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Integrated responses of crustaceans inhabiting estuaries to the challenges of feeding and digestion in low salinity

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INTEGRATED RESPONSES OF CRUSTACEANS INHABITING ESTUARIES TO THE CHALLENGES OF FEEDING AND DIGESTION IN LOW SALINITY

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ABSTRACT

Integrated Responses of Crustaceans Inhabiting Estuaries to the Challenges of Feeding and Digestion in Low Salinity

by

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Estuaries are highly productive and serve as vital habitats for numerous decapod crustacean species. However, the environmental conditions within estuaries are often highly dynamic and subject to large changes in salinity and temperature that occur on seasonal and tidal scales. Not all of the species occupying these habitats are adept in coping with changes in these environmental conditions. This dissertation describes the influence of low salinity conditions on the 1) habitat preference, 2) feeding behaviour and 3) digestive physiology of crustaceans inhabiting estuaries. I have primarily focussed on a weak osmoregulator, the Dungeness crab, *Cancer magister*, but have also compared some aspects with the blue crab, *Callinectes sapidus*, an efficient osmoregulator. Recordings of temperature, salinity and depth using archival data tags affixed to crabs in the field showed that adult *Cancer magister* spent the majority of time in deep water where they were not subjected to stressful salinity or temperature conditions. When crabs did enter into the estuary, these forays often corresponded to times of increased food abundance and crabs avoided challenging temperature and salinity conditions by exploiting the estuary during nocturnal high tides when salinities were higher and temperatures lower. In the laboratory, experiments in temperature and salinity gradients

showed that *Cancer magister* alters thermal and salinity preference behaviour in response to a food stimulus. Thus, while *Cancer magister* primarily avoids challenging environmental conditions, they may forage in these areas during times of increased food abundance. Since crabs may be foraging in low salinity, the effects of low salinity and starvation on feeding behaviour of *Cancer magister* were examined. The likelihood of feeding, the amount of food consumed and the time spent feeding were all reduced in low salinity. However, these responses were partially overridden by starvation. Removal of the sinus gland (the potential source of inhibitory hormones that regulate feeding) revealed that changes in feeding behaviour result from hormonal regulation rather than physiological limitation. Although crabs regulate food intake in response to hyposaline exposure, they may be exposed to low salinity conditions at any point in the digestive cycle. Therefore the effects that low salinity has on digestive physiology and how these effects were influenced by osmoregulatory ability were examined. In *Cancer magister*, exposure to low salinity post-feeding resulted in a prioritization towards the responses to low salinity, resulting in a reduction in oxygen uptake that corresponded to a reduced rate of gut contraction and an increase in gastric evacuation time. These reductions also corresponded with a delay in digestive enzyme secretion and a subsequent reduction in the post feeding increase in circulating free amino acids in the hemolymph. In contrast, *Callinectes sapidus*, an efficient osmoregulator, displayed a summation of the metabolic responses to low salinity exposure and digestion. Accordingly, digestive processes continued unabated in low salinity, resulting in a build up of free amino acids in the hemolymph.

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CHAPTER 1

INTRODUCTION

Background and Rationale

In order to survive, all animals must feed. However, feeding and subsequent digestion are associated with a general increase in metabolic parameters known as specific dynamic action (SDA). The effects of SDA are well known and usually controlled for in physiological experiments: animals are either starved prior to experimentation or not fed during experiments. However, in nature, animals do not starve themselves prior to environmental perturbations. If the energetic costs of digestion and assimilation of a meal are large, will an animal be able to balance the demands of other physiological systems? – Maybe not! Recently, a number of studies have been published examining the effects of digestion on other physiological systems (e.g. Legeay and Massabuau, 2000; Bennett and Hicks, 2001; Farrell *et al*, 2001; Robertson *et al*, 2002, Hicks and Bennett, 2004; McGaw, 2006a,b,c; Thorarensen and Farrell; 2006; Bernatis *et al*, 2007). This is exemplified by the recent work of Legeay and Massabuau (2000) and McGaw (2006), wherein postprandial crabs cannot maintain physiological mechanisms in low salinity, leading to an increased mortality rate.

The digestive and osmoregulatory physiology of decapod crustaceans have received a great deal of attention (Mantel and Farmer, 1983; Pequeux, 1995). These investigations have primarily focused on isolating individual physiological processes in order to further our understanding. However, to truly appreciate the intricacies of physiological costs in an ecologically relevant context, it is necessary to integrate

relevant environmental conditions as well as considering behaviours that may influence the physiology of an animal because in combination, their effects may be very different (Robertson *et al.*, 2002).

The decapod crustacean is an ideal choice for ecophysiological studies because they are highly mobile and in the course of their daily and seasonal migrations are likely to encounter many different microhabitats. Decapod crustaceans are relatively easy to collect in the field or purchase from suppliers. Most species are fairly hardy and survive well in laboratory conditions. Estuary inhabitants in particular are excellent models for such investigations due to the broad scale changes in the parameters of their physical environment.

Crustaceans inhabiting estuaries exhibit a wide range of osmoregulatory abilities and strategies. For example the spider crab, *Libinia emarginata* may be seasonally found in estuaries (O'Brien *et al.*, 1999), but is classified as an osmoconformer (Cornell, 1980). In this case exposure to low salinity may impart high levels of physiological stress. Conversely, the blue crab *Callinectes sapidus* is a very efficient osmoregulator that can survive in fresh water (FW) for extended periods (Hill *et al*., 1989). Intermediate between these two is the Dungeness crab, *Cancer magister* (Hunter and Rudy, 1975) which is classed as weak regulator.

