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The precise release time of crustacean hyperglycaemic hormone (CHH) in response to environmental stressors in the shore crab Carcinus maenas

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The precise release time of crustacean hyperglycaemic hormone (CHH) in response to environmental stressors in the shore crab *Carcinus maenas*

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Keywords: crustacean hyperglycaemic hormone, shore crab, Carcinus maenas, stress, haemolymph, hyperglycaemia

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Literature Review

Abstract

The crustacean hyperglycemic hormone (CHH) is a neuropeptide involved in numerous biological processes, such as glycogen mobilisation, osmoregulation and stress response. CHH belongs to a superfamily of peptides, including moult inhibiting hormone (MIH), gonad inhibiting hormone (GIH/VIH), and mandibular organ inhibiting hormone (MOIH), which play a vital role in glucose and ion regulation in crustaceans. Despite extensive research on CHH structure, gene expression and locations, studies have predominantly overlooked its adaptive significance in carbohydrate regulation and rapid release patterns during stress response. This literature review examines existing research on CHH, revealing knowledge gaps in understanding the immediate CHH release response. The proposed study aims to quantify CHH release during the initial stages of stress response, such as 10 minutes, providing insights into organism adaptation to changing conditions and neuropeptide release patterns. Various stressors will be incorporated into a single study to examine the influence of different environmental factors on CHH release in shore crabs, with green shore crabs being used as the main type of crab due to their higher tolerance to environmental stressors. Red shore crabs will be tested under hypoxia conditions, offering insights into potential differences in stress response at different molt cycle stages.
Three main methods have been used to measure CHH in crustaceans: radioimmunoassay, enzyme-linked immunoassay (ELISA), and time-resolved fluroroimmunoassay (TR—FIA). The chosen method for the proposed study is the TR-FIA for its higher sensitivity and precision. This research will significantly contribute to a deeper understanding of CHH's role in stress response and adaptation mechanisms in crustaceans, potentially offering insights into neuropeptide release in insects as well.

Introduction

The crustacean hyperglycemic hormone (CHH) is a functionally diverse neuropeptide closely related to the moult inhibiting hormone (MIH), gonad inhibiting hormone (GIH) also known as vitellogenesis inhibiting hormone (VIH) and the mandibular organ inhibiting hormone (MOIH) (Chen et al, 2020). The X-organ sinus gland complex in the eyestalks was the first tissue in crustaceans to be identified in producing these neuropeptides however studies have shown that a small volume is also produced in other tissues such as the endocrine tissue in the gut (Chung et al, 1999).

One signature of these peptides in this family are presence of 6 cysteine residues that form 3 intra-molecular disulphide bridges. The CHH peptide consists of 72-73 amino acids and MIH/GIH/MOIH peptides consist of 77-78 amino acids (Soyez, 1997). The amino acid sequences determined that MIH and GIH/VIH have closer similarities to each other than that of CHH in regards of sequence, length, and termini modifications where MIH and GIH are free at both ends, but CHH remains blocked (Kegel et al, 1991).

Half a decade later, MOIH was characterised from the crab *Cancer pagurus* which found that there were 2 biochemically different sequences which differed by 1 amino acid (Wainwright et al, 1996). The sequences uncovered between the different peptides allowed them to be divided into 2 groups; Type 1, which consists of CHH, and Type 2, which consists of MIH, GIH/VIH and MOIH (Chen et al, 2020).

Type I peptides can be classified as peptides with a cryptic sequence or precursor related peptide and a diabasic cleavage site upstream of the mature peptide sequence. The precursor related peptide consisting of 33-38 amino acids (Soyez, 1997). However, Type 2 has been characterised by the lack of precursor related peptide and includes a glycine residue at position 5 after the first cysteine (Chen et al, 2005).

Non-crustacean peptides have been identified allowing the CHH family to be expanded earning its status of a superfamily. The first of these non-crustacean peptides was the Ion transport peptides (ITP) which was first found in the desert locust *Schistocerca gregária* where it was isolated from the corpora cardíaca (Meredith et al, 1996). ITP was initially thought to be specific to non-crustaceans after further being found in other species of Hexapods but additionally in nematodes and chelicerates (Christie, 2008). However, extra members were uncovered in non-Malacostraca crustaceans such as phyllopods, copepods and remipedes. And appears to be exclusive in those species due to the absence of other characterised CHH peptides. (Christie, 2014)

The iso form of ITP presents closer to that of CHH than that of type 2 isoforms due to the presence of PRP and the diabasic cleavage site. This led to ITP being included in the type 1 group. However, a type 3 group was created which would consist of all the various ITP isoforms due to phylogenetic studies placing these iso forms at the base of type 1 and 2 groups. (Lacombe et al, 1999)
There has been further expansion of the superfamily from the addition of Latrobe tin peptides and helical arthropod-neuropeptide-derived (HAND) toxins that are present in spider and centipede venoms. (Undheim et al, 2015)

CHH has a multifunctional role within crustaceans and is involved in a number of biological processes. Early studies show that CHH mobilises glycogen reserves in target tissues, for example in the muscle and in the hepatopancreas, which regulates the activity of the enzymes glycogen synthase and glycogen phophorylase, which in turn leads to hyperglycaemia.
An RNAi approach in the crayfish *Procambarus clarkii* has been more recently used to characterise the metabolic roles of CHH and has indicated that CHH has more diverse effects on the hepatopancreas and muscle where the two are regulated differently. (Seldmeier, 1987)
In the hepatopancreas, CHH causes lipolysis and decreases glycogen accumulation allowing in higher levels of glucose resulting in glycolysis. Lipids in glucose from these processes move into the hemolymph where they are taken up by the muscle and further utilised by further glycolysis and the tricoarboxylic acid cycle (TCA) which results in greater levels of ATP. Alongside this is the stimulation of the pento-phosphate pathway (PPP) which increases the levels of nucleotide biosynthesis and an elevation in amino acid biosynthesis. Furthermore, the process of glycolysis, the TCA cycle and the pent-phosphate pathway is driven by nicotinate and nicotinamide metabolism which involves the production of two nicotinamide co-enzymes NAD^+ and $NADP^+$ which are used by these processes in the muscle. (Nagai et al, 2011)

One of the first reports that indicated CHH was involved in osmoregulation was in the American lobster *Homarus americanus* which found that the osmoregulatory capacity was increased in eyestalk ablated animals when kept at low salinity when they were injected with sinus gland extracts. D-Phe^3-CHH variant has been shown to restore both the Na^+ concentration and hemolymph osmoregulatory in eyestalk ablated American lobster and the freshwater crayfish *Astacus leptodactylus* stimulating the trans-epithelial electrical potential difference in the posterior gills as was seen in the crab *Pachygrapsus marmoratus* and in the freshwater crayfish *Cherax quadricarinatus*. The gills in the shore crab *Carcinus maenas* was tested and high affinity CHH binding sites were identified and when treated with CHH the levels of glucose and cGMP significantly increased in the tissue. However, it has been noted that in the Christmas Island Red Crab, *Gecardcoidea natalis*, CHH had no effect on Na^+-ATPase, K^+-ATPase or V-ATPase activity but still stimulated Na^+ transport across the gill epithelia.

A study quantifying the physiological role of CHH at a specific life stage found that releasing gut-derived CHH into the hemolymph during the pre-moult and ecdysis stages of the moult cycle stimulated water and ion uptake into the crab. This caused the body to swell which was needed for the ecdysis to be successful and produced an increase in the size of the animal during the post-moult stage. While this study focused on the physiological role of CHH and the effects it caused in regards to osmoregulation, the molecular targets on which CHH acts were not directly addressed.

These functional roles presented by CHH had led it to be considered that the hormone acts physiologically as a stress hormone. It has been observed that CHH induces hyperglycaemia when the animals have been exposed to different environmental stressors such as extreme temperatures, hypoxia and organic/inorganic pollutants. This metabolic response is modest likely for assisting the animal in acclimatising to the new environment.
Studies into the Christmas Island Blue Crab, *Discoplax celeste*, and the Christmas Island Red Crab, *Gecarcoidea natalis*, highlight the practical use of CHH in an eco-physiological context. Both species of crabs undertake an annual migration at the start of the wet season towards the sea but remain largely inactive during the dry season. In the studies, the crabs were forced to undergo 10 minutes of exercise which as expected produced a rapid release of CHH followed by an increase in glucose levels in the hemolymph (Morris et al, 2010; Turner et al, 2013).
However, it was found that the levels of hemolymph CHH in the migrating animals in the wet season were significantly lower than in the dry season. This was the opposite as to what was to be expected as it would be thought that a higher metabolic burden when migrating would increase the CHH released. This decrease during the wet season demonstrates the negative feedback loop which occurs when CHH is inhibited by the high released levels of glucose. This inhibitory effect could be caused by the animals continuing to feed throughout the migration as a need to conserve the glycogen stores that will be utilised in the return migration. The lack of negative feedback loop in the dry season could also be metabolically advantageous when the animals have limited foraging activity and need CHH to stimulate glucose release. One other reason for an increase in the CHH levels in the dry season could be linked with the ions regulation activity of CHH as the conservation of ions and water would be needed during that time of year (Morris et al, 2010).

Carcinus maenas, also commonly known as the shore crab, has its native range along the coastline of the north east Atlantic from Norway, around the British Isles, Iceland, and even down the west coast of Africa to Mauritania (Lewis, 2011). In these areas it is considered to be an important species both ecologically and economically due to the species being the most common intertidal decapod crustacean with it being a popular choice for commercial fishing to be used as bait or food. However, concerns have been raised regarding the decrease in the population of shore crabs in recent years due to overfishing with calls to focus on sustainability (Morris-Webb et al, 2007). Outside of these native populations *Carcinus maenas* is considered to be an invasive species in several locations around the world. The coastline of the mid-Atlantic United States is considered to have two lineages. The crabs were first discovered in 1817 which were presumed to have been carried over on the outside of wooden ship hulls from south-central Europe. From here they spread southward with the limit seemingly being Chincoteague Bay in Virginia, further spread south from here is limited by temperature and predators, the main one being the blue crab *Callinectes sapidus* (deRivera et al, 2005). The second lineage was associated with an introduction most likely from Norway in the 1980s and began a northward spread starting in Nova Scotia then further travelling to Canada and Newfoundland (Darling et al, 2008). A population descended from this second lineage was further found on the west side of the United States in San Francisco Bay, California in 1989 with the transmission route most likely being from crabs being trapped in seaweed packed into baitworm shipments (Cohen et al, 1995; Bagley, 1999). In the 12 years following the discovery the eastern Pacific population had travelled northward by 1500km and had settled in British Colombia and

Vancouver Island, Canada and most recently have been observed in the Salish Sea (Behrens Yamada et al, 2017)
The populations of shore crabs in the northwestern Atlantic and the northeastern Pacific regions have been the most widely studied of the invasive populations however many other populations have spread across more continents with the main ones being located in Argentina, South Africa and Australia. The earliest introduction in these other three locations was thought to be in the late 1800s in Australia from contaminated hulls. Over 100 years later in 1999 it is thought that a small number of individuals from this population were introduced to Atlantic Patagonia, Argentina from ballast water (Vinuesa, 2007).
Along the costal habitats where *Carcinus maenas* is located they tend to occupy sheltered, wave protected areas in the sublittoral and intertidal zones, such as estuaries and harbours or other rock and seaweed concentrated areas. (Young and Elliott, 2020) Due to lacking adequate vertical tenacity or a sufficient ability to grip onto rock substrate, shore crabs have an increased mortality rate of 80-90% when they are subjected to wave swept open shore coastlines and so are not usually founds in those types of environments (Moksnes et al, 1998). Adult crabs migrate across the sublittoral and intertidal zones with the changing tides for foraging and from this they are regularly exposed to fluctuating levels of temperatures, salinity and hypoxia with it being observed and noted in numerous studies that the species is highly tolerant to these variations (Young and Elliott, 2020)
Shore crabs are eurythermal, meaning they are able to tolerate short term temperatures between 0^{o}C to 35^{o}C. Adult crabs can evaporatively cool their bodies by 2^{o}C if they become exposed on mud flats at low tide allowing them to be more resistant to high air temperatures (Ahsanullah and Newll, 1977). In addition to this shore crabs have higher levels of heat shock proteins such as HSP70 compared to other species which do not reside in intertidal zones which assists in cellular defences against proteotoxic stress (Madeira et al, 2012). However, the crabs do face some limitations in their life cycles at various temperatures. Even though they can tolerate temperatures of up to 35^{o}C breeding can only take place up to a maximum of 26^{o}C and the brooding of eggs cannot exceed 18^{o}C (Naylor, 1965). Following this, 10^{o}C is the minimum temperature needed for growth which is also close to the lower limit temperature for feeding which sits at 7^{o}C, though it has been theorised that the feeding behaviour itself does not cease because of these temperatures but it is the overall activity of the crab which is reduced (Ropes, 1968). It has been reported that crabs from colder environments such as Sweden do exhibit feeding below 7^{o}C in the autumn months but not in spring, however the crabs from these cold tolerant Scandinavian lineages still cease to feed at 3^{o}C as they near the lower levels of temperature tolerance (Eriksson et al, 1975). These restrictions for breeding and feeding explain why there are no expanding populations in tropical or arctic environments despite attempts at introductions (Carlton and Cohen, 2003).

Alongside being eurythermal *Carcinus maenas* is also classed as euryhaline. The species is able to survive long term at a salinity as low as 4‰ (McGaw and Naylor, 1992) Adults have a short-term tolerance to high salinities of 54‰ but normally reside in salinities of 27-40‰ (Ameyaw-Akumfi and Naylor, 1987) With high salinities the shore crab is considered to be an osmotic conformed but changes to an osmotic regulator at extreme low salinities (Henry et al, 1999) Temperature can also be a factor in the tolerance levels in shore crabs. At 15‰, Brokhuysen, 1936 found that at temperatures of 0.6^{o}C shore crabs could only survived for thirty days but at 9.7^{o}C no deaths occurred. In another study is was reported that shore crabs were frequently found in salinities of 1.4-3.2‰ at temperatures of 8-11^{o}C and usually stayed in these conditions for up to 8 hours at a time. Whereas crabs which were exposed to salinities at 1-2‰ at 6-10^{o}C died rapidly but at 3‰ they could survive for up to 34 days (Perkins et al, 1969).

Adult shore crabs have been found to be able to survive out of water for at least 10 days if they are at a moderate temperature and have access to moisture (Darbyson, 2006) As a response to a lack of oxygen in water the crabs are able to breathe air by a process called ‘bubbling’, the direction of the

scaphognathite beat is reversed causing air to enter the bronchial chamber via the exhale to openings and exit via the inhalant Milne-Edwards openings (Ried and Aldrich, 1989). It has been hypothesised that this ability allowed shore crabs to be introduced from the population along the eastern coastline of the United States to the western coastline in California by the shipments of live bait worms packed in wet seaweed (Carlton and Cohen,2003). Adult crabs are less able to adapt to to lower levels of oxygen when their rate of oxygen consumption is high (Aldrich, 1986). However, in conditions of declining oxygen, the degree of respiratory independence is dependent on the level of activity by the crab meaning inactive crabs can can maintain a respiratory independence of 60-80 mmHg (Taylor, 1976) During high tide the crabs migrate into the intertidal zone to forage, the few which remain or get trapped in rock pool environments at low tide experience hypoxia, the act of reducing the level of activity when in extreme hypoxia aids in allowing the crab to continue to function (Jungblut et al, 2018; McMahon, 1988).

Different stages of the molt cycle can be denoted by the colour of the carapace. Newly molted crabs are green in colour (green crabs) which transitions through to red (red crabs) before the cycle is repeated again. Green and red crabs exhibit differences in environmental tolerances. Green crabs are more tolerant of low salinity that red crabs, this can be seen in the shore crabs' native range where green crabs are found more commonly in intertidal and estuarine habitats whereas red crabs reside more in the subtidal zone and the saline waters of the open shore (Baeta et al, 2005) These differences in tolerances is a result of a greater gene expression in green crabs than in red crabs of the osmoregulatory genes controlling the presentation of Na+/K+ ATPase and cytoplasmic carbonic anhydrase (Himes et al, 2017).
This lack of tolerance in red crabs compared to green crabs is also mirrored in hypoxia environments. When red crabs experience hypoxia conditions in their environment they leave the water and begin the bubbling behaviour with a much higher oxygen tension. This means the red crabs are unable to compensate for hypoxia as easily as green crab and succumb more rapidly to anoxic conditions (Ried and Aldrich, 1989).

The aim of this literature review is to examine the existing body of research on the stress induced release of the crustacean hyperglycemic hormone and the methods used to measure the CHH levels. By reading these studies, the objective is to identify the most effective and reliable techniques for quantifying CH, as well as uncovering any knowledge gaps or inconsistencies in the current understanding of CHH's role in stress response. This review will then serve as a foundation for the development an experiential design that can further advance our knowledge of neuropeptides, particularly CHH, and the biological mechanisms that they regulate.

Previous Research Relating to Stress Conducted on the Crustacean Hyperglycemic Hormone

While many studies have explored CHH structure, gene expression and where it is expressed but little on the adaptive significance of CHH in carbohydrate regulation in respect to release patterns or quantifying the levels of released CHH.
Many early studies Regarding stress exposures focused on the hyperglycemic response and glucose levels in the hemolymph. Hyperglycemia has been reported in many species of crustaceans such as the intertidal crab, *Chasmagnathus granulate*, When undergoing emersion (Santos and Colares, 1986), The giant prawn, *Macrobranchium rosenbergii*, When subjected to cold shock (Kuo and Yang, 1999), and the response can even be stimulated in the freshwater prawn *Macrobranchium kistensis*, the crab *Barytelphusa canicularis* and the crab *Scylla serrata* when exposed to metals such as copper, mercury and cadmium (Nagabhushanam and Kulkarni, 1981; Machele et al, 1989; Reddy and Bhagyalakshmi, 1994). The glucose levels that arise from hyperglycemia can indicate when CHH is released it does not pinpoint exactly the release time or the release content as measurable

hyperglycaemia can take up to 30 minutes to occur. Measuring CHH instead of glucose would allow us to see how the CHH content in both the sinus gland and hemolymph Varies when subjected to stressor.

In the edible crab, *Cancer pagurus*, the levels of CHH have been measured following emersion stress (Webster, 1996). These levels were recorded by taking samples of hemolymph at 30-minute intervals over a period of four hours. It was found that within this first hour CHH increased rapidly then started to level off but still increased slowly. This slow increase after the one-hour mark could be contributed to the variable responses of each individual crab. As this significant increase within the hour suggested that CHH was released in quick succession from exposure to the emersion stressor, a second test was carried out which measured levels of CHH every 15 minutes over the course of an hour and concurrently measured the levels of glucose in these hemolymph samples. A rapid increase in CHH was observed at 15 and 30 minutes after which the levels became variable again. The glucose levels did begin to increase within the first 30 minutes but showed a significant increase from the initial value after 45 minutes this lines up with previous research which suggests that an increase in CHH produces measurable hyperglycemia within 15 to 30 minutes of the initial release time (Keller and Andrew, 1973).
The average size of the crops in this study was approximately 0.75 kg which was larger than the crabs used in previous studies, this allowed larger and repeat samples to be taken from each individual.

A later study by Chang et al 1998 aimed to quantify CHH in the lobster, *Homarus americanus*, following the various stressors such as temperatures of 23^{o}C and 28^{o}C, emersion and exposure to salinities of 50% and 150% sea water. The time scale for the temperature stresses and emerging were measured every hour over the course of four hours. But emerging was measured every two hours over the course of six hours. The emersion condition had the same outcome as that of Webster 1996 which showed an increase within the first hour however the level of CHH continued to increase and there were significant differences from the controls at 2 h ($P<0.01$) and at 4 h ($P<0.001$). Thermal stress again showed a significant increase at both temperatures at 1 h however this increase was not as great as the one seen for emersion, and as was to be expected the higher temperature of 28^{o}C showed a larger increase over 23^{o}C. At the two hour mark for 23^{o}C there was a further increase as has been seen before again significantly different than the controls however at 28^{o}C there was a decrease.

Following on from Lorenzon et al, 2000 The variation in the levels of CHH in the shrimp *Palaemon elegans* was measured following Exposure to copper, mercury, and a bacterial contaminant lipopolysaccharide (Lorenzon et al, 2004). In Lorenzon et al, 2000 sublethal concentrations of mercury, cadmium and lead had produced significant hyperglycemic responses in the same species however at the highest concentrations no hyperglycemic response was recorded. However, the shrimps exposed to copper and zinc show a hyperglycemis response at both sublethal levels and at the highest concentrations. It was found that high concentrations of Cu^{2+} (5 mg l^{-1}) caused a significant increase ($P<0.05$) in CHH levels at 30 minutes to 2 h which after it decreases at 3 h. Lower Cu^{2+} (0.1 mg l^{-1}) concentrations did not affect the levels of CHH which remained the same as the resting control levels. Hg^{2+} (0.5 mg l^{-1}) exposure followed the same pattern, CHH levels in the hemolymph had a significant difference ($P<0.05$) from the control levels and increased up until 2 h and again decreased at 3 h but still stayed significantly different. The highest Hg^{2+} (5 mg l^{-1}) concentration also induced a significant difference ($P<0.001$) at 1 h but this increase was not as high as the 0.5 mg l^{-1} concentration. At 2 h this concentration proceeded to decrease yet still had a significant difference ($P<0.05$) with the controls, this then further decreased at 3 h. The Hg^{2+} (0.1 mg

l^{-1}) concentration followed the same trend as the Hg^{2+} (5 mg l^{-1}) concentration but at a lower intensity, though there was still a significant difference between ($P<0.05$) between the resting control levels. The highest concentration not causing the greatest increase suggests that exposure to Hg^{2+} is not related to the dose type. This could potentially be explained by the high toxicity of Hg potentially affecting the functionality of the preprp-CHH by altering the configuration of the peptide preventing its ability to bind therefore the higher the concentration the less CHH would be able to bind in the ELISA reducing the level of detection (Lacombe et al, 1999).

Over the past decade more studies have been looking into how the roles of CHH affects crustaceans in regards to their life history outside of a laboratory. Morris et al, 2010 studied the significance of CHH in the Christmas Island Red Crab when they exhibited their natural behaviour of their annual breeding migration. The crab species they used Was a good model species for the study as they underwent a migration that was part of their natural behaviour so it would allow researchers to see how CHH naturally acts when there is a long-term stress on the body. The contrast in the environment of the dry season and the wet season meant that it would be possible to see if there was any difference in the way CHH responds to two very different environments.
Hemolymph samples were taken from the crabs in the field during the dry season at the point when the crabs were inactive and then again at the start of the wet season when the crabs had begun their migration. Typically, circulating CHH levels ranged from 20 to 40 pmol l^{-1}, though when the migrating and digging phases were occurring in the wet season there were instances where CHH titres fill below 10 pmol l^{-1}. Laboratory experiments were undertaken to determine what effect short, intense exercise had on the levels of CHH glucose and lactate. The crabs were exercised for 10 minutes and then left to rest for 110 minutes. The samples were then taken at 0, 5, 10, 20, 30, 60 and 120 minutes. The levels of CHH increased and was significantly different from the controls at 10 minutes whereby it then decreased from that time onwards.
The initial studies measured the CHH levels in the hemolymph usually across the course of a few hours however in Morris et al, 2010 when studying the effects of 10 min exercise showed the quantity of CHH released within the first 10 minutes of being subjected to the stressor, something which had not been widely focused on in the past.

The proposed research to carry out would be to see when CHH is first released when the crab comes into contact with a stressor and quantify the CHH to see the amount which is produced and compare it to different stressors. This would be able to be compared with the levels seen after a few hours. CHH being a neuropeptide hormone acts quickly in response to stressors. Examining the release of CHH at a short response time, such as 10 minutes, can provide insights into the initial stages of the stress response and help understand how organisms quickly adapt to changing conditions. With different stressors compiled together it would be able to set a basis to see how different species of crustaceans react to the stressors and how they have adapted to the various environments they have evolved into. As crustaceans are the easier animal to test how neuropeptides react, the research could potentially give an insight into how neuropeptides are released in insects.

<u>Previous Methodologies</u>

Stressing of animals/taking the blood:

Chang et al 1998 achieved Hypoxic stress via emersion by placing the lobsters in two litre jars without any water. Stress from the thermal conditions was created by transferring the lobsters from sea water they had been acclimatised to into 1.2 litres of aerated sea water which had been set to the correct stress temperatures previously chosen. It was important for the water to be aerated as it was static which reduces oxygen and carbon dioxide diffusion but also at higher temperatures meant that there was less oxygen in the water which would cause the lobsters to undergo extra stress from

hypoxia. Creating 50% seawater is done by mixing seawater with distilled water in a ratio of 1:1 to create an equal mix. To create seawater with a higher salinity at 150%, seawater is mixed with commercial salt.
Hemolymph samples can be taken using a hypodermic needle and syringe in the joint at the base of the crab's leg. 200 ul of hemolymph is the most common volume taken per sample for the use of measuring CHH. It is important to ensure the volume collected is not too large so as to not cause excessive stress or harm to the crab during the process.

Laboratory analysis:
There have been three main methods used to measure CHH; radioimmunoassay, enzyme-linked immunoassay (ELISA) and time-resolved fluoroimmunoassay (TR-FIA). Identifying the most sensitive technique for detecting low levels of CHH is crucial as it ensures accurate measurements of even the smallest fluctuations in CHH levels within the hemolymph. This is particularly important when investigating the physiological responses of crabs to various environmental stressors, where subtle changes in CHH levels may provide valuable insights into the crabs' stress responses and adaptability. Moreover, a highly sensitive technique can help minimise the amount of hemolymph required for the analysis reducing stress on the sampled crabs. Additionally, identifying the equipment needed to perform the chosen technique is essential to ensure that the necessary resources are available for conducting the experiments. By selecting the most appropriate and sensitive technique the quality and reliability of the data can be maximised while minimising potential harm to the crabs under study.

Previous to these methods, the determination of hormones in both the tissues and blood relied on bioassays which had limited accuracy and insufficient sensitivity (Jaros and Keller, 1979). A radioimmunoassay was established to determine the amount of CHH contained within the sinus gland. 960 *Carcinus* sinus glands was combined with Freunds complete adjuvant then injected into rabbits through the foot pads and intradermal injections. These rabbits then received 8 s.c. booster injections of 80 sinus gland equivalents combined with incomplete Freunds adjuvant. 1.3 µg of pure *Carcinus* CHH was then iodinated by using the chloramine T-method with 1 mCi ^{125}J in a volume of 130 µl. The ^{125}J- antigen was then separated from the free ^{125} J by gel filtration on Sephadex G-25 and Sephadex G-50. It was found that with the control serum less than 5% bound with ^{125}J-CHH whereas with the excess of antiserum 73-76% bound with the ^{125}J-CHH. From this new assay it was found that there was a hormone content of 1.2 ±0.3 µg per gland. It was concluded that this test was suitable for measuring CHH as it was faster and more accurate than the previous bioassay, the detection limit for the bioassay being around 30 ng whereas the radioimmunoassay was around 50 pg (Jaros and Keller, 1979).

The enzyme-linked immunoassay method regarding CHH was first developed by Keller et al, 1994 to quantify CHH released in *Cardisoma carnifex* by a single X-organ sinus gland. IgG fractions were prepared using protein A/Sepharose chromatography. It was assumed that the epitope of *Cardisoma* CHH was significantly differently from that of *Carcinus* CHH so the antibodies from two different rabbits were needed, with a different antibody of each epitope from each rabbit, in order to obtain a cross-reactivity of the *Cardisoma* CHH in the assay. However, it was found that using either antibody from each animal or mixed in equal volumes *Cardisoma* CHH showed a strong cross-reactivity. Unpublished results from Keller R. further supported this when a radioimmunoassay was performed, which depends on a single epitope recognition, and *Cardisoma* CHH had again a strong cross-reactivity when a standard *Carcinus* CHH assay was used.
Initially the microtiter plates were prepared and activated with 0.25% glutaraldehyde in 0.1 mol^{-1} sodium phosphate buffer, left to rest for 4 hours at 37^{o}C and washed with the sodium phosphate

buffer. Equal volumes of IgG from both rabbits were diluted to 20 µg ml^{-1} using the sodium phosphate buffer and incubated. This was followed by washing then 400 µl of 2% bovine serum albumin solution was added along with the sodium phosphate buffer and 0.02% sodium azide and again left to rest for 1 h at room temperature. *Carcinus* CHH was diluted with phosphate-buffered saline which contained 0.1% Tween 20 and used as the standards. The samples were diluted with the same phosphate-buffered saline and were assayed in duplicate. The plate was then incubated in a refrigerator for 24 h and then washed with the phosphate-buffered saline. The second set of antibodies were biotinylated with the use of Amersham RPN 28 then diluted using the phosphate-buffered saline to 2 µg ml^{-1}. Again, the plate was left to rest and was washed with the buffer. 25 µl fractions were created containing 125 µg of avidin and biotinylated horseradish peroxide and 62.5 µg of enzyme which were then added to 250 µl of phosphate-buffered saline and left to react for 1 h at room temperature. Samples containing 0.06 – 0.18 µg of peroxide were created from a dilution with phosphate-buffered saline and added to the wells and left to incubate for 1 h before being washed 8 times. To take the readings, a solution of 0.04% 2, 2'-azino-di-3-ethylbenzthiazolinesulfonate in a 0.5 mol l^{-1} sodium phosphate/citrate buffer containing 0.003% H_2O_2 was added to the wells and absorption readings were taken using a Biorad model 2550 EIA reader.

The study showed that the ELISA method had a greater sensitivity than that of a radioimmunoassay as it was commented that the lower limit of detection for *Carcinus* CHH was 1pg, with the usable range for the assay being between 1 pg and 50 pg. Even though using radioimmunoassays remains a less expensive method, the ELISA has the greater sensitivity and also does not require special precautions and licensing since radioactive substances are not used.

This same method was used by Chang et al, 1998 when quantifying CHH in the lobster, *Homarus americanus*, however biotinamidocaproate N-hydrozysuccinimide ester was used as the biotinylated reagent and it was mixed with a borate buffer instead of using phosphate-buffered saline. A streptavidin peroxidase solution was used after this step-in replacement of the peroxide used by Keller, 1988. Chan et al, 1999 commented that these changes to the ELISA proved sensitive enough to measure the levels of CHH produced from repeat sampling from the same animal and that one advantage to using an ELISA over a radioimmunoassay was that the samples of hemolymph could be assayed directly with extraction or hormone enrichment procedures.

A time-resolved immunoassay was first used in regards to CHH to investigate the adaptive significance of CHH in the migratory activities of the Christmas Island Red Crab *Gecarcoidea natalis*. The antiserum for *G. natalis* CHH was created with the same method as in previous studies using a single rabbit and injecting 10 nmol CHH at intervals of 30 days. The first injections used Freund's complete adjuvant and the remains on using Freund's incomplete adjuvant. The samples first needed to be purified. Using a vacuum manifold 200 mg Strata-X polymeric reverse-phase cartridges were conditioned with isopropanol and water, hemolymph samples which had been diluted with water in a 1:1 ratio and passed through. This was followed by washing of the cartridges after which the peptides were eluted with 60% isopropanol and samples were dried on a vacuum centrifuge. Plates were coated with 100 µl/well 10 µg ml^{-1} anti-CHH IgG in 0.1 mol l^{-1} sodium bicarbonate buffer and left to rest overnight at 4°C. The plate was then washed with this same buffer and incubated with blocking buffer for 1 h. Once blotted the CHH dilution standards and samples dissolved in assay buffer were added in duplicate and left overnight. Washing then occurred with Delfia buffer and again incubated with Europium-labelled streptavidin which had been diluted with assay buffer to 100 ng ml^{-1}. After the final wash, enhancement solution was added, and the plate underwent orbital shaking. A PerkinElmer Victor^2 1420 instrument was used to read the time-resolved fluorescence of

the europium which allows the user to see the levels of CHH in the samples at specific times (Morris et al, 2010).

This method was preferred over using a radioimmunoassay as 1-2 ml of hemolymph would have been needed to be taken for a radioimmunoassay compared to <100 µl in a TR-FIA due to its lower sensitivity level. This would potentially prevent repeat samples from being taken from individuals. Circulating CHH levels in *Gecardoidea natalis* had not previously been recorded so an assay with a greater detection limit would have been needed as the levels may have been at very low levels. The same method had first been used to measure MIH in the American Crayfish, *Procambarus clarkia*, and it was found to be 50-fold more sensitive than the ELISA for the MIH of *Carcinus* maenas (Nakatsuji and Sonobe, 2003). The sensitivity and precision of the TR-FIA being $<50x10^{-18}$ moles per well gave it a greater advantage over using the other methods stated.
Some limitations which has been found when using TR-FIA in other studies outside of crustaceans. Background fluorescence from components in samples such as proteins can cause the results to be affected and become inaccurate. However, the use of lanthanide chelates such as europium and time resolved detection systems reduces this background interference as the europium decays over a longer period of time.

Out of the three methods discussed, I have elected to use a time-resolved immunoassay as it routinely gives consistent and accurate results in a safe and controlled manner. Bangor University also holds the equipment needed so I shall be able to carry it out without the need to source additional equipment or rely on the previously used methods.

Conclusion

This literature review was conducted with the aim of examining existing research on the crustacean hyperglycemic hormone (CHH) and identifying potential knowledge gaps. It became evident that the majority of previous studies measured the release of CHH over the course of several hours, leaving a gap in understanding the immediate response of CHH release. This presents an opportunity to investigate CHH release within a 10 minute window and compare it to the levels observed over longer time periods.
The proposed study can further compile various types of stressors into a single study to examine how different environmental stressors experienced by shore crabs influence CHH release This approach will shed light on the stress response and the organism's ability to adapt to changing conditions. Green shore crabs an serve as the primary subject for this study, given their higher tolerance to environmental stressors. However, red shore crabs can be tested under hypoxia conditions to provide insights for further research into potential differences on the stress response at various stages of the molt cycle.
By addressing the immediate response of CHH release and integrating a variety of stressors, this study will significantly contribute to a deeper understanding of the role CHH plays in the stress response and adaptation mechanisms in crustaceans.

Research Paper

The release of the crustacean hyperglycemic hormone (CHH) in response to environmental stressors in the shore crab *Carcinus maenas*

Abstract

The crustacean hyperglycemic hormone (CHH) is a multifunctional neuropeptide involved in various biological processes, including glucose recognition, lipid mobilisation, osmoregulation, ionoregulation, and ecdysteroid synthesis. This study aimed to determine the effects of different environmental conditions on the release levels of CHH in green shore crabs, *Carcinus maenas*, within a 10 minute period, CHH release being an indicator that a stress response has occurred. The crabs were exposed to various conditions simulating their natural habitat, such as heat shock, cold shock, salinity, and hypoxia. It was found that CHH release in crabs varies depending on environmental conditions with heat shock, cold shock and hypoxia causing significant changes in CHH levels. Notably, significant increases in CHH levels were observed at the 5 min mark for temperatures of 30^{o}C, 25^{o}C, 5^{o}C and for the hypoxia condition. No significant changes were seen in response to low salinity, possibly due to the osmoregulatory response taking longer than the 10 min measurement period. Additionally, red shore crabs exhibited higher CHH levels under hypoxia compared to green crabs, suggesting that red crabs are less able to adapt to the stress of hypoxia. Further research could potentially compare temperature and salinity conditions on red crabs against that of green crabs.

Understanding CHH release within 10 minutes of a crab being exposed to environmental stressors can advance our knowledge of the neuropeptide's role in stress response and help determine how quickly shore crabs can mobilise the hormone. This research also provides insights into the function of similar hormones in insects and other invertebrates, due to the similarities between neuropeptides in these species.

Introduction

The crustacean hyperglycemic hormone (CHH) is part of a superfamily of functionally diverse neuropeptides. These neuropeptides are mainly produced by neurosecretory perikaryal in medulla terminalis X-organs located in the eyestalk and stored and released from the sinus gland (Chen et al, 2020). However, it has been shown that the hormone can be expressed in other tissues, the ecdysis associated surge of CHH is released from endocrine tissue in the gut (Chung et al, 1999). The other closely related peptides to CHH are the moult inhibiting hormone (MIH), the gonad inhibiting hormone (GIH) also known as the vitellogenesis inhibiting hormone (VIH) and the mandibular organ inhibiting hormone (MOIH) which can be divided into two groups based on their gene structure and characteristics with Type I including CHH and Type II including MIH,

GIH/VIH and MOIH (Chen et al, 2020). The main sequence feature that links these peptides are two arginines, an aspartic acid, phenylalanine residue and six cysteine that pair together to form three disulfide bridges with CHH having the C- and N- terminally blocked (Bocking et al, 2002).

CHH is a hormone which is notably pleiotropic and has a multifunctional role in the biological processes found in crustaceans. Apart from its first discovered role in the regulation of glucose (Parvathy, 1972), it is also involved in lipid mobilisation (Santos et al, 1997), osmo and ionoreguation (Kleinholz, 1976), regulation of ecdysteroid synthesis (Chang et al, 2010) and is a key player in the ecdysis behavioural programme (Chung and Webster, 2003).

In response to stress conditions, carbohydrate regulation occurs in order for energy to be provided. For this to happen CHH targets tissue in the hepatopancreas and the muscle with different processes taking place in each tissue to increase the production of energy. In the hepatopancreas, glycolysis and lipolysis take place (Li et al, 2017). In the muscles, glycolysis, the TCA cycle, stimulation of the pentose phosphate pathway flux and an increase in amino acid biosynthesis allow more carbohydrates to be utilised (Li et al, 2019). The metabolic effects of CHH relies on and is driven by the metabolism of the nicotinamide coenzymes NAD+ and NADP+, also known as the nicotinate and nicotinamide metabolism. As CHH targets the tissue in the hepatopancreas and muscle it decreases the glycogen accumulation which results in an increase in levels of glucose. In the hepatopancreas lipids are also produced through lipolysis. The glucose and lipids move from the hepatopancreas to the haemolymph where it is then taken up by the muscle. The stimulation of nicotinate and nicotinamide metabolism by CHH provides NAD^+ and $NADP^+$ which are the nicotinamides which drive glycolysis, the TCA cycle and the pentose phosphate pathway. This then results in an increased ATP supply which can be used by the crab in reaction to any environmental stressors (Li et al, 2017). An outline of this process is demonstrated in Fig.1.

When the gene expression of CHH is silenced, it has been observed to have a significantly negative impact on the glycolytic flux process (Li et al, 2017). The metabolic effects produced by CHH are not limited to glycolysis with synthesis of NAD+ and adenine

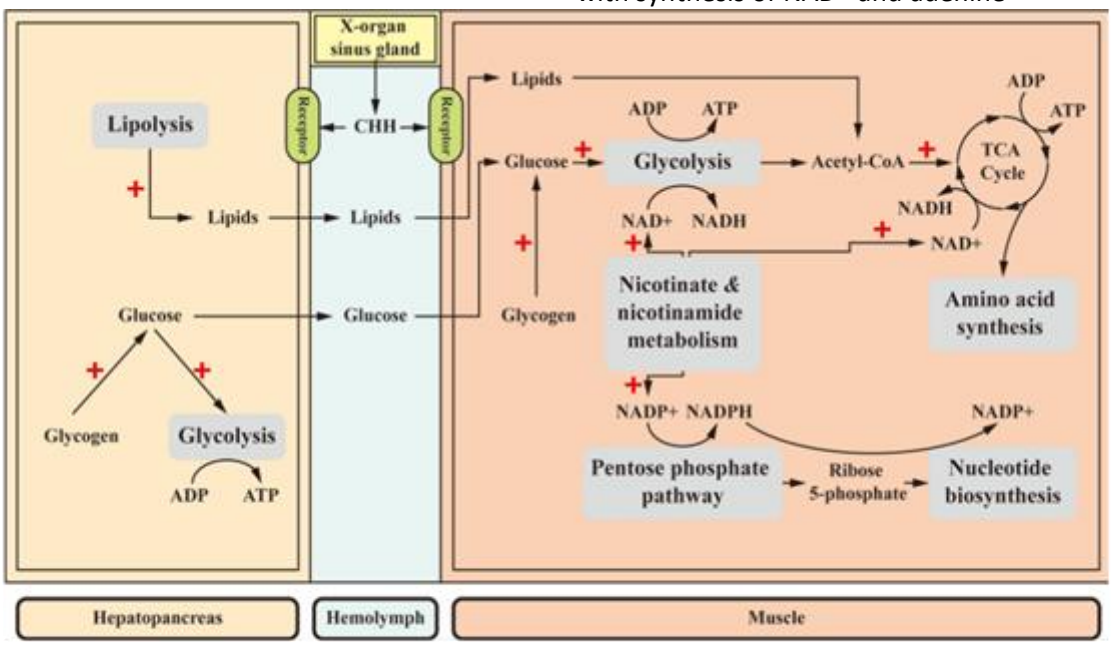

Fig. 1. The metabolic pathways induced by CHH in the hepatopancreas and the muscle. (Li et al, 2017) (Li et al, 2019) Diagram created and taken from Chen et al, 2020

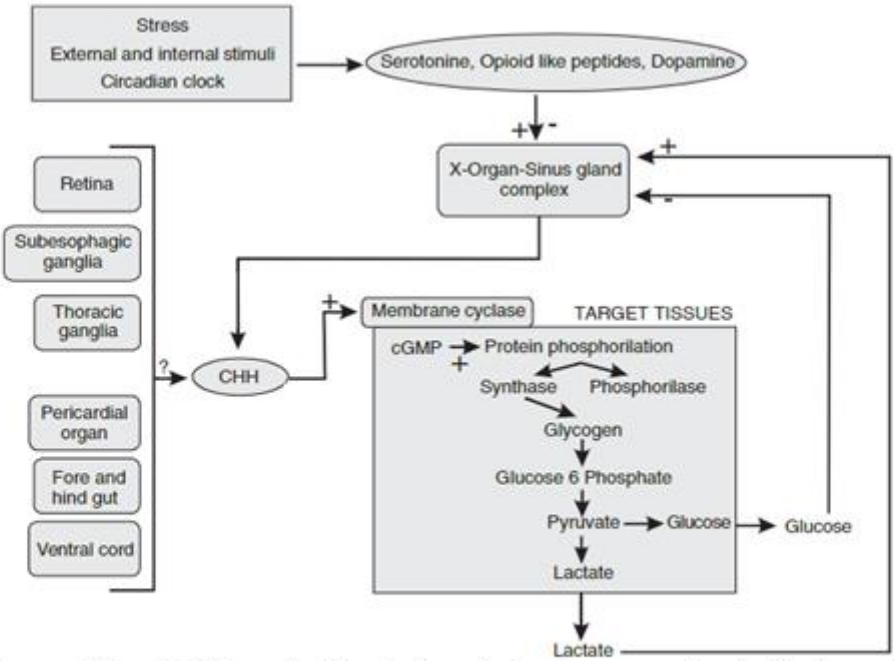

Fig. 2 Illustration of the regulation of CHH by a dual feedback control system: a negative feedback caused by glucose and a positive feedback by lactate as described by Santos and Keller, 1993a. Illustration taken from Fanjul-Moles M.L., 2006.

ribonucleotides being important factors needed to support the metabolic roles. Li et al, 2017 noted that 5-phosphoribosyl 1-pyrophosphate is a product of 5-phosphoribosyl 1-pyrophosphate synthesis and is a precursor in the synthesis of ribonucleotides and $NADP^+$, so it could be possible that CHH could regulate these biosynthetic pathways by affecting certain enzymes such as 5-phosphoribosyl 1-pyrophosphate synthase.

Adaptive mechanisms of CHH have been suggested to work via a dual feedback control system (Santos and Keller, 1993a). Glucose hemolymph produces a negative feedback loop with the CHH-producing neurons in the X-organ sinus gland complex. Once the CHH release has induced hyperglycemia, the D-glucose activates a K^+ current causing the hyperpolarisation of the CHH secreting cells and the release of CHH is inhibited. After, hypoglycemia occurs the CHH secreting cells depolarise which induces the release of CHH and allows glucose to be mobilised from glycogen (Glowik et al, 1997). Lactate produces a second feedback loop but this time it is a positive feedback loop, the increase in lactate as a result of an increase in glycolytic flux could cause a release in CHH which in turn stimulates glycogenolysis (Santos and Keller, 1993a). This process can be seen illustrated by Fig. 2.

Additional evidence shows that another function of CHH being regulation of osmoregulation and ionoregulation. When injected with CHH the freshwater crayfish, *Astacus leptodactylus* showed a greater increase in hemolymph osmolality and Na^+ concentration which was also seen in the American lobster, Homarus americanus (Serrano et al, 2002). In the crab, *Pachygrapsus marmoratus*, CHH was found to prompt an Na+ influx and a trans-epithelial electrical potential difference in the posterior gills (Spanings-Pierrot et al, 2000) and was then seen to restore Na^+ and K^+ levels in the hemolymph of the freshwater crayfish, *Cherax quadricarinatus*, after the levels had decreased following exposure to low temperature and high salinity stress (Prymaczok et al, 2016). The role CHH plays in osmoregulation and ionoregulation is also important when crustaceans undergo ecdysis to enable growth and an increase in animal

size. When the shore crab, *Carcinus maenas*, has been injected with physiologically relevant levels of CHH it was seen that ecdysis occurred sooner than in saline injected control crabs, then when even greater levels of CHH were injected, the crabs became trapped in their old exoskeleton due to the expansion in the new tissue. It was suggested that CHH stimulated ion and water uptake in the crab as ecdysis relied on a high-water uptake in the gut which was needed to cause swelling in the body (Chung et al, 1999).

Other neuropeptides belonging to this family have also been found in non-crustacean species. The first of these peptides to be identified was the ion transport peptide (ITP) which was isolated from the desert locust, *Schistocerca gregaria* (Audsley et al, 1990). In the same insect, ITP is released from the *corpora cardiaca* and drove water reabsorption by prompting the ileum to transport Cl^- ions from the lumen into the hemolymph (Audsley et al, 1992). Further studies on the primary structure and cDNA found that it was similar in structure to CHH and was therefore placed in the Type I group (Meredith et al, 1996). ITP has been shown to respond to osmotic stress and has an impact on osmoregulation similarly to CHH. In the fruit fly, *Drosophila melanogaster*, it was suggested that ITP promotes water and ion reabsorption in the ileum and rectum when urine enters the hindgut and mixes with the gut contents, which is directly analogous to the situation in *Carcinus maenas*. It was also found that ITP enhanced water retention in the fruit fly by encouraging thirst, leading to an increase in water uptake by increased drinking, and by inhibiting the need to eat dry food, this combined helped reduce the risk of dehydration (Galikova et al, 2019).

These hormones in invertebrates can be a struggle to test for in real time, however, crustaceans are the easiest of species to use when required for the hormones to be measured. With the similarities between the neuropeptides in insects and crustaceans, experiments regarding these hormones in crustaceans could be used as a basis to theorise how similar hormones in the same family could act in insects and other invertebrates.

The crustacean hyperglycemic hormone is a vital neuropeptide involved in various biological processes in crustacean. Despite its importance, there is limited knowledge about the precise timing of CHH release in response to environmental stressors. The study aims to investigate the exact time of CHH release and the magnitude of its increase within a 10 minute window after exposure to different environmental stressors in the shore crab, *Carcinus maenas*. A short timeframe of 10 minutes has been chosen to better understand the rapid response mechanisms of shore crabs to environmental stress and how CHH plays a role in their immediate physiological adaptations and provide valuable insights in the early stages of stress response.

The following hypotheses can be drawn prior to conducting the study:

H0: There will be no significant increases in the CHH levels at the 5 min and 10 min mark after exposure to temperatures of 20^{o}C, 25^{o}C and 30^{o}C.
H1: There would be a slight increase in the levels of CHH at 20^{o}C due to the moderate increase in temperature, there would be a greater increase than this at 25^{o}C due to this temperature being a significant increase from the control temperature and then the greatest significant increase would occur at 30^{o}C as this would be an extreme increase in temperature compared to the control group.

H0: There will be no significant difference in CHH levels at the 5 min and 10 min mark after exposure to temperatures of 10^{o}C and 5^{o}C.
H1: There would be a slight increase in the levels of CHH at the 10^{o}C mark similar to that seen at 20^{o}C as both are a 5^{o}C difference from the initial acclimatised temperature.
A significant increase would be seen at 5^{o}C

compared to that of the control group due to the temperature being close to the lower survivable limit.

H0: There would be no significant difference in CHH levels at 5 min and 10 min when exposed to a salinity of 10% seawater compared to the control group.
H1: There would be a significant difference compared to that of the control group due to the high osmotic stress of being close to the lower tolerance level which would prompt the rapid release of CHH to help regulate ion balance and osmoregulation.

H0: There would be no significant difference in the CHH level at the 5 min and 10 min mark when crabs were exposed to hypoxia conditions compared to the control group.
H1: There would be a significant difference in CHH levels at 5 min rising further at 10 min compared to that of the control group with the level of CHH increasing the most out of all the conditions.

H0:There would be no significant difference between the CHH levels at 5 min and 10 min in red crabs exposed to hypoxia compared to the control group.
H1: There will be a significant increase in the levels of CHH at the 5 min and further at the 10 min mark, there will be a greater increase compared to the green crabs in hypoxia as observations have reported that red crabs are less tolerance to environmental stressors so more CHH would need to be released to aid the crab in adapting to the new environment.

Materials and Method

Animals:
The Shore Crab, *Carcinus maenas*, was the choice species for this study as it is a crab species which is commonly found around the coasts of the UK.

Green shore crabs (N=12 per condition) in the early inter-moult stage were used in each of the conditions. Red shore crabs (N=12) in the late inter-moult stage were exposed to the hypoxia condition only. Eyestalk ablate crabs (N=5) were tested at the 30^{o}C temperature to demonstrate that although the majority of CHH is synthesised in the X-organ, a minor proportion of CHH is also present in the gut region of the crab.

Hemolymph samples (200 µl) were taken at the base of the leg with a hypodermic needle and syringe at 0, 5 and 10 minute intervals. Samples were then immediately placed on ice. Conditions in which the samples were taken are as follows: 1) 30^{o}C 2) 25^{o}C 3) 20^{o}C 4) 10^{o}C 5) 5^{o}C 6) 10% seawater 7) hypoxia.

Crabs were housed in a holding tank filled with salt water which was an exact replica of the water conditions of their natural environment in the Menai Straits. Prior to each experimental sessions, the crabs were acclimatised in water taken from the holding tank that was cooled to 15^{o}C, in order to mimic the environmental conditions they would naturally experience. No crabs were sampled twice as multiple sampling events can cause additional stress to the crabs which can cause further release of CHH which may not accurately reflect the response to the intended experimental conditions in the subsequent samplings.

Heat shock conditions were established by immersing a small tub filled with water from the crab holding tank into a larger tank containing hot water. The temperature of the water in the small tub was adjusted by adding hot or cold water to the tank until the desired temperature was achieved. Cold shock conditions were then placed in the small tub which contained water from their original tub. This method of heating and cooling the holding tank water ensured that salinity was not diluted by the additional water or ice used for temperature control. Alternatively, heating rods or a water bath could be employed to raise the water temperature instead of manually adjusting the water in the tank. However, it was found that heating rods took an extended period of time to heat the

water and a water bath was not available in the laboratory aquarium. Thus, the described method was chosen as a practical and efficient way to manipulate water temperature.
The 10% seawater salinity was created by mixing one part water from the original crab holding tank with nine parts freshwater. The water temperature was then measured and was brought to 15^{o}C so it matched the water temperature the crabs would normally reside in.
Hypoxia conditions were induced by placing the crabs in a shallow tray out of water and encouraging them to move continuously for the duration of 10 minutes. This exposure to air and sustained physical activity would stimulate a low oxygen environment during a high oxygen demand for the crabs, as their gills are not designed for efficient gas exchange in the air.

There was a risk of decreasing the oxygen concentration at the higher temperatures and stressing the crabs with hypoxia alongside the heat stress conditions. Oxygen was passed through the tanks throughout to prevent this from occurring.

Laboratory experiments:
The purification method and analysis method are the same as the one used in Morris et al, 2010. First, the samples needed to be purified. Samples were thawed on a centrifuge (10 min, 4^{o}C, 10,000G), then 200µl of haemolymph samples were diluted 1:1 with water. Using a 16-port vacuum manifold 3ml 100% isopropanol and 10ml water were passed through Strata-X 3um Polymeric Reverse Phase 200mg/3ml tubes (Phenomenex) at 5 InG. This was followed by haemolymph samples, then washed with 10ml water. The tubes were then left to dry completely for 1 minute before 30ml 50% isopropanol was passed through and dried on a vacuum centrifuge. As samples were drying, plates were coated with 100µ l/well 10µg ml^{-1} anti-CHH IgG in 0.1mol l-1 sodium bicarbonate buffer pH 9.3 and left overnight at 4^{o}C. Plates were then washed 3x with sodium bicarbonate buffer and left for 1hr with 300µl/well blocking buffer. A standard CHH dilution series (50 fmol-50 amol per 100µl) was added in duplication to the first two rows of wells and a duplication of the purified hemolymph samples dissolved in assay buffer were placed in the remaining rows of wells. Plates were then left overnight at 4^{o}C. Next, the plates were washed (5x) using wash buffer and incubated (6hr, room temperature) in 100µl/well biotinylated anti-CHH IgG (5µg ml^{-1}). Again, the plates were washed (5x) and incubated (2hr, room temperature in darkness) in 100/well Europium-labelled streptavidin (PerkinElmer) diluted in assay buffer (PerkinElmer) to 100 ng ml^{-1}. Plates were washed (5x) and 50µl/well enhancement solution (PerkinElmer) was added, then underwent orbital shaking (2 minutes) using a shaking machine. A PerkinElmer Victor^{2} 1420 instrument and software for data analysis measured the time resolved fluorescence of the Europium in the wells of the plates.

Statistical Analysis:
Analysis and descriptive statistics were performed using SPSS 28.0. Paired t-test was the choice of statistical test performed to find the significant differences between samples. SigmaPlot 13.0 was the software used to create the graphs.

A standard curve is shown in Fig. 3 and was made up from pre-determined concentrations of CHH (50 fmol-50 amol per 100µl). Three orders of magnitude were covered and the assay detection limit was less than 50 amol/well.

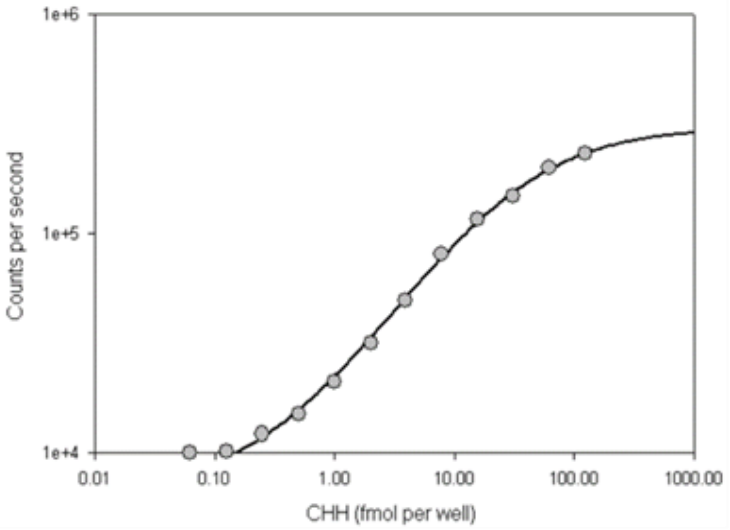

Fig. 3. The standard curve made from pre-determined concentrations of CHH (50 fmol amol per 100ul)

Results

The experiments were undertaken to determine the effects different conditions had on the release levels of CHH within a 10 minute period. The conditions were chosen to mimic conditions which the crabs would experience in their natural habitat. The laboratory crabs used were acclimated to temperatures of 15°C, a temperature similar to that of their natural environment (Klassen and Locke, 2007).

Heat Shock

The results for the heat shock condition are shown in Fig.4. These temperatures were used to reflect temperatures the crabs could experience in the higher intertidal waterline and should they become caught in rockpools (Rob Mc Allen et al, 1999).

At 30°C there was a significant increase ($p = 0.01$) from 0 min to 5 min in the treated crabs (t=0, 21.8 ± 4.78 pmol^{-1} to t=5, 53.6 ± 6.83 pmol^{-1}), there was then an increase from 5 min to 10 min (53.6 ± 6.83 pmol^{-1} to 61.2 ± 3.04 pmol^{-1}) however, there was no significant difference ($p>0.1$) in the increase. Significant differences ($p<0.005$) are present between the control and treated crabs at 5 min (control, 16.4 ± 4.6 pmol^{-1}; treated, 53.6 ± 6.83 pmol^{-1}) and also at 10 min ($p=0.001$, control, 17.3 ± 5.12 pmol^{-1}; treated, 61.2 ± 3.04 pmol^{-1}). As expected, there was no significant difference in the levels of CHH at 0 min between the control and treated crabs.

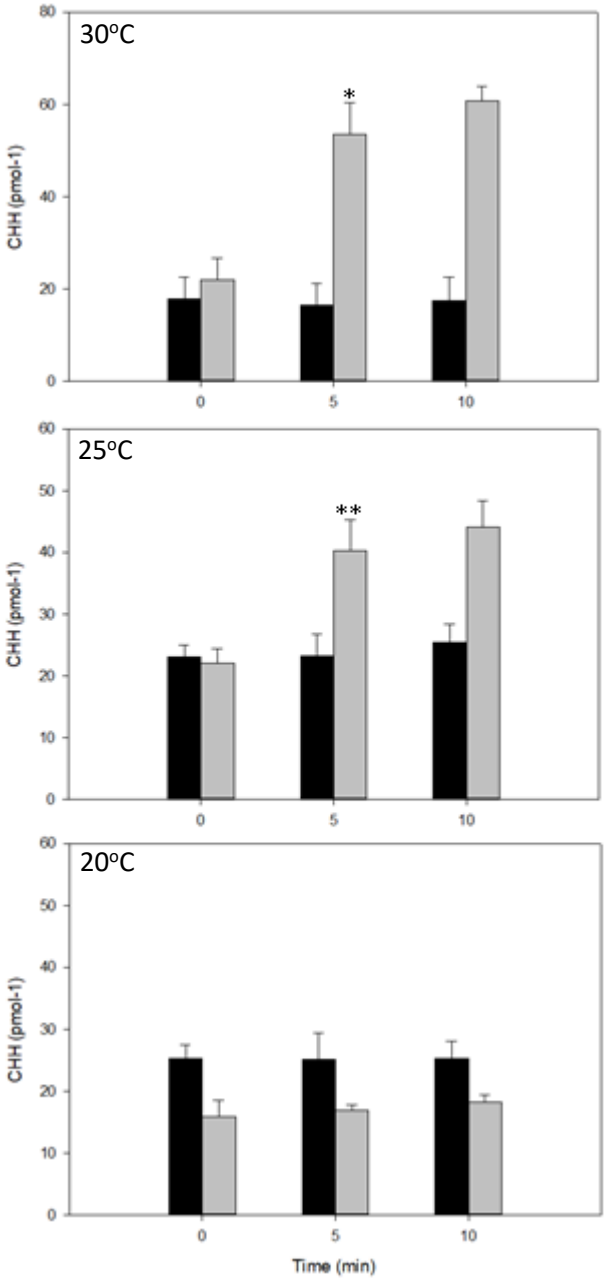

Fig. 4. Heat shock at temperatures 30°C, 25°C and 20°C. Black columns show the control crabs kept at their lab acclimated temperature of 15°C, grey columns show treated crabs (means + s.e.m). Asterisks show the significance of the increase from the previous time, *p<0.5, **p<0.005, ***P<0.001

At 25°C there was again a significant increase ($p<0.005$) at the 5 min mark from 0 min (t=0, 22.0 ± 2.36 $pmol^{-1}$ to t=5, 40.3 ± 4.94 $pmol^{-1}$) and an increase from 5 min to 10 min (40.3 ± 4.94 $pmol^{-1}$ to 43.9 ± 4.37 $pmol^{-1}$) however this was not a significant increase ($p>0.4$) and was a smaller increase than at 30°C between the same times. As was found at 30°C, there are significant differences found between the control and treated crabs at 5 min ($p<0.005$, control, 19.1 ± 1.16 $pmol^{-1}$; treated, 40.3 ± 4.94 $pmol^{-1}$) and 10 min ($p<0.05$, control, 24.9 ± 3.64 pmol-1; treated, 43.9 ± 4.37 $pmol^{-1}$) but no significant differences ($p>0.5$) at 0 min.

The results at 20°C show a different trend than is seen at 25°C and 30°C. There are no significant increases ($p> 0.4$) between the levels of CHH at 0 min to 5 min and 5 min to 10 min for either the control crabs or the treated crabs. However, there is a significant difference ($p< 0.05$) in the levels of CHH between the control crabs and the treated crabs at each individual time. The reason for the elevated levels of CHH in the control crabs could be attributed to the crabs becoming stressed from handling when haemolymph samples were being taken.

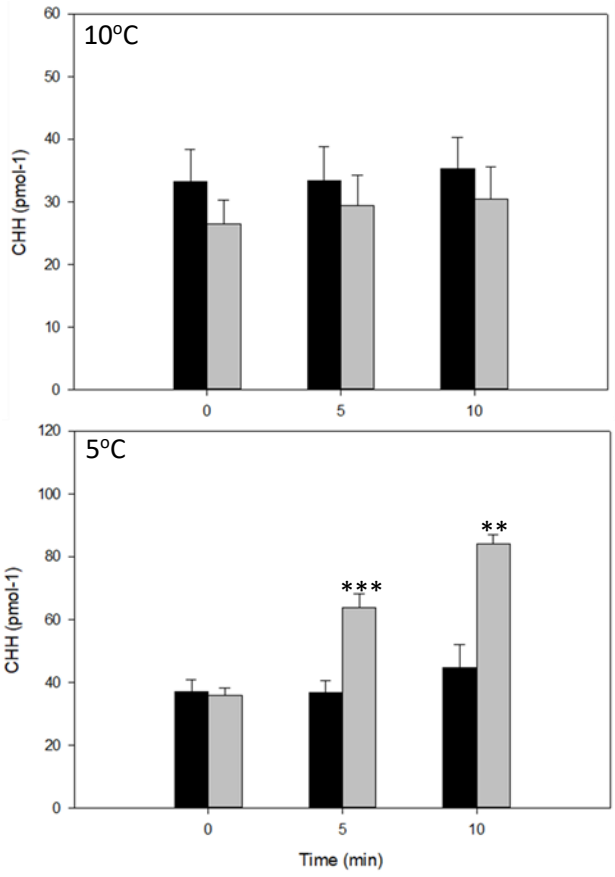

Fig. 5. Cold shock at temperatures 10°C and 5°C. Black columns show the control crabs kept at their lab acclimated temperature of 15°C, grey columns show treated crabs (means + s.e.m). Asterisks show the significance of the increase from the previous time, *p<0.5, **p<0.005, ***P<0.001

Cold Shock

Fig.5 shows the results which were obtained from the cold shock conditions. Only two temperatures were tested here as it was in keeping with the 5°C changes between temperatures whilst not exceeding the lower survivability limit of the crabs (Eriksson and Edlund, 1977).

The trend at 10°C is similar to that at 20°C. There was no significant increase ($p>0.1$) in the levels of CHH between each time for the control crabs or the treated crabs, with levels remaining at an almost constant rate. However again, there is a significant difference ($p<0.05$) between the control crabs and treated crabs at each time. This could again be attributed to the control crabs being stressed from the handling.

At 5°C the treated crabs seem to have a stronger reaction similar to the 30°C and 25°C temperatures. In the treated crabs there was a highly significant increase ($p=0.00$) from 0 min to 5 min (35.8 ± 2.23 $pmol^{-1}$ to 63.7 ± 4.53 $pmol^{-1}$) and again another highly significant increase ($p<0.005$) from 5 min to 10 min (63.7 ± 4.53 $pmol^{-1}$ to 84.2 ± 2.81 $pmol^{-1}$). There was a highly significant difference between CHH levels in the control and treated crabs at both 5 min ($p=0.00$; control, 38.9 ± 4.53 $pmol^{-1}$; treated, 63.7 ± 2.23 $pmol^{-1}$) and 10 min ($p<0.005$; control, 48.8 ± 8.49 $pmol^{-1}$; treated, 84.2 ± 2.81pmol-1). While the increase in the CHH levels for this temperature follows a similar trend to that seen at 30°C and 25°C, the levels of CHH released in the treated crabs are higher than the levels of CHH released in the treated crabs at the heat shock temperatures.

Salinity

In this condition the treated crabs were subject to 10% seawater which can be seen in Fig.6.

This condition did not show a large increase in the levels of CHH in the treated crabs from 0 min to 5 min ($p=0.2$; 17.4 ± 1.33 pmol-1 to 20.7 ± 2.88 pmol-1) and levelled off at 5 min to 10 min ($p>0.5$; 20.7 ± 2.88 pmol-1 to 21.4 ± 3.92 pmol-1). Even though there was an increase at the 5 min and 10 min mark this was not a significant difference ($p>0.1$) compared to the levels of CHH in the control crabs ($t=5$; control, 15.4 ± 1.06 $pmol^{-1}$; treated, 20.7 ± 2.88 $pmol^{-1}$) ($t=10$; control, 15.6 ± 1.18 $pmol^{-1}$; treated, 21.4 ± 3.92 $pmol^{-1}$). Due to there not being a notable significant reaction from the crabs in this salinity, tests at 20% SW or 30% SW were not attempted as it was concluded that a minimal reaction would take place. Lower salinities were not tested as 10% SW was already at the extreme lower level of survivability for the crabs.

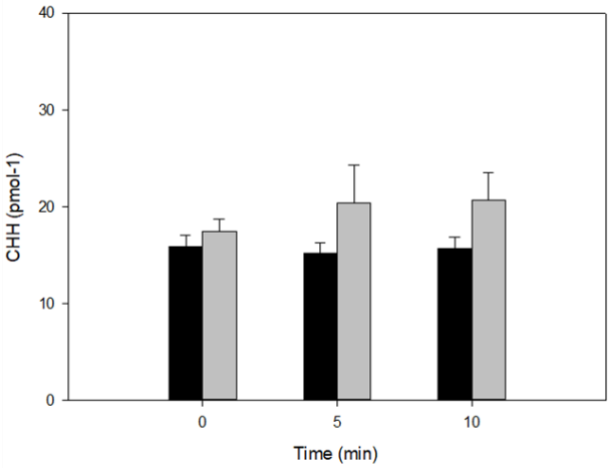

Fig. 6. Salinity at 10% SW. Black columns show the control crabs kept at their lab acclimated temperature of 15°C, grey columns show treated crabs (means + s.e.m). Asterisks show the significance of the increase from the previous time, *p<0.5, **p<0.005, ***P<0.001

Hypoxia

Crabs could naturally be exposed to a degree of hypoxia when they migrate into the intertidal zone at high tide and potentially trapped in a rock pool (Agnew and Taylor, 1986).

The release levels seen in Fig. 7 were similar to levels seen at 30°C and follows the same trend. There is a highly significant ($p<0.005$) increase from 0 min to 5 min (24.3 ± 2.63 $pmol^{-1}$ to 44.2 ± 4.58 $pmol^{-1}$) which is then followed by a less significant increase ($p<0.05$) to 10 min (44.2 ± 4.58 $pmol^{-1}$ to 58.9 ± 7.02 $pmol^{-1}$). This increase at the 5 min mark is a greater significant difference ($p=0.001$) between the control levels than the 30°C (control, 18.2 ± 1.06 $pmol^{-1}$; treated, 44.2 ± 4.58 $pmol^{-1}$) and there is a greater significant difference ($p=0.00$) at 10 min (control, 20.1 ± 1.93 $pmol^{-1}$; treated, 58.9 ± 17.02 $pmol^{-1}$).

Red shore crabs were also tested with this condition to explore how the release and levels of CHH would change at a different stage in the moult cycle. It was found that the

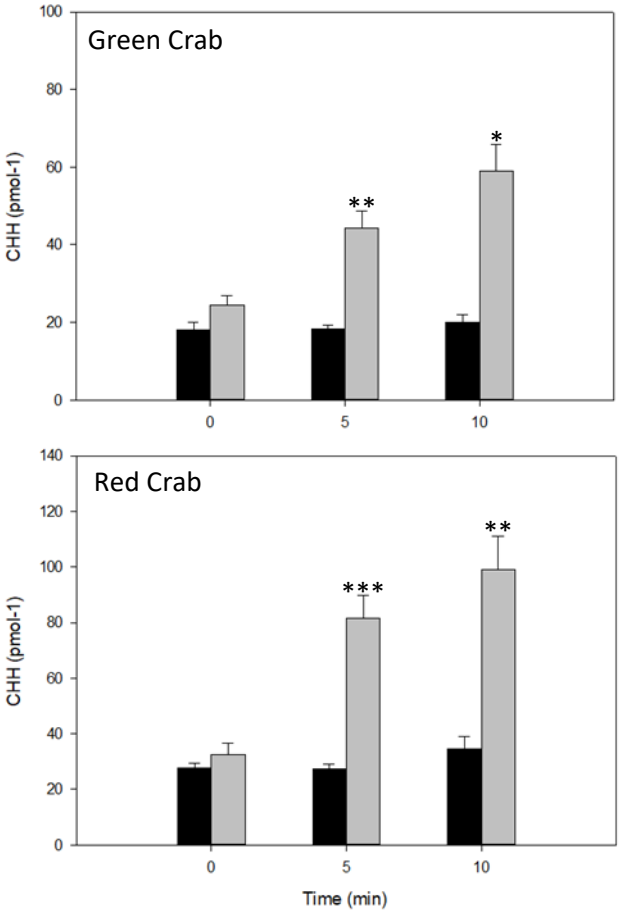

Fig. 7. Hypoxia for green shore crabs and red shore. Black columns show the control crabs kept at their lab acclimated temperature of 15°C, grey columns show treated crabs (means + s.e.m). Asterisks show the significance of the increase from the previous time, *p<0.5, **p<0.005, ***P<0.001

highest levels of CHH were released out of all the other conditions tested. There was the highest significant increase (p=0.001) from 0 min to 5 min (32.5 ± 3.99 $pmol^{-1}$ to 81.7 ± 8.21 $pmol^{-1}$) and also for 5 min to 10 min (p<0.005; 81.7 ± 8.21 $pmol^{-1}$ to 109.1 ± 6.78 $pmol^{-1}$). The significant difference between the control and treated crabs at 5 min and 10 min had the same p values as the green crabs in hypoxia (t=5; p=0.001; control, 28.2 ± 1.29 $pmol^{-1}$; treated; 81.7 ± 8.21 $pmol^{-1}$) (t=10; p=0.00; control, 34.6 ± 4.28 $pmol^{-1}$; treated, 109.1 ± 6.78 $pmol^{-1}$). Compared to the green crabs in hypoxia there was a highly significant increase at 5 min (p=0.00) and 10 min (p=0.001) for the red crabs.

Eyestalk Ablated Crabs

The levels of CHH were tested in eyestalk ablated crabs against treated crabs at 30°C. This allowed it to be shown that even though the majority of CHH is produced in the X organ, there is a small percentage of CHH which is produced in a different area of the crab. At 0 min there was no significant difference (p>0.9) between the control, eyestalk ablated or treated crabs. At 5 min, as expected the levels of CHH in the treated crab increased significantly (p<0.01; 17.3 ± 3.48 $pmol^{-1}$ to 55.1 ± 6.12 $pmol^{-1}$) this was a significant difference (p= 0.05) between the eyestalk ablated value which had not increased significantly (p>0.5; 16.8 ± 2.08 $pmol^{-1}$ to 16.9 ± 2.53 $pmol^{-1}$). At 10 min there was no significant difference (p>0.5) between the eyestalk ablated crabs and the control, nor was there a significant increase (p>0.5) in the eyestalk ablated crabs from 5 min (16.9 ± 2.53 $pmol^{-1}$ to 17.9 ± 3.98 $pmol^{-1}$). The further increase in the treated crabs created an even more significant difference (p<0.05) between the eyestalk ablated value. While the increase in the levels of CHH at 5 min to 10 min in the eyestalk ablated crabs was not significant, the small increase still shows that there is an area in the anatomy of a crab that can still respond to stressors in the environment however is unable to respond as effectively as the X organ.

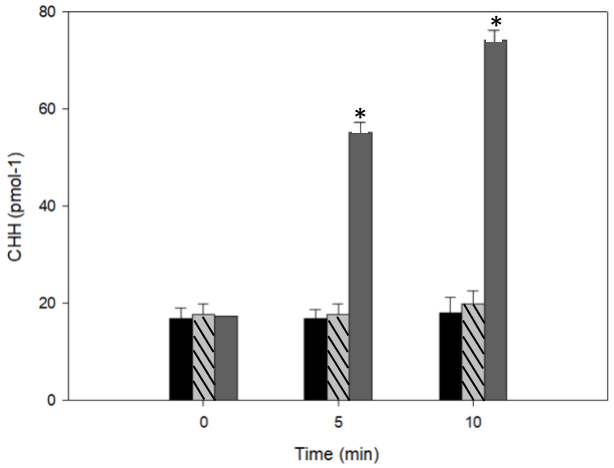

Fig. 8. CHH levels in eyestalk ablated crabs. Black columns show the control crabs kept at their lab acclimated temperature of 15°C, striped columns show eyestalk ablated crabs and grey columns show treated crabs (means + s.e.m). Asterisks show the significance of the difference between the eyestalk ablated crabs and the treated, *p<0.5

Discussion

Shore crabs were the choice species of crab as they were easily accessible due to the proximity of the Menai Straights and due to their high levels of tolerance. Shore crabs are able to withstand temperatures from 3°C to 35°C (Eriksson and Edlund, 1977). Being efficient osmoregulators meant that they had a tolerance for very low % sea water salinity, allowing for 10% SW to be tested (McGaw et al, 1999).

In the heat shock condition, the results at 30°C and 25°C followed the expected hypothesis where the levels of CHH had a significant increase before the 5 min mark. At the two higher temperatures of 30°C and 25°C this response was expected as *Carcinus maenas* need to react to thermal changes in their natural environment due to being an intertidal organism (Crothers, 1968).

The effects of thermal stress on CHH measured in Chung and Webster, 2005 found that the response was asymmetrical with the hyperthermic temperatures of 5°C and 10°C having significant increases at 5 min, whereas the temperatures in the hypothermic region only had the significant increase at 30°C and

35°C. However, in this study the opposite asymmetry was found with 25°C and 30°C having a significant increase but only 5°C having a significant increase. Both studies found that at 20°C there was no significant increase. A greater hyperglycemic response occurred at lower temperatures in the tropical palaemonid prawn, *Macrobranchium rosenbergii*, which supported the results found in Chung and Webster in that conditions outside of the normal environmental experience elicited more of a significant increase in the levels of CHH (Kou and Yang, 1999). The significance of the CHH increase in the hypothermic temperatures could be explained by the fact that *Carcinus maenas* is an intertidal organism which encounters regular daily changes to the temperatures in its environment due to the changing tides (Klassen and Locke, 2007). *Carcinus maenas* exhibit refuge behaviour when exposed to higher temperatures and regularly move to covered areas to reduce any effect of evaporative loss, CHH could be released as the crab exhibits this behaviour in order for energy to be provided via hyperglycemia enabling the crab to quickly move away from the stressor. The 20°C temperature was a 5°C increase from the water the crabs had been acclimated in and as has already been stated did not give a significant increase in CHH levels. This was also found by Chang, 2005 who also commented that there were no significant changes to haemolymph CHH following a 5°C temperature increase in the lobster *Homarus americanus*.

Interestingly, the results found in the low salinity stressor did not produce a large response from the crabs with there being a slight increase at 5 min however this was not a significant increase. This then prompted this condition to be repeated again with a new set of crabs however this did not yield different results. This was surprising as the low salinity was close to the lethal limit (Cohen and Carlton, 1995). The crabs would also experience different salinities in their natural environment so would be expected to have a response. CHH has been found to be involved in osmoregulation with the gill membrane having the highest receptor density compared to other binding sites (Chung and Webster, 2006). Significantly increased transepithelial potential difference and an increased Na+ influx of about 50% occurred when physiologically relevant CHH or higher levels were injected (Chung et al, 1999). A further low salinity stress test showed the Pt-CHH2 gene responded quickly to this type of stress and remained at a high level in the gills. Similar results to this were also reported in the mud crab, *Scylla paramamosain*, and the swimming crab, *Portunus trituberculantus*, where CHH2 gene expression was highly observed in low salinity stress (Miaoan et al, 2012; Sun et al, 2019). These increases in CHH can be explained by the fact that osmoregulation is a very energy demanding process and CHH increases the availability of metabolised energy through the increase of glycogenolysis which can then be employed by the ion-exchange pumps in the gills (Spanings-Pierrot et al, 2000).

A study by Turner et al, 2013 found that two forms of CHH were present and were utilised throughout different parts of the year in the Christmas Island Blue Crab, *Discoplax celeste*. CHHa significantly increased the Na^+ uptake in the gills in the dry season and CHHb significantly increased the Na^+ uptake in the wet season. In the dry season, Na^+ was in a higher concentration due to the lower volume of urine being produced and filtered into the brachial chambers, CHHa would be acting on the gills at this time to maximise the Na^+ uptake, whereas in the wet season the volume of urine produced and excreted was higher meaning a greater number of ions would be lost, an increase in CHHb was needed to increase the rate of Na^+ uptake to compensate. Turner et al, 2013 stressed the importance of the need to minimise salt loss especially during monsoon downpours which

can cause salt depletion in the environment. From this it would be expected that the levels of CHH in low salinity would increase to allow *Carcinus maenas* to conserve ion loss which raises questions as to why it did not happen in two separate instances. However, in Chung and Webster, 2006 when the measurement of CHH in *Carcinus maenas* was taken at 30 min after exposure to dilute seawater no elevated titres of CHH were seen. It was theorised that because *Carcinus maenas* was a strong hyper regulator it was able to adapt quickly to the dilute conditions. They then further theorised that a rapid release of CHH could occur, which would not be able to be detected at 30 min due to the short half-life of CHH, to initiate a signalling cascade but would not need prolonged elevated levels of CHH for any long-term adaptations. The results in this current study show that a release of CHH does not in fact occur when *Carcinus maenas* is exposed to dilute seawater. This contrast in the lack of release of CHH compared to other studies could be explained by the fact that CHH release could be related to life history strategy as also suggested in Chung and Webster, 2005. The Christmas Island Blue Crab, *Discoplax celeste*, used in the study of Turner et al, 2013 is a terrestrial crab which resides in freshwater springs inland then undertakes a breeding migration to the ocean in February to March (Turner, 2014). One of the challenges terrestrial crabs face is the loss of ions rather than the loss of water, to combat this the gill number and planar gill surface area is reduced compared to that of water breathing crabs and is primarily used for the regulation of ions and the reprocessing of urine (Morris S, 2001). The high urine rates in *Discoplax celeste* would be energetically expensive and would require large amounts of CHH to be produced to avoid the loss of ions in the freshwater something which would not be seen in *Carcinus maenas* as it spends its life cycle in a higher salinity marine environment. The stress response at a low salinity is also not seen at a transcriptional level. Towle et al, 2011 found that when *Carcinus maenas* was exposed to 10ppt seawater, genes encoding for stress proteins remained largely unaffected. They concluded that this could be because the species was well adapted for high tolerances. However, it has been commented that the limited ability to osmoregulate and therefore limited metabolic adaptation to salinity stresses would only increase CHH by a small amount which goes against the argument that *Carcinus maenas* doesn't react due to being able to adapt well (Chang et al, 2005).

The lack of response from CHH can also be explained due to a delay in Na+/K+ ATPase activity which causes osmoregulatory changes. At low salinities crustaceans must change from osmoconformity to osmoregulation in order for the hemolymph osmolarity to be maintained above that of the surrounding seawater (Henry, 2005). Na+/K+ is a trans-membrane protein localised to the basolateral membrane in the gill, believed to assist in the active transport of Na+ ions into the hemolymph from the branchial intracellular compartment by generating an electrochemical gradient (Henry et al, 2012). When *Carcinus maenas* was transitioned from 32 ppt to 10 ppt an increase in Na+/K+ ATPase activity has been observed at 24 hr – 48 hr post transfer (Henry et al, 2002). Energy would be needed to be provided in the form of ATP to assist in the active transport of ions, CHH release would aid in this process by regulating glucose levels in the hemolymph (Spannings-Pierrot et al, 2000). This activity of Na+/K+ ATPase at least 24 hr after transfer to a low salinity gives an indication that CHH would not be able to be measured with the 10 min window in this study as it would not yet be released. The effect of CHH has on osmoregulation is not a process which is fully understood so further research could be undertaken to compare how the levels of CHH leading up to and beyond a 24 hr period compares to Na+/K+ ATPase activity when a crab has been exposed to an environment of extreme salinity.

As expected, there was a significant increase at 5 min for the hypoxia condition. It is typical for glucose to be released from carbohydrate stores by CHH in the anaerobiosis of crustaceans so a substrate can be provided for glycolysis and lactate formation (Chang et al, 1998). The results here are in line with Webster,1996, Chang et al, 1998 and Chang, 2005 with Webster, 1996 finding a significant increase after 15 min and Chang, 2005 after 20 min. It has been shown that CHH released in response to a hypoxia stressor only induces release from the eyestalk neuroendocrine cells, confirmed by Chung and Webster, 2005 which found no evidence of hypoxia-induced CHH in eyestalk ablated crabs.

Red shore crabs which had spent a longer time in the inter-moult stage were also tested in the hypoxia condition to give a basis for further research into how different stages of the moult cycle would affect the release of CHH in different conditions. Interestingly, red crabs had the most significant increase at 5 min out of all the other conditions tested, with a further significant increase again at 10 min. Shore crabs migrate daily into the intertidal at high tide whereas some individuals remain at low tide (Crothers, 1968). At this point it is likely the crabs can be exposed to hypoxia, mainly if the crabs are trapped in rockpools (Agnew and Taylor, 1986). Observations in Crothers, 1968 saw that red crabs were less common in the intertidal area which led him to hypothesise that red crabs were not able to adapt to hypoxia changes as easily as green crabs. Later studies found that when there was lower oxygen tension, red crabs extracted less oxygen from water than green crabs and were less able to compensate for the hypoxia (Aldrich and Reid, 1989; Reid and Aldrich, 1989). In response to hypoxia, *Carcinus maenas* has been observed to exit from water and perform the behaviour 'bubbling'. This is a process in which the crab takes air into the water filled branchial chamber to increase oxygen availability (Reid et al, 1997). Reid and Aldrich, 1989 saw that when oxygen free nitrogen was added to water to lower the oxygen tension red crabs escaped from the water and began the 'bubbling' behaviour earlier than green crabs. This same study also observed that the red crabs died more easily and rapidly than green crabs in the hypoxic conditions, this explains why in their natural environment they avoid the intertidal zone. The highly significant increases in the CHH levels for the red crabs could now be explained by the fact that the red crabs are less able to adapt to the stress of hypoxia. Further research could now be undertaken to see if other stressors such as thermal shock and salinity produce the same levels of CHH release. It could be theorised that the CHH levels could be higher as it has been found that red crabs are not as good as green crabs at maintaining haemolymph osmolarity in low seawater concentration and migrate away faster to higher salinities (Reid et al, 1993; McGaw and Naylor, 1992).

The majority of the significant increases occurred at the 5 min mark but there were three further significant increases at the 10 min mark at 5^{o}C and for both the green crab and red crab hypoxia. While these increases could be from having a stronger reaction to the stressors, CHH would not increase further due to the hormone being released episodically with the circulation half-life of 5-10 min (Chung and Webster, 2005). An increase at 5 min followed by increases at 10 min is reflected in other studies also measuring CHH and observed that the levels of CHH peaked at 10 min after exposure to a stressor followed by a decrease (Webster et al, 2012).

This study set out to explore and determine the precise release time of the crustacean hyperglycemic hormone when *Carcinus maenas* was exposed to the different environmental stressors of temperature, salinity and hypoxia. For the green shore crabs there were significant increases at the 5 min min mark for temperatures of 30^{o}C, 25^{o}C, 5^{o}C

and for the hypoxia condition. No significant changes were seen in response to low salinity which most likely is due to a osmoregulatory response being produced long after the 10 min measurement period. Red shore crabs were tested in the hypoxia condition to give an initial research insight into whether there are life stage specific differences in the levels and release time of CHH. Further research could potentially be conducted to compare temperature and salinity conditions on red crabs against that of green crabs. Measuring CHH within 10 min of a crab being exposed to an environmental stressor can advance our understanding of the neuropeptide in several ways. Assessing CHH levels within a short timeframe can help us to determine the role of CHH in the crabs' stress response and aid us in understanding how quickly shore crabs can mobilise the hormone, if there are any time dependent variations in the release pattern and give a deeper understanding of the different mechanisms through which CHH is released and what other mechanisms it can affect, this in turn can show how it contributes to their adaptation and survival in changing environments. This can also provide a basis to allow us to compare the stress response between different crab species potentially providing insights into the evolutionary and developmental aspects of stress response. There could also be potential to use the rapid release of CHH as a bio marker for environmental monitoring. Assessing CHH levels in shore crabs could provide information of the health of coastal ecosystems and their susceptibility to environmental stressors.

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