minutes with TBS-T, then washed twice more for 5 minutes with TBS-T. The blot was probed for 1 hour at room temperature with the second antibody, a horse radish peroxidase labeled anti-rabbit antibody (Amersham), at 1:10000 dilution in 0.5% milk-powder TBS-T. At the end of the incubation the membrane was removed from solution and washed as before followed by two final 5 minute washes in TBS.

The second antibody was visualized by an enhanced chemiluminescence kit (ECL; Amersham). The blot was incubated in a 1:1 mixture of ECL reagents (Amersham) for 1 minute at room temperature. The excess liquid was removed and the blot wrapped in plastic-wrap, then exposed for 5 minutes to Kodak X-OMAT RP XRP-1 film. The protein content of immuno-positive bands was not quantified.

5.3 Results

The results from a western blot of crude eyestalk extract prepared from *Cancer magister* tissue probed with antiserum directed against *Carcinus maenas* CHH are shown in figure 5.1. Several immunopositive bands were identified. In addition to a prominent band present at approximately 7.5 kDa, immunopositive material was apparent in a large band at 16.5 kDa. Smaller amounts of immunopositive material were observed at 15, 28, 35 and 39 kDa. The arrow indicates the position of CHH-like peptide present in *C. magister* ESE. Quantification of CHH by densitometry was not performed due to the lack of control material.

5.4 Discussion

Antiserum directed against CHH from one species is known to be immunoreactive for CHH of another species of the same infraorder (Leuven et al., 1982; Keller et al., 1985; Keller and Sedlmeier, 1988). This is observed for crude ESE of *C. magister* probed with *Carcinus maenas* anti-CHH antibodies, in which western blotting revealed the presence of several immunopositive substances. A prominent band of approximately 7.5 kDa was determined to be the most likely candidate to represent CHH based on size, quantity of material and absence of other immuno-reactive substances within the expected size range for CHH.

Within the sinus gland, CHH is known to represent a large proportion of total protein (Keller et al., 1985). Therefore, crude extracts prepared from this tissue were expected to be enriched for this protein. Mass determination of CHH from gel electrophoresis and column chromatography are known to underestimate the size of CHH peptides (Kegel et al., 1989; Soyez et al., 1990). Consequently, the molecular weight of 7.5 kDa determined here is not likely to be reflective of the true molecular weight of this neuropeptide. Nevertheless, the determined mass is in close approximation to 6.7 kDa, an earlier mass determination of CHH from *C. magister* by Kleinholz and Keller (1973) using column chromatography.

To date, all of the studies of the crustacean hyperglycemic hormone have noted the presence of more than one hyperglycemic peptide within a species for CHH of *C. magister* (Chung and Webster, 1996; Kleinholz and Keller, 1973; Keller et al., 1985). The results obtained here indicate the presence of only one immunoreactive peptide in the expected size range. The putative CHH band identified is not as well defined as desirable and could, therefore, obscure the presence of two peptides of very similar molecular weight. The low ratio of saline to tissue homogenized in the crude eyestalk extract preparation resulted in an extremely elevated protein content (data not shown).

The presence of higher molecular weight immunopositive material in the crude eyestalk extract is not surprising given that CHH is processed from a pre-prohormone (Newcomb, 1987; Weideman et al., 1989; Tensen et al., 1991b; De Kleijn et al., 1994; De Kleijn et al., 1995). The presence of pro-CHH as well as pre-pro-CHH in the crude eyestalk extract prepared here is likely and could account for some of the additional immunopositive bands observed, especially the dark band at 16.5 kDa or the faint band at 15 kDa, both of which approximate the pro-CHH weights of 13.3 kDa and 14 kDa calculated for *C. maenas* (Weideman et al., 1989) and *C. carnifex* (Tensen et al., 1991b), respectively. The CPRP and signal peptide are more variable in content and length than the region coding for CHH (De Kleijn et al., 1994; De Kleijn et al., 1995). Differences in these sequences could account for

larger or smaller pre-pro-CHH and pro-CHH in *C. magister*. Additionally, in some species a large degree of cross reactivity has been observed for other members of the CHH/MIH/GIH family with CHH antibodies (Meusy and Soyez, 1991). This is not surprising in light of the high degree of sequence homology shared by these peptides (Van Herp, 1998; Lacombe et al., 1999). Another source of high molecular weight immunopositive material in the crude eyestalk extract could be the result of CHH associations with other proteins. Kallen et al. (1990) proposed that in the hemolymph CHH is associated with carrier proteins. While this is unlikely for this crude eyestalk extract preparation, the possibility merits consideration.

5.4.1 Summary

The results of western blot analysis of the crude eyestalk extract prepared from isolated *C. magister* eyestalks used in the current investigation has substances which are immunopositive to anti-CHH antibodies from *Carcinus maenas*. A CHH-like peptide of approximately 7.5 kDa was identified.

Chapter Six: Characterization of Nucleotide Sequences Coding for CHH in *Cancer magister*

6.1 Introduction

Many studies have published both complete and partial primary amino acid sequence information for CHH peptides from species belonging to several decapod infraorders and one from a non-decapod species. On the other hand, cDNA sequence information has only recently become available and, in the case of genomic DNA, sequences have only been reported for two species. Both types of nucleotide information have relied on screening of, in the former case, cDNA, and in the latter case, genomic DNA, libraries with CHH specific probes.

Complete cDNA sequence information is available for the brachyuran, *Carcinus maenas* (Gromoll and Weideman, 1990), the astacideans, *Homarus americanus* (De Kleijn et al., 1995) and *Orconectes limosus* (De Kleijn et al., 1994), and the Penaeid (shrimp), *Penaeus japonicus* (Ohira et al., 1997). The sequences exhibit a high degree of homology, over 50%, in the CHH coding region, but have much more variable signal peptide and CPRP coding regions (Soyez, 1997; Van Herp, 1998).

Genomic DNA sequence information is available for CHH from *Carcinus maenas* (Dircksen et al., 2001) and the shrimp, *Metapenaeus ensis* (Gu and Chan, 1998). The results from these studies indicate that CHH is encoded by multiple gene copies and each copy is comprised of several exons and introns. In both species, the first exon codes for a portion of the signal peptide and the second for the remainder of the signal peptide, the CPRP and the first 40 amino acid of the mature CHH peptide. The third exon of *C. maenas* codes for the N-terminal portion of an isoform of CHH which is only found in the pericardial organ (Dircksen et al., 2001). This has not been identified in the shrimp CHH gene. The final exon of both species codes for the N-terminal region of the major CHH isoform (Gu and Chan, 1998; Dircksen et al., 2001). Introns are of variable length. Interestingly, the described gene structure is very similar to that of MIH from the crab, *Charybdis feriatus* (Chan et al., 1998).

Although a number of early studies focused on *Cancer magister*, full amino acid and cDNA sequences have not been characterized. Kleinholz (1975) reported the amino acid composition of CHH for this species, but not the primary sequence. More recently, a small portion of the coding region, between amino acids 30 and 67 of the mature peptide, was sequenced by RT-PCR amplification of reverse transcribed mRNA isolated from frozen eyestalks of *C. magister* (Sharrock, 1999). Due to the lack of information, an investigation of the cDNA and DNA sequences was undertaken. The cDNA coding sequence(s) was investigated by RT-PCR, 3′ and 5′ rapid

amplification of cDNA ends (RACE) and the genomic DNA sequence investigated by PCR amplification. The results of these studies are outlined below.

6.2 Methods

6.2.1 DNA isolation

DNA was isolated from hepatopancreas tissue by a protocol modified from that of Taggart et al. (1992) and Strauss (1997). Fresh or frozen tissue (10-100 mg) was digested overnight (12-18 hours) at 50° C in a microcentrifuge tube containing 120 µl/10 mg tissue digestion buffer (100mM NaCl, 10 mM Tris-Cl pH 8.0, 25 mM EDTA, pH 8.0, 0.5% (w/v) SDS and 0.1 mg/ml proteinase K). The DNA was separated into an aqueous phase by adding an equal volume of 25:24:1 phenol/chloroform/isoamyl alcohol (buffered phenol was prepared according to Ausubel et al. 1997). Samples were thoroughly mixed and centrifuged for 3 minutes at 12 000 *g*. The aqueous layer was removed to a new microcentrifuge tube and re-extracted with an equal volume of 24:1 chloroform/isoamyl alcohol. Samples were centrifuged as before and the aqueous layer removed to a new tube. DNA was precipitated by the addition of a ½ volume of 7.5 M ammonium acetate and 2 volumes of 100% ice cold ethanol. The ethanol supernatant was discarded and the pellet washed with 70% ethanol, allowed to dry, and re-suspended in TE buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA, pH 8.0, and dH2O). The final DNA concentrations and purity were measured spectrophotometrically at 260 nm and 280 nm (Ultraspec 3000, Pharmacia).

6.2.2 RNA Isolation for RT-PCR

Total RNA was isolated from frozen eyestalks using a modified guanidium isothiocyanate silica-gel membrane technique (RNeasy, Qiagen). Eyestalks were thawed for 10 minutes prior to dissection. The exoskeleton and photoreceptive portions were removed using sterile dissecting instruments leaving nervous and muscle tissue. This tissue was ground under liquid nitrogen with a mortar and pestle. Samples (20- 30 mg) were placed into microcentrifuge tubes containing 600 µl guanidium isothiocyanate solution for homogenization by shearing through a 20-guage syringe and 1 ml needle. The resulting lysate was centrifuged for 15 seconds at 10 000 rpm (mini-centrifuge, Shelton) and the pellet discarded. The supernatant along with 70% ethanol was applied to an RNeasy spin column and re-centrifuged. The column was subsequently washed several times before eluting the RNA by centrifugation with DEPC treated water.

6.2.3 cDNA Synthesis

Total RNA (4 µl) was reversed transcribed with 2.5 pmol of primer DP4 (CTT CTT GCC A/GAC CAT CTG T/GAC CT) using 200 U cloned Moloney murine RNase H$^-$ reverse transcriptase (Superscript^{TM} II, Gibco) for 50 minutes at 42° C. The final reaction volume was 20 µl and contained 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 2 mM dNTP, 10 mM DTT and sterile dH_2O. The reaction was terminated by heating to 70° C for 15 minutes and stored at - 20° C.

6.2.4 Primers

Primers were designed for use in cDNA synthesis, 3′ RACE, RT-PCR and PCR. A small section of *Cancer magister* CHH (corresponding to amino acids 31 to 68), had been previously sequenced using degenerate forward and reverse primers, DP3 and DP4 respectively by RT-PCR (Sharrock, 1999). This cDNA sequence information was used to design the forward and reverse gene specific primers, A1F and A1R respectively. Another forward degenerate primer, HSP2, was designed based on a region of high amino acid and nucleotide identity in the CPRP coding region of the pre-prohormone. Sequences for *Carcinus maenas*, *Cancer pagurus*, *Homarus americanus* and *Orconectes limosus* were used in designing this primer (Gromoll and Weideman, 1990; Chung et al, 1998; De Kleijn et al., 1994; De Kleijn et al., 1995). The information from *Cancer pagurus*, a congeneric species to *C. magister*, and *Carcinus maenas* were heavily favoured in the primer design. For primer sequence information and location please see figures 3.1 and 3.2 and table 1.

6.2.5 RT-PCR and PCR

The section of cDNA corresponding to pro-CHH CPRP amino acid 7 to CHH amino acid 75 was amplified with degenerate primers HSP2 and DP4. The section of DNA corresponding to CHH amino acids 32 to 66 was amplified with gene specific primers, A1F and A1R (see figures 3.1 and 3.2 and table 1 for further details). Amplifications were performed as outlined in Table 2. Reactions were carried out in a total volume of 50 µl containing 20mM Tris-HCl (pH 8.4), 50 mM KCl, 0.2 mM dNTP, 1-3 mM $MgCl_2$, 1.4 µM primers, 0.5 –1 µg cDNA or DNA, 2.5 U DNA polymerase (cloned *Thermus aquaticus* Taq, Gibco) and dH_2O. Reactions were covered with 50 µl sterile mineral oil. Amplified products were separated by 1% agarose gel electrophoresis and visualized UV illumination after ethidium bromide (EtBr) incubation.

Figure 6.1. Depiction of mRNA of CHH with putative splice sites marked. SP, signal peptide, CPRP, CHH-precursor-related-peptide, CHH, crustacean hyperglycemic hormone. Arrow directions indicate position of forward and reverse primers. White boxes represent potential splicing sites based of genomic DNA information for CHH from *Metapenaeus ensis* (Gu and Chan, 1998) and *Carcinus maenas* (Dircksen et al., 2001).

Table 6.1 Nucleotide sequences for primers used in cDNA synthesis and amplification reactions.

Primers
Forward Primers
Degenerate primers
DP3 GTG TGT GA(C/G) GA(T/C) TGT TAC ACC CTC TA
HSP2 GGA (C/A)GG ATG GAG (C/A)GG CTT CTT GC
Gene Specific Primer
A1F AAT TCA TAT GTG GCC ACC GCG TG
Reverse Primers
Degenerate Primer
DP4 CTT CTT GCC (A/G)AC CAT CTG (T/G)AC CT
Gene Specific Primer
A1R GGC ATA CTT GTC AAA CTC TTC CA

Table 6.2 RT-PCR and PCR amplification protocol

Denaturation	Amplification	Termination
1.5 min 94° C	30 x 0.75 min 94° C denaturation 0.5 min 55° C or 65° C annealing 1.5 min 72° C elongation	10 min 72° C

6.2.6 Rapid Amplification of cDNA Ends (RACE)

Total RNA was isolated using a 3′ RACE kit (Gibco, BRL) according to the manufacturer's instructions. cDNA template was synthesized from mRNA using SuperScript™ RNase H⁻ reverse transcriptase and an adapter primer (AP; GGC CAC GCG TCG ACT AGT ACT TTT TTT TTT TTT TTT T) directed to match the poly-A tail at the 3′ end of mRNA transcripts. The reaction was performed in a final volume of 10 µl and contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 500 nM AP, 500 µM each dNTP and 1-5 µg RNA. The reaction was allowed to proceed at 42° C for 50 minutes after which time the reaction was terminated by heating to 70° C for 15 minutes. Original mRNA templates were removed by treatment with RNase H for 20 minutes at 37° C.

cDNA was amplified with a gene specific primer, in this case GSP1 (GTG TGT GAC/G GAT/C TGT TAC ACC CTC TA) located between amino acids 22 and 30 along with an anti-sense primer, AUAP (GGC CAC GCG TCG ACT AGT AC), that pairs with the AP of the template cDNA. Control cDNA and control primers were amplified alongside *C. magister* cDNA to test the PCR protocol. The PCR reaction was carried out in a total volume of 50 µl containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 nM GSP1, 200nM AUAP, 200 µM dNTP each, 2 U *Taq* DNA polymerase and 2 µl (~0.4 µg) of cDNA. The cDNA was amplified according to the protocol outlined in table 1 with a Perkin Elmer thermal cycler. Amplified products were analyzed by agarose gel electrophoresis.

Table 6.3 RACE amplification protocol

Denaturation	Amplification	Termination
3 min 94° C	30 x 0.75 min 94° C denaturation 0.5 min 65° C annealing 2 min 72° C elongation	10 min 72° C

6.2.7 Gel electrophoresis

PCR products were separated on a 1% agarose gel using a horizontal electrophoresis system (Easy-cast, Owl Scientific). The gel was prepared from agarose powder (Gibco) dissolved in TBE buffer (0.09 M Tris-borate and 0.002 M EDTA, pH 8.0). Amplification products and base pair ladders (100 bp, and 1 kb both obtained from Gibco) were combined with loading buffer (30% glycerol, 0.25% bromphenol blue in TBE) and electrophoresed at 60-80 V for 1 to 1½ hours (VWR 105 powerpack). Bands were visualized by UV illumination after a 20-30 minute incubation of the gel in dH_2O with 0.5 μg/ml ethidium bromide.

6.2.8 DNA purification

DNA fragments of expected size were excised from the gel and purified according to the Glass MAX® DNA Isolation Spin Cartridge System (Gibco). The excised band was placed in a microcentrifuge tube containing 6 M sodium iodide (NaI) and heated to 50° C until all the agarose had melted. The resulting solution was then applied to the GlassMAX Spin Cartridge, which binds DNA preferentially in the presence of NaI, and centrifuged 13 000 rpm for 20 seconds. Following centrifugation the membrane was washed to remove contaminants and, in the final step, DNA was eluted in sterile dH_2O by centrifugation at 13 000 rpm for 20 seconds.
Purified DNA was quantified spectrophotometrically at 260 and 280 nm (Ultraspec 3000, Pharmacia) and sent with the appropriate primers to the NAPS unit located at the University of British Columbia for dideoxynucleotide sequencing.

6.3 Results

RACE amplification of the 3′ end of *Cancer magister* mRNA did not yield repeatable results. The amplification products of one reaction are shown in figure 6.3. Two products, 600 and 3100 bp in length, can be seen in lane 6. The prominent band at 720 bp seen in lane 3 represents the product of a control cDNA amplification reaction indicates that *Taq* polymerase was functional. The absence of products in lane 4 indicates that contaminants were not present in the amplification reactions. Attempts at increasing the amount of product and successfully re-amplifying products of the same size failed. Similar results were obtained for 5′ RACE. Data not shown.

The product of RT-PCR amplification of *C. magister* cDNA with degenerate primers HSP2 and DP4 is shown in lane 4 of figure 6.4. The band at approximately 360 bp, indicated with an arrow, is within the expected size range based on sequence information for the same region of CHH from other species. Attempts at increasing reaction yield and nucleotide sequencing were unsuccessful.

PCR amplification with gene specific primers of genomic DNA isolated from *C. magister* hepatopancreas tissue yielded two products. Arrows in figure 6.5 indicate the positions, approximately 690 and 790 bp, of these products. These results provide support for the presence of an intron located in the DNA coding sequence corresponding to mature CHH amino acid position 32 to 66 and the presence of two coding genes for this species. Attempts to sequence DNA purified from these bands were unsuccessful.

6.4 Discussion

The results from 3´RACE and 5´RACE were inconclusive. Only very faint bands of inconsistent size were identified in both cases. RT-PCR amplification of a portion of *C. magister* CHH located between the nucleotides corresponding to amino acid 7 of the CPRP and amino acid 75 of CHH resulted in an approximately 360 bp product, according to agarose gel electrophoresis. Sequence information *for C. maenas*, *C. pagurus*, *O. limosus* and *H. americanus* (Weideman et al., 1989; De Kleijn et al., 1994; De Kleijn et al., 1995; Chung et al., 1998) indicates that amplification of the equivalent section should result in a 330 bp product. Confirmation that the observed product was in fact cDNA coding for CHH was attempted by nucleotide sequencing. Although spectrophotometry of the gel amplification product indicated that the isolation step was successful, nucleotide sequencing reactions were unsuccessful.

Amplification of *Cancer magister* genomic DNA with gene specific primers yielded results suggesting the presence of an intron in the coding sequence of CHH. Based on previous cDNA sequencing results for *C. magister*, amplification with primers directed against the nucleotide sequence corresponding to amino acids 32 to 39 (A1F) and 59 to 66 (A1R) of the CHH coding region a product of 102 bp were expected. The results here indicate amplification of 690 and 790 bp products. The reported genomic DNA sequence information indicates the presence of an intron located between the nucleotides coding for amino acids 40 and 41 of the mature peptide (Gu and Chan, 1998; Dircksen et al., 2001). The introns present in the genes coding for CHH from *Metapenaeus ensis* are reported to be between 200 and 700 bp in length (Gu and Chan, 1998). Data in not available for *C. maenas* introns. The results obtained here for *C. magister* indicate the presence of an intron of approximately 600 to 700 bp located in the region coding for amino acids 32 to 66 of CHH. Attempts to confirm these results by nucleotide sequencing were unsuccessful.

The results obtained for *C. magister* also suggest that more than one CHH coding gene may be present in this species. The presence of two PCR amplification products resulting from reactions employing gene specific primers may attest to the presence of two CHH genes. *Homarus americanus* and O*rconectes limosus* have both been determined to have at least two CHH coding genes (De Kleijn et al., 1994; De Kleijn et al. 1995), *Carcinus maenas* has at least 4 (Dircksen et al., 2001), *Penaeus japonicus* 5 (Ohira et al., 1997) and *Metapenaeus ensis* 8 (Gu and Chan, 1998).

Although it has been suggested that the observed presence of two CHH isoforms in brachyurans is the result of post-translational modification (Chung and Webster, 1996; Chung et al., 1998), the recent results obtained for *C. maenas* indicate that gene duplication and divergence may account for the observed isoforms (Dircksen et al., 2001). Evidence for the presence of two CHH peptides and,

therefore, the possibility of two coding genes, in *C. magister* was initially provided by Kleinholz and Keller (1973). Although their results allowed for the possibility of post-translational modification of the N-terminal as the source of the two isoforms, the results of Keller et al. (1985) remarked on the presence of two CHH peptides in this species distinguished by their amino acid composition. These authors determined, by HPLC purification and amino acid composition analysis, that in one of the two CHHs, an extra alanine was present while in the other form, an extra valine was present, suggesting differences in the nucleotide and, therefore, gene coding sequence. In order to confirm the presence of two coding genes in *C. magister* attempts to purify and sequence the 690 and 790 bp bands were undertaken but these were unsuccessful.

6.4.1 Summary

Although sequence analysis results were equivocal, PCR amplification reactions of genomic DNA indicate the presence of an intron in a region previously identified by genomic sequencing of *M. ensis* and *C. maenas* CHH genes. Additionally, the results suggests the presence of more than one CHH coding gene.

Chapter Seven: General Discussion and Conclusions

7.1 Hemolymph glucose regulation in decapod crustaceans

A role for CHH in regulating hemolymph glucose has been supported for some time. Early investigators proposed that this hormone functioned in an analogous manner to vertebrate glucagon and acted to stimulate glycogenolysis and inhibit glycogenesis at target tissues facilitating glucose release to the hemolymph (Huggins and Munday, 1968; Ramamurthi et al., 1968; Keller, 1977; Keller et al., 1985). The data obtained here for *Cancer magister* indicates that eyestalk factor(s) are capable of eliciting hyperglycemia but do not do so *via* the proposed mode of action: mobilization of carbohydrate stores in hepatopancreas and/or muscle tissue (Keller and Andrew, 1973; Sedlmeier and Keller, 1981; Keller and Sedlmeier, 1988). As mentioned earlier, the view that hemolymph glucose regulation constitutes the primary physiological role of CHH has been called into question (Hohnke and Scheer, 1970; Keller and Sedlmeier, 1988; Santos and Keller, 1993a). The data reported here for *C. magister* lend support to this contention.

In vertebrates, only very small fluctuations in blood glucose concentrations are observed and these are tightly regulated by two antagonistic hormones; insulin and glucagon (Randall et al., 1997; Marieb, 1998). Large increases, 9- to 10- fold, were observed in hemolymph glucose concentrations of *C. magister.* Similar oscillations in circulating glucose of decapod crustaceans have been reported, not only under stressful conditions (Schmitt and Santos, 1993), but also during normal diurnal fluctuations (Reddy et al., 1981). Additionally, the activity of other factors (other than CHH) proposed to be involved in hemolymph glucose regulation are ill defined. Roles for the catecholamines serotonin, dopamine and octopamine in eliciting hyperglycemia have been proposed but, whether or not these amines act directly or indirectly, *via* effects on CHH release, is unclear (Bauchau and Mengeot, 1966; Kuo and Yang, 1990; Lüschen et al., 1993; Kilham, 1997; Hague, 2000). The presence of a peptide with hypoglycemic activity was proposed by Rangneker et al. (1961) but has never been confirmed (Lüschen et al., 1993). Dopamine and the opioid peptide, leucine-enkephalin, have been proposed to possess hypoglycemic activity but, again, this may be the result of effects on CHH secretion (Rothe et al., 1991; Hanke, et al, 1992; Hanke et al., 1993; Sarojini et al., 1995; Glowik et al., 1997).

One of the most salient features of decapod crustaceans is their open circulatory system. By necessity, the blood volume of these systems is much greater than that of closed circulatory systems.

In fact, the hemolymph of decapods is estimated to account for up to 30% of body mass (Gleeson and Zubkoff, 1977). Comparatively low resting glucose, 0.5 mM or less (Honhke and Scheer, 1970), and the large hemolymph volume of decapods may allow for greater tolerance of increases in glucose concentrations characteristic of these animals. Consequently, tight regulation of circulating carbohydrates is not necessary and the dedication of CHH, a hormone present in enormous quantities, to this physiological role seems unlikely.

7.2 The physiological role of CHH

Animals which experience frequent hypoxic exposure in their habitat exhibit adaptations to reduced oxygen availability, most notably, metabolic reduction. Energy production by anaerobic metabolism is markedly less efficient than aerobic energy production and requires high glycolytic rates and large fuel stores (Hochachka, 1988). The increase in glucose utilization observed during decreases in ambient oxygen tension in hypoxia intolerant species is termed the Pasteur effect (Hochachka and Somero, 1984). Anoxia tolerant species such as turtles, goldfish and mussels do not exhibit the Pasteur effect because of their ability to reduce metabolic rate and glucose utilization (De Zwaan, 1983; Storey, 1987; Storey, 1988; Mehrani and Storey, 1995). Emersed hypoxia tolerant decapod crustaceans exhibit metabolic depression (Teal and Carey, 1967; deFur and McMahon, 1984; Hill et al., 1991a). In light of this information, it was of interest to investigate the role of CHH in carbohydrate utilization and metabolic rate reduction in emersed *Cancer magister*.

In fact, data supporting a role for CHH in reducing metabolic rate have been reported and include studies investigating glycogen mobilization and oxygen consumption. Rao et al. (1991) suggest that decreases in tissue glycogen content of decapods in response to eyestalk ablation is indicative of the presence of a restraining factor. In its absence, metabolic rate increases and, consequently, fuel sources are utilized at accelerated rates. Similarly, increases in whole animal oxygen consumption of eyestalk ablated brachyurans was hypothesized to implicate an eyestalk factor in reducing metabolic rate (Reddy, 1990; Rao et al., 1991; Santos et al., 1987). Results of oxygen consumption from *in vitro* studies were more complicated. Although the data supports a role for CHH in decreasing metabolic rate, as evidenced by reduced oxygen consumption in the presence of purified hormone, this was dependent on the presence of glucose in the incubation medium (Santos and Gonçalves, 1992). Likewise, reduced glucose release by isolated tissues in response to ESE treatment was glucose dependent (Telford, 1975; Santos et al., 1988).

In regards to the current investigation, the hypotheses of Santos and Keller (1993a) are most relevant. These authors proposed that if the role of CHH is to promote a Pasteur effect (mobilization of glucose for anaerobic metabolism), then in its absence, glucose should not be mobilized and hemolymph

glucose and lactate levels should remain low. Conversely, if the role of CHH during emersion is to reduce the Pasteur effect, the absence of the hormone should be characterized by higher lactate accumulation due to increases in glycolytic flux and reduced hyperglycemia as a result of enhanced glucose utilization. The results obtained here are consistent with a Pasteur effect inhibiting role for eyestalk factors in *C. magister*. The absence of circulating eyestalk factors (anterior aorta ligated animals) was characterized by reductions in carbohydrate stores during emersion. Similar changes were absent, except for highly oxidative heart tissue, in non-ligated crabs. Since glycogen content was not observed to increase in any of the other tissues of ligated animals and hemolymph glucose concentrations did not increase significantly, these results suggest that the released glucose was metabolized.

7.3 Metabolic rate reduction

Investigations of metabolic reduction under a variety of conditions as well as in different species indicate that the underlying mechanisms are conservative and include: post-translational modification of glycolytic enzymes, reversible enzyme association with particulate fractions in the cytosol, and allosteric regulation by specific metabolites (Storey, 1987; Storey, 1996). Targets of post-translational modification by phosphorylation include glycolytic enzymes such as phosphofructokinase (PFK), pyruvate kinase (PK) and glycogen regulatory enzymes such as glycogen phosphorylase. Disassociation of commonly occurring assemblages of glycolytic enzymes with cytoskeletal components, as well as glycogen regulatory enzymes and glycogen particles in the cytosol, reduces the activity observed when aggregated (Storey, 1996). Regulation of metabolic rate *via* PFK, an important molecule in controlling the use of carbohydrates for biosynthetic processes, occurs via allosteric activation by fructose-2,6-bisphosphate (F-2,6-P_2) (Neely and Morgan, 1974; Kraus-Friedmann, 1984; Storey, 1988;). Levels of this metabolite are known to fluctuate in response to hormonal input, starvation and anoxia (Storey, 1996). The activity of CHH on glycogen phosphorylase points to an obvious mechanism by which CHH may elicit a reduction in metabolic rate. Interestingly, eyestalk ablation studies demonstrate that a factor(s) present in the sinus gland may also effect the activity of glycolytic and oxidative enzymes (Reddy, 1990; Rao et al., 1991).

7.4 Conclusions

In this study, the occurrence of a hyperglycemic factor(s) in the eyestalks of *Cancer magister* was confirmed physiologically by ESE injection and anterior aorta ligation. Heart, hepatopancreas and muscle tissue were identified as targets of eyestalk factor(s) under control conditions. During emersion stress, hepatopancreas and muscle tissue were identified as the primary targets. However, it

is important to note that these results could be due to a more generalized effect of abrogating flow through the anterior aorta. Further investigations of the effects of ligation on circulating hormone titers, as well as on physiological parameters such as oxygen consumption and cardiorespiratory function, will be required to elucidate the cause of the observed responses. Investigations of the mode of action of eyestalk factors in eliciting hyperglycemia did not support the working hypothesis proposed by earlier investigators. In fact, the data obtained here support the alternate hypothesis that the physiological role of hyperglycemic eyestalk factor(s) is to reduce metabolic rate *via* effects on glycogen synthase and metabolic enzymes at target tissues. Results of immunological investigation suggest that the active factor from eyestalks was CHH. Confirmation of these results is required and could be accomplished by quantifying circulating hormone titers in both non-ligated and anterior aorta ligated animals under control and experimental conditions.

Overall, this preliminary investigation of carbohydrate regulation in *Cancer magister* suggests that anterior aorta ligation is a useful technique for manipulating circulating concentrations of eyestalk factors and that emersion stress in *C. magister* constitutes an appropriate model system.

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