In decapod crustaceans low salinity exposure typically results in modulation of cardiac, respiratory, and behavioural parameters that are thought to be compensatory mechanisms for maintaining the ionic composition of the haemolymph (McGaw and McMahon, 1996; McGaw and Reiber, 1998). Efficient osmoregulators such as *Callinectes sapidus* and *Carcinus maenas* show an increase in heart rate, respiration and

locomotor activity with dilution of the medium, facilitating increased oxygen uptake and active ion transport (Taylor, 1977; McGaw and Naylor, 1992; Piller *et al.*, 1995; Hume and Berlind, 1996; McGaw and Reiber, 1998; McGaw *et al.*, 1999). Weak osmoregulators such as *Cancer magister* tend to show mixed responses: oxygen uptake is unaffected by dilution of the medium (Brown and Terwilliger, 1999), however, there is an increase in heart rate (McGaw and McMahon, 1996). There is also a transient increase in locomotion (McGaw *et al.*, 1999). The lobster, *Homarus americanus*, shows a reduction in heart rate similar to osmoconformers (Cornell, 1973, 1974) when acutely exposed to low salinity (Dufort *et al.*, 2001). However, this is also accompanied by an increase in oxygen uptake (Jury *et al.*, 1994). The osmoconformer *Libinia emarginata* exhibits a decrease in heart rate and locomotion with hyposaline exposure (Cornell, 1973, 1974; McGaw *et al.*, 1999).

The specific dynamic action following feeding is characterized by an increase in oxygen uptake above the resting rate and declining over a period of hours to a few days (Whiteley *et al.*, 2001). In crustaceans, SDA is generally accepted to encompass all aspects of feeding and digestion including food handling, mechanical breakdown of food and protein synthesis. In the blue crab, *Callinectes sapidus* and the green crab, *Carcinus maenas*, the postprandial peak in oxygen uptake occurs within 4h of ingestion of a meal (McGaw and Reiber, 2000; Houlihan *et al.*, 1990). This increase in oxygen uptake remains above prefeeding levels for approximately 2 days following feeding (Wallace, 1973; Houlihan *et al.*, 1990; McGaw and Reiber, 2000). It is thought that oxygen uptake remains elevated even after the gut has been cleared of food to support the costs associated with protein synthesis (Houlihan *et al.*, 1990). The scope and duration of SDA

can vary with temperature, meal type, meal size and body size of the animal (Secor and Faulkner, 2002).

Investigations into the physiological costs of osmoregulation have typically been carried out on starved animals to avoid the confounding effects of SDA on other systems. Recent work has shown that the digestive state of an animal can alter both its physiological and behavioural reaction to environmental perturbations. In *Carcinus maenas*, fed animals are less able to tolerate hypoxia and low salinity concurrently (Legeay and Massauau, 2000). The increase in heart rate typically associated with exposure to decreased salinity in *Cancer magister* is reduced when crabs are postprandial, and the extent to which heart rate is depressed varies inversely with the severity of salinity exposure (McGaw, 2006c). The inability of animals to balance the demands of digestion with competing physiological demands will result in a prioritization to one response or the other. If animals are able to balance the demands, a summation will result, and both responses will continue unabated (Bennett and Hicks, 2001).

Crustaceans are highly mobile organisms. By virtue of their ability to sense changes in the surrounding environment, they are able to avoid challenging conditions. When faced with stressful environmental conditions, crabs may use behavioural mechanisms to avoid or mitigate the use of more costly physiological mechanisms. It is therefore necessary when investigating physiological costs to account for how changes in behaviour may affect the actual costs that are imparted on the animal in the field. In low salinity conditions, decapod crustaceans may exhibit avoidance behaviours; exploiting the spatial heterogeneity of salinity conditions within estuaries and reducing physiological costs (Jury *et al.*, 1995). In the field, it has been shown that on a seasonal

scale, crabs tend to avoid areas with high frequencies of low salinity conditions (Stone and O'Clair, 2001). Through the use of these and other behavioural mechanisms, crabs may be able to avoid or cope with conditions that impart high physiological costs. Conversely, behavioural mechanisms may also make it possible for crabs to exceed threshold tolerances determined in the lab, allowing for the exploitation of areas that were not otherwise thought to be available.

The role of behaviour with respect to the risks of increased mortality has been examined in a number of settings (for review see Lima and Dill, 1990). The most pertinent to the proposed study is that of feeding in the presence of predation (Abrahams and Dill, 1989). In this case, an energetic equivalence between the risks associated with predation in a given patch and the benefits that can be gained from foraging in that patch is expressed. If there is sufficient benefit, animals are more likely to feed in areas with high levels of predation (Abrahams and Dill, 1989). A similar train of thought can be applied to crabs faced with foraging in low salinity environments. If the energy gained from foraging in low salinity areas is high, it may outweigh the concurrent costs of osmoregulation and digestion. This essentially, is foraging theory as it pertains to exposure to physiological costs.

Choice of Species

The Dungeness crab, *Cancer magister* is a commercially important species found in sandy bays and estuaries of the NE Pacific, from Alaska to California (Pauley *et al.*, 1986). *Cancer magister* is classified as a weak hyperosmoregulator, and although this

species is found in estuaries, it is unable to tolerate salinities below 12 ‰ (Hunter and Rudy, 1975). *Cancer magister* is able to maintain haemolymph osmolality slightly above that of the medium in dilute seawater (SW), and does so in salinities below 24‰ (Brown and Terwillinger, 1992). The osmoregulatory ability of this species is slightly higher than other cancrid species, such as *Cancer irroratus* and *Cancer borealis*, that also hyperosmoregulate in dilute SW (Charmantier and Charmantier-Daures, 1991). Its presence in estuaries, combined with its poor osmoregulatory ability makes *Cancer magister* an ideal candidate for examining how behaviour can be used to help mitigate the costs associated with digestion in low salinity. The blue crab, *Callinectes sapidus*, is a very efficient osmoregulator that is found in estuaries in the Western Atlantic and Gulf of Mexico (Hill *et al.*, 1989). In contrast to *Cancer magister*, *Callinectes sapidus* is able to inhabit freshwater, and maintains the osmolality of the hemolymph well above the external medium (Tan and van Engle, 1966). There has been a great deal of research on the ecology and physiology of *Callinectes sapidus* and *Cancer magister*, so much so, that they can almost be described as model organisms. While many other species exhibit similar ecological and physiological characteristics, information on their biology is relatively scarce. *Cancer magister* and *Callinectes sapidus* are also relatively large, and their anatomy has been well studied, making them easy to work on. Because of the fisheries that each of these crabs supports, they are easy to collect in the field and readily accessible from local suppliers, something that is of key importance when studying marine organisms in the desert! While both of these species live and feed in estuaries, their differences in their physiological responses to low salinity exposure make them

excellent choices for comparative studies examining the effects of low salinity exposure on digestive processes.

Dissertation Plan

This dissertation is divided into 3 sections, each consisting of 2 studies.

Salinity and thermal preference:

Question 1 What are the fine scale movements of Dungeness crabs in the field with relation to salinity and temperature?

Question 2 How do starvation and the availability of food affect salinity and thermal preference?

To answer these questions I carried out the following studies:

1. I developed and refined a new methodology for the use of archival data tags to record the microhabitat conditions experienced by free ranging crabs in estuaries. Archival data tags (DST-CTD, Star-Oddi, Iceland) were affixed to free-ranging *Cancer magister* in the field and monitored fine-scale changes in the salinity, temperature and depth that the crab was experiencing.
2. I used a combined field and laboratory study based on the techniques developed in (1) to determine the salinity and thermal preference of Dungeness crabs, and

how these preferences may be affected by food availability. In the laboratory, I used salinity and temperature gradients reflective of the range of habitat conditions experienced in (1) to determine how preference behaviours changed following 5 or 21d starvation, in the presence and absence of food.

I reasoned that since exposure to low salinity or high temperature imparts a physiological challenge, crabs will show a general avoidance of these conditions. In the field, this will result in crabs exploiting the spatial heterogeneity of challenging environmental conditions within the estuary. However, preference behaviours may be altered by the presence of a food stimulus.

Feeding behaviour in low salinity:

Question 1 Is the rate and likelihood of feeding affected by the salinity of the external medium, starvation period or the amount of time spent in low salinity?

Question 2 Are changes in feeding behaviour during low salinity exposure the result of physiological limitation or hormonal regulation?

To answer these questions, I carried out the following studies:

1. I investigated the effects of the level and duration of low salinity exposure, as well as starvation on the likelihood and rate of feeding. Changes in feeding

behaviour were examined in response to exposure to 100, 75 or 50% SW for 0.5, 6, or 24 h, following 2, 5, or 21 d starvation.

2. I investigated the role of the neuroinhibitory hormones produced in the sinus gland in the regulation of feeding behaviour. Changes in feeding behaviour for crabs which had the sinus gland (the possible source of inhibitory neurohormones) removed were compared with those of intact animals.

I reasoned that since *Cancer magister* is unable to balance the physiological demands of digestion and osmoregulation, crabs will be unlikely to feed in low salinity conditions. If crabs do feed in low salinity conditions, they will consume less food as a means of mitigating the metabolic demands associated with digestion. Previous work by Sears and Rittschof (1991) has shown that feeding behaviour in response to satiation is regulated by hormones originating in the eyestalks. Based on this, I reasoned that if changes in feeding behaviour take place soon after exposure to low salinity, they may also be regulated by a similar hormonal mechanism rather than physiological limitation.

Digestion in low salinity:

Question 1 How does osmoregulatory ability affect a crab's ability to balance the metabolic demands of concurrent osmoregulation and digestion, and how are these changes manifested in the rate of gut contraction and gastric evacuation?

Question 2 Do changes in metabolic responses and mechanical digestion translate to extracellular and intracellular digestive processes?

To answer these questions, I carried out the following studies:

1. I investigated changes in oxygen uptake, mechanical digestion and gastric evacuation in two species of differing osmoregulatory ability digesting a meal while exposed to low salinity. Changes in oxygen uptake were measured using a flow through respirometer and the mechanical processing (foregut contraction rates and food transit time) of a radio-opaque meal was followed *in vivo* using a fluoroscope.
2. For these species, I also investigated changes in digestive enzyme activity, circulating free amino acids and digestive efficiency while exposed to low salinity. Changes in protease activity were determined both in the gut fluid and the hepatopancreas using a fluormetric assay. The concentration of circulating free amino acids in the hemolymph was determined using high performance liquid chromatography.

I reasoned that since *Cancer magister* cannot balance the demands of osmoregulation and digestion, this species will prioritize metabolic responses towards those of osmoregulation. As a result of this, digestive processing will be restricted. In contrast, since *Callinectes sapidus* is an efficient osmoregulator, this species will be able to sum the demands of osmoregulation and digestion, and digestive processes will continue unabated.

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CHAPTER 2

DEVELOPMENT OF FIELD METHODOLODY

Abstract

This study presents a methodology for combining archival data storage tags (DSTs) and ultrasonic transmitters to investigate the microhabitat conditions of adult *Cancer magister* (Dana), inhabiting an estuary. The temperature, salinity and depth experienced by free-ranging Dungeness crabs was recorded at 10min intervals for periods ranging from 1 week to 8months. Crabs were tracked using a hydrophone and tags were recovered via concentrated trapping or returned by recreational fishers for a reward. These methods led to a return rate of 50%. Representative CTD tag data showed that the conditions recorded at fixed stations within the estuary were not reflective of those experienced by free ranging crabs, but rather crabs were able to orient and avoid low salinity within the estuary. The prevalence of low salinity exposure was linked to times of increased food availability within the estuary, suggesting that crabs were entering the shallows of the estuary to forage. The techniques employed in this study demonstrate that DSTs are a viable means of determining the microhabitat conditions of crustaceans inhabiting highly variable environments.

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Introduction

The field of biologging has seen great advancements in recent years as satellite technology and reductions in the size of sensors have made monitoring the habitat conditions experienced by pelagic and benthic marine animals more practical. Multi-sensor tags can record environmental, behavioural and/or physiological data simultaneously, providing key insight into an otherwise unknown realm (Cooke *et al*., 2004; Block, 2005). While there have been substantial advancements, among marine organisms the majority of these studies have focussed on mammals or large pelagic fishes (Block, 2005). Until recently the large size and exceptionally high cost of the tags used in these studies has prevented the broad scale application of this technology to smaller organisms.

Recent technological advances leading to the miniaturization of commercially available, multi-sensor archival data storage tags (DSTs) has allowed their application in studies involving smaller vertebrates and invertebrates. Through the combined use of telemetric tags for tracking and DSTs it is now possible to compare the movements of animals within a particular habitat as well as the environmental conditions that they face in 'real time'. Previous studies describing the potential for combining ultrasonic transmitters and DSTs have not explicitly outlined methodology for maximizing returns using these techniques, especially in areas where there is not a commercial fishery for the species in question (Freire and Gonzalez-Gurriaran, 1998; Gonzalez-Gurriaran *et al*., 2002; Wolcott *et al*., 2003).

The influence of environmental conditions such as temperature and salinity on the physiology of decapod crustaceans has been well studied in laboratory conditions (for

reviews see Pequeux, 1995, Whiteley *et al*., 1997; McMahon, 2001). These environmental factors can present significant physiological challenges. A number of studies have examined movements of crustaceans in relation to these environmental conditions based on measurements taken at fixed locations within a particular habitat (Stevens *et al*., 1984; Gunderson *et al*., 1990; Watson *et al*., 1999; Jury *et al*., 1995; Bell *et al*., 2003; Rewitz *et al*., 2004), or manual readings taken in the vicinity of tracked or captured animals (Shirley and Wolcott, 1991; Stone and O'Clair, 2001, 2002; Rewitz *et al*., 2004). However, little is known about the actual scales of exposure to physiologically challenging conditions (Cooke *et al*., 2004) and the influence that behaviour may have on the duration and severity of exposure (Wolcott and Wolcott, 2001). This is important because the range of conditions used in laboratory studies may not accurately reflect those experienced in the field. For crustaceans inhabiting estuaries, changes in the salinity and temperature regimes within the estuary may have significant effects on their energetics (Guerin and Stickle, 1997; Whitely *et al*., 2001; Normant and Lamprecht, 2006) and distribution (Barnes, 1967; Watson *et al*., 1999; Rewitz *et al*., 2004).

One such estuarine inhabitant found in the NE Pacific is the Dungeness crab, *Cancer magister*. Despite living in habitats subject to frequent episodes of low salinity, *Cancer magister* has been classed as a weak osmoregulator (Englehardt and Dehnel, 1973) and is unable to tolerate salinities below 12‰ (Cleaver, 1949). Because of this relatively low tolerance of hyposaline conditions, alterations in the salinity regime within an estuary can have substantial impacts on the physiology of this species (McGaw and McMahon, 1996; McGaw, 2006) which may affect the crabs' ability to fully exploit

hyposaline environments (Barnes, 1967). A number of studies have sought to determine the preferred habitat conditions and distribution of *Cancer magister* by tracking the movements of crabs fitted with ultrasonic tags (Smith and Jamieson, 1991; Stone and O'Clair, 2001, 2002) or by carrying out trawl surveys and comparing estimates of abundance within an area to the local oceanographic conditions (Stevens *et al*., 1984; Gunderson *et al*., 1990). These studies have provided valuable insights into patterns of estuary use; however, little is known about the microhabitat conditions that individual free-ranging crabs experience.

In this paper, we present a methodology for combining miniaturized DSTs and ultrasonic telemetric tags to monitor the movements of *Cancer magister* in relation to depth, temperature and salinity. Since *Cancer magister* can detect differences in salinity as low as 1‰ (Sugarman *et al*., 1983) and are highly mobile, behavioural adjustments may create different spatial and temporal scales of exposure for free ranging crabs compared with those observed at fixed stations within the estuary. By using the methodology described here, we were able to record oceanographic conditions (temperature, depth and salinity) that individual crabs were exposed to in the field. Representative data collected for one individual over an entire year are presented.

Methods

Adult male *Cancer magister* were trapped in the Sarita river estuary, Barkley sound, British Columbia (49º 01.94'N, 125º 18.34' W) and transferred to the Bamfield Marine Sciences Centre, where they were held in running seawater (32 ± 1‰; 12 ± 1ºC)

for one week prior to release. Crabs were subsequently released in the main river channel of the Sarita River estuary. The Sarita River estuary is located within Numukamis bay and consists of extensive tidal flats with subtidal eelgrass (*Zostera marina*) beds at shallower depths. A steep slope towards the seaward side (Fig. 2.1) extends to a maximum depth of 120m. Deep water, narrow outlets to the east and west, and a structured, large cobble substrate (circles) likely served to limit emigration from the study area. Crabs were released in July and August 2004, and from July to December 2005.

Previous studies using DSTs to examine the movements and habitat conditions of crustaceans have been restricted to temperature and depth sensors. The DSTs used in this study are unique in that they also incorporate a sensor to measure conductance (salinity). These sensors have only recently (2001) been miniaturized for such applications and have not been widely used in smaller marine organisms (Walker *et al*., 2004; Fukuwaka *et al*., 2005). The temperature, depth and salinity that each crab experienced was recorded in real time using archival data storage tags (DST-CTD, Starr-Oddi, Iceland). At a programmed sampling interval of 10 min, the DSTs were able to hold 300 d of data and had a 14-month battery life. Tags weighed 12 g in water and measured 15 x 46 mm. Each tag was inserted into Tygon® tubing of the same internal diameter as the external diameter of the tag and secured inside the tubing with silicone sealant. This allowed the tag to be easily removed upon capture, and prevented the tag from being damaged. A printed message on the tag could be easily read through the tubing, informing captors of a $50 reward and providing return instructions. Crabs were also fitted with coded ultrasonic transmitters (CT-82-2E, Sonotronics Inc., Tucson AZ) that emitted a unique signal for each crab and had a battery life of 14 months. This allowed individuals to be

identified and tracked using a directional hydrophone. Ultrasonic transmitters weighed 9 g, measured 54 x 16 mm and were inserted into tubing in the same manner as the DSTs.

Data tags and transmitters were affixed to the dorsal side of the carapace using A788 Splash Zone epoxy (Z-Spar Inc., Los Angeles CA). Large adult crabs that had recently moulted (identified by shells that had not fully hardened and few epibionts), were used in order to provide the longest possible intermoult period (Wainwright and Armstrong, 1993). The area of the carapace where the epoxy was applied was abraded to maximize adhesion. Moulding the epoxy allowed the tags to be mounted at an upward angle of approximately 45º immediately posterior to the apex of the carapace (Fig. 2.2). This means of attachment prevented them from being fouled by mud and sand when crabs buried in the substrate (McGaw, 2005). Once the tags were mounted, crabs were housed in flowing seawater for a period of 24h, a sufficient time for the epoxy to cure. The entire attachment including both tags and the epoxy weighed less than 30 g in water and was approximately 3-4% of the animal's body mass.

Using a directional hydrophone and receiver (Sonotronics DH-4 and USR-96), individual crabs could be located from a small boat at distances up to 1.5 km and their location determined to within 15 m. This allowed crabs to be recovered by focussed trapping efforts. Tagged crabs were also returned by recreational fishermen. Notices advertising the study were posted at local marinas to alert fishermen, and a $50 reward was offered as an incentive for the return of intact data tags. Once tags were recovered, data was retrieved from the DST using a communication box and Seastar software (StarrOddi, Iceland).

Processing of large data sets (up to 40 000 readings) was accomplished using spreadsheet macros for microclimate data as described by Sinclair (2001). Low salinity exposure was considered to be any exposure lasting greater than 10 minutes (one sampling interval) at a salinity of less than 24‰ (75% SW). This salinity was chosen because *Cancer magister* actively regulate their hemolymph osmolality below this level (Engelhardt and Dehnel, 1973) and exhibit an increase in heart rate (McGaw and McMahon, 1996), which is indicative of a stressful environment (McMahon, 1999).

Oceanographic conditions within the estuary were recorded at 3 fixed stations during June and October 2005 for a period encompassing a tidal series of spring and neap tides. High discharge rates coupled with a large amount of debris in the estuary prevented recordings during the winter months. The stations were approximately 300 m apart at depths of 27, 5, and 2 m below mean low low water (MLLW) and were arranged along a transect running east to west in the main river channel (Fig. 2.1). Station A (27 m depth) was located at the mouth of the estuary where the bottom drops away rapidly, Station B (5 m depth) was located within the estuary and Station C (2 m depth) was located above station B, further inside the estuary. DSTs were attached to fixed anchors and temperature, salinity and depth were recorded at 10min intervals. Tags were elevated approximately 10cm above the substrate, emulating the conditions that crabs would experience at this location.

Results

Estuary

Decreases in salinity with the Sarita estuary occurred at regular intervals and corresponded with low tide (Fig. 2.3). The severity of low salinity within a tide series was inversely proportional to tidal height and varied with season. In June, Station A was originally deployed at a depth of approximately 27 m, however, shortly thereafter the anchor was moved to a depth of about 12m below MLLW. This is likely the result of an errant fisherman assuming that the float was attached to a trap and not a brick; thus the remainder of readings from the deep station for June were taken at this depth. At the estuary mouth (station A), temperature ranged from a minimum of 10.7ºC to a maximum of 18.6ºC, and salinity ranged from 30.1‰ to 19.4‰ (Fig. 2.3a). At station B, temperature ranged between 18.4ºC and 11.5ºC, and salinity ranged from 31.3‰ to 20.9‰ (Fig. 2.3b). Further into the estuary at station C, temperature ranged from 18.7ºC to 12.7ºC, and salinity ranged from 30.4‰ to 19.6‰ (Fig. 2.3c). As the tide receded (depth decreased), the proportion of warm, fresh water in the estuary increased resulting in a decrease in salinity and a concomitant increase in temperature. The magnitude of individual temperature or salinity events was not consistent across stations.

During the October tidal series, there were only slight changes in temperature, ranging from 12.4ºC to 9.8ºC; these showed little correspondence to tidal height (Fig. 2.3 d-f). The salinity conditions at the mouth of the estuary (station A), remained constant, ranging between 29.9‰ and 28.7‰ (Fig. 2.3d). The changes in salinity further within the estuary at stations B and C were more severe. At station B, salinity ranged between 34.2‰ to 7.18‰ (Fig. 2.3e) and at station C, the salinity ranged from 30.4‰ to 1.7‰

(Fig. 2.3f). As with during the June tidal series, decreases in salinity were associated with low tides. The most severe decreases in salinity corresponded to the largest tidal fluxes.

Tagging

A total of 54 deployments were made, of which 27 tags were recovered, giving a 50% return rate. Of the 27 returns, 70% (19) were returned by recreational fishers in the area and 30% (8) were recovered by focussing trapping efforts based on tracking data. The length of tag deployment ranged from 5 to 220 d, with a mean deployment time of 44.3 ± 14.8 d and a median deployment time of 16d. Few tags were recovered between January and April, likely due to inclement weather, which restricted both recreational fishing and directed trapping efforts.

For one individual, Crab A, nearly an entire year of data was collected (Fig. 2.4a). This individual was first released on 08/30/05. After 90d at large, the crab was recaptured on 11/29/05 by a recreational fisher. Data from the DST was downloaded and the crab was allowed to recover in the lab for 25d, following which it was re-released in the estuary. This deployment lasted 220d, and the crab was recaptured on 07/31/06.

Following its initial release directly into the main river channel on 08/30/05, Crab A remained in the shallows of the estuary for 2d. During this time the crab was exposed to salinity below 75% SW for a total of 40min, reaching levels as low as 2.2 ‰ (Fig. 2.4b). These exposures corresponded with increases in temperature to a maximum of 17.9 ºC. Thereafter, the crab retreated into deeper water for a period of 28 d. During this time, the crab remained at depths between 30 and 45m, with the exception of a few brief

(<3 h) forays into water as shallow as 3m and salinity levels remained above 75% SW. While in deeper water, there were episodes (lasting up to 11 d) during which oscillations in temperature and depth varied with tidal height.

Between 10/01/05 and 10/16/05 Crab A migrated into shallow water (< 24 m) and made a series of 8 forays into the littoral zone during nocturnal high tides, each lasting between 6 and 18h (Fig. 2.4c). These movements were associated with an increase in temperature from 10.7ºC up to 13.7ºC and a corresponding decrease in salinity to a minimum of 16.2‰. On 10/16/05, the crab was exposed to salinities ranging from 18.2 to 19.9‰ for a period of 13 h at a depth of 17 m. Following this exposure the crab retreated to depth. Between 10/16/05 and recapture on 11/29/05, the salinity ranges encountered by the crab were highly variable and low salinity exposures of 10 min to 6 h duration occurred at depths of up to 45 m. The temperature remained relatively stable during this time.

Following capture and data upload, Crab A was re-released into the main river channel on 12/23/05, where it remained at depths between 1.5 and 6.6 m for a period of 50d (Fig. 2.4d). During this time, the crab was exposed to frequent and severe bouts of low salinity. Exposures ranged from 10min to 143h, with a mean exposure time of 4.4 ± 2.5h. The minimum recorded salinity during this period was 5.2‰. On 02/11/06 the pressure sensor on the DST malfunctioned and no longer recorded accurate depth measurements. From this point until 05/17/06, the crab was not exposed to salinities below 75% SW. However, in mid-April, the crab was exposed to decreased salinities approaching 75% SW and a corresponding increase in temperature for a period of 16d. From mid May until its capture in late July, the crab was exposed to short bouts of low

salinity below 75%SW along with corresponding increases in temperature. Based on data from other crabs, such exposure to low salinity and increased temperature is indicative of movement into shallower water

Another crab, Crab B, was also released on 08/30/05 into the main river channel. In the 3d following release, Crab B was exposed to 4 episodes of low salinity with the most severe reaching a minimum of 1.6 ‰ (Fig. 2.5a). Following this initial low salinity exposure, the crab retreated to depths greater than 20m and only made 2 brief forays into shallower water during the next 25 d. Between 09/27/05 and 10/16/05, Crab B made a number of forays into the shallows (<15 m) (Fig. 2.5b). The timing of this migration corresponded to that observed for Crab A. Coincidentally, Crab B was also re-captured on 11/29/05 by a recreational fisherman; but was never subsequently captured following re-release.

Discussion

A number of articles detail the habitat preferences of decapod crustaceans and the influence of low salinity (Stevens and Armstrong, 1984; Stevens et al., 1984; Gunderson et al., 1990; Smith and Jamieson, 1991; Stone and O'Clair, 2001). However, only a few of these articles report the microhabitat conditions that animals are experiencing in the field (Freire and Gonzalez-Gurriaran, 1998; Gonzalez-Gurriaran *et al*., 2002; Wolcott *et al*., 2003). The results of the current study show that the conditions which free ranging crabs experience are different than those measured at fixed locations within the estuary. For example, during the fall (Sept.-Nov.) Crab A made a series of 8 forays into shallow

water and these migrations corresponded to nocturnal high tides. This pattern of estuary use by adult *C. magister* also occurs in juveniles (Holsman *et al*., 2006). Such migrations would provide the least amount of salinity stress due to the correspondence between tidal height and salinity, while minimizing visual predation (Stevens *et al*. 1984; Gunderson *et al*., 1990). While previous studies employing trawl sampling during daytime low tides (Stevens and Armstrong, 1984; Gunderson *et al*., 1990) have provided relative measures of prevalence, these studies may not accurately represent the degree to which adult crabs exploit shallow areas of the estuary. Additionally, trawls do not account for the frequency of individual forays into areas of low salinity. Data presented here suggest that adult crabs migrate in and out of littoral and shallow sublittoral zones with the tide, usually only spending brief periods in low salinity. Patterns of estuary use may therefore not be as clear as is indicated by more infrequent techniques that sample over shorter periods and it is essential that investigators show caution when drawing conclusions about habitat preference without considering the influence of behaviour (Wolcott and Wolcott, 2001).

Methodology

Like many brachyurans, *Cancer magister* frequently buries itself in the substrate when inactive (Bellwood, 2002; McGaw, 2005). Our preliminary experiments showed that tags mounted parallel to the carapace were fouled with sediment when crabs buried. This resulted in inaccurate salinity measurements due to decreased conductivity across the sensor (Curtis and McGaw, Unpublished obs.). Fouling of the tag was manifested in the data as rapid and severe decreases in salinity. This is easily overcome by comparing

the rate of change in the estuary (accounting for locomotory ability) with that observed in the data. In order to reduce the frequency of sediment fouling, Z-spar epoxy was used to mould the attachment and DSTs were mounted at an upward angle of approximately 45º relative to the carapace (Fig. 2.2). The use of a marine epoxy also allowed for the least amount of emersion time for the animal while tags were being affixed because the epoxy cured underwater. Previous studies have used a variety of methods for attaching tags to the carapace of decapod crustaceans, these methods have included "fast drying" epoxies (Smith and Jamieson, 1991), wiring (Wolcott and Hines, 1990) and even tape (Watson *et al*., 1999). The combination of Z-spar epoxy and Tygon® tubing used in this study was more effective because it provided a permanent attachment that protected the tags from damage while still allowing them to be easily removed for data transfer.

Adult Dungeness crabs typically moult once per year (Wainwright and Armstrong, 1993). To maximize the length of deployment, recently moulted, adult crabs (>150mm carapace width), bearing no epibionts were used. This ensured the longest possible intermoult time (Wainwright and Armstrong, 1993), and subsequently the longest possible tag retention. In southern British Columbia and northern Washington, large adult crabs usually moult between late spring and early fall (Orensanz and Gallucci, 1988; Curtis and McGaw, Unpublished obs.). This restriction on the timing of deployments combined with high natural mortality rates likely contributed to the scarcity of recaptured individuals during winter and spring (Smith and Jamieson, 1991). The low median deployment time is likely the result of increased recreational fishing pressure during the summer months. Delaying the release times may have alleviated this, but would also reduce the potential time at large. In support of this, the majority of returns

were in the summer; however most of the longer deployments came from crabs that were released in the late summer.

Ultrasonic signals could typically be detected at distances greater than 1.5km, and individual crabs could be located to within 15m under ideal conditions. Nevertheless, the distance at which crabs could be located was restricted during inclement weather, likely due to changes in the signal to noise ratio caused by turbulence in the water (Baras and Lagardere, 1995). While the bathymetry of the site likely limited emigration, it also made the recovery of tagged individuals by SCUBA following death or ecdysis difficult. In a shallower system where recovery by SCUBA is feasible, return rates may be further increased.

Most studies involving the tagging of crustaceans (mark-recapture and data storage tags), have relied on intensive commercial fisheries to bolster return rates. Despite this intense effort, the average return rate for these studies is approximately 23% (for examples see: Cronin, 1949; Smith and Jamieson, 1990; Fitz and Wiegert, 1992; Watson *et al.*, 1999; Smith *et al*., 2001; Gonzalez-Gurriaran *et al*., 2002; Bell *et al*., 2003; Turner *et al*., 2003; Wolcott *et al*., 2003; Aguilar *et al*., 2005; Yamada *et al*., 2005). Studies employing DSTs to monitor the habitat preferences of decapod crustaceans are rare, likely due to the cost associated with this technology and the low return rates of other tagging studies (Cooke *et al*., 2004). DSTs and transmitters have been used to measure the depth and temperature experienced by *Maja squinado* as they make seasonal migrations (Freire and Gonzalez-Gurriaran, 1998; Gonzalez-Gurriaran *et al*., 2002). These studies garnered high return rates (up to 68%), but also relied on an intense commercial fishery for returns. The current study is unique in that it incorporates a

salinity sensor and a much finer sampling interval to examine the conditions experienced by crabs in a highly variable habitat. The use of ultrasonic tracking to locate animals followed by concentrated trapping efforts in the vicinity increased the returns, suggesting that this is a viable means of tag recovery in the absence of a commercial or recreational fishery. Combining a relatively high reward for returned tags with our directed trapping lead to a high return rate of 50%. During the course of this study a few tagged crabs were reported as being captured, but were never returned for reward. The return rate would have been increased had we acquired these tags. Of the tags returned, the data could not be retrieved from 2 because the membrane on the front of the tag had been punctured and the tag flooded with seawater. The depth sensor on the tag which recorded the data for Figure 2.4 ceased to function and depth data are not available for the latter portion of the deployment; however the temperature and conductivity sensors continued to function. Only one tag showed evidence of biofouling (by a small bryozoan), and this did not appear to affect the performance of the sensors. While the cost associated with this technology has been reduced in recent years, it is still an impetus to the employment of this technology on the scales of other mark-recapture techniques.

Representative Data

The Sarita river estuary remained stratified in the summer and fall (Fig. 2.3). As the tide receded, a lens of freshwater travelled seaward towards the mouth of the estuary. The severity of decreases in salinity was greater in October and corresponded to increased rainfall. The data presented here show that during these times the extent of low salinity was restricted to depths shallower than about 27m below MLLW. However,

between the June and October sampling periods a portion of freshwater flow from the river switched to another channel and flow through what was the main channel was reduced. Therefore the extent of low salinity in the estuary may be greater than the data suggest.

During the summer months, forays into warmer low salinity water were infrequent (Fig. 2.4a). This is in accordance with data from other crabs that were at large during the same period in 2004 and 2005 (Curtis and McGaw, In prep.). An increase in temperature (up to a maximum of 15ºC) leads to increased metabolic and growth rates in juvenile *C. magister* (Gutermuth and Armstrong, 1989; Kondzela and Shirley, 1993). Above 15ºC, the increased energy expenditure associated with respiration limits growth and reduces survival (Kondzela and Shirley, 1993). Because temperatures in the Sarita river estuary regularly exceeded 15ºC during the summer months (Fig. 2.3) this may have accounted for the scarcity of forays into shallower water. Alternatively, Stevens *et al*. (1984) have linked the prevalence of *Cancer magister* in estuaries with prey abundance. In the laboratory, crabs are more likely to enter into physiologically challenging conditions when a food stimulus is present (Curtis and McGaw, In prep.), suggesting that the number of forays may be linked to relative food availability. In support of this assumption, both Crab A and Crab B made more frequent and prolonged migrations into shallow water in early October (Fig. 2.4b, 2.5a). These migrations corresponded to the first instances of spawned out salmon carcasses appearing in the Sarita River estuary which has an annual run of approximately 150 000 fish (Ochman, Pers. comm.). It has previously been suggested that *Cancer magister* may enter into areas of low salinity to

forage on salmon carcasses at river mouths (Sugarman *et al*., 1983) and the data presented here appear to substantiate this claim.

Following re-release in late December 2005, Crab A remained in the shallows of the estuary for 50d and was exposed to salinities as low as 5.2‰. In January 2006, rainfall was more than double the average for that month (www.climate.weatheroffice.ec.gc.ca), possibly contributing to the severe salinity exposures. *Cancer magister* can remain buried in the sediment for prolonged periods during the winter months. However, the depth ranges experienced by the crab did not mimic the tidal cycle indicating that the crab was mobile rather than simply remaining buried in one place. The crab may have remained in the shallows to forage despite the severity of low salinity exposures (Sugarman *et al*., 1983). In the laboratory, we have found that *Cancer magister* feeds less frequently in low salinity; however, as the time since their last meal increases, crabs are more likely to enter into low salinity and feed (Curtis and McGaw, 2005). This contradicts previous reports that adult *C. magister* retreat into more stenohaline areas during times of high run-off (Stevens and Armstrong, 1984; Stone and O'Clair, 2002). Coho salmon finish spawning in late December in the Sarita river (Ochman, Pers. comm.) and with the increased runoff, a large amount of detritus and potential prey items were washed downstream into the estuary (McGaw, Unpublished obs.). This again supports the idea that migrations into the estuary are influenced by an increase in food availability (Stevens *et al*., 1984).

Despite forays into the estuary throughout the year, adult *Cancer magister* appear to spend the majority of their time at depth where temperature and salinity conditions are more stable. While the lower temperatures at depth may reduce metabolic rate and

subsequently growth, it has been suggested that adult crabs are better physiologically adapted to low temperatures (Gutermuth and Armstrong, 1989). Therefore unless there is a gain, such as increased prey availability in the estuary, it would be beneficial for crabs to remain in stable conditions rather than venturing into more ephemeral areas where they will be challenged by higher temperatures or low salinity.

On several occasions, crabs were subjected to particularly severe low salinity conditions that exceeded their physiological tolerance (Cleaver, 1949). Following these exposures crabs retreated to deeper water. Jury *et al*. (1995) reported similar movements for lobsters in response to a freshet following a hurricane. Similar preference behaviours are observed in the laboratory; *Cancer magister* can detect haloclines and avoid low salinity conditions (Curtis and McGaw, In prep.). However, one must be careful about inferring movements in the field solely based upon laboratory experiments. In the lab sharp gradients existed over small spatial scales; in the field where salinity gradients occur on larger scales such directional orientations may not be possible (Bell *et al*., 2003). Nevertheless, the results presented here suggest that within the Sarita River estuary, crabs are able to orient to, and avoid low salinity conditions.

This study has demonstrated a methodology for combining miniaturized multi-sensor DSTs, with ultrasonic transmitters for tracking. This work improves upon previous studies (Freire and Gonzalez-Gurriaran, 1998; Gonzalez-Gurriaran *et al*., 2002), by examining highly variable habitat conditions at a fine sampling scale in the absence of an intense commercial fishery. DSTs provides a viable alternative to well developed telemetric methods that have successfully been used to monitor the habitat conditions, physiological and behavioural variables of crabs in estuaries (Hines, 2007). To

continuously monitor habitat conditions experienced by crabs using telemetry on estuary wide spatial scales, crabs must be continuously monitored (Wolcott, 1995). If manual monitoring is used, only a few animals can be at large at once and the duration of deployment is limited by the persistence of the investigator and available boat time. Conversely, to record data from a larger number of individuals or for longer durations, complex hydrophone arrays must by employed (Giacalone *et al*., 2006) Telemetric tags are limited in the amount of data that they can transmit (Wolcott, 1995) and the ability to detect the signal may be limited in complex habitats (Giacalone *et al*., 2006). DSTs however, are not without faults and the key limiting factor is the need to recover the tags in order to retrieve the data. This can be overcome by combining telemetric tracking tags with DSTs to improve return rates. Another major limiting factor is the high cost. While telemetric tags fabricated in the laboratory are inexpensive, the hydrophone arrays necessary to carry out a study of similar scale using telemetry are equally costly.

Despite the relatively high cost of DSTs, the high recapture rates attained in this study make this technique a viable and exciting option for monitoring previously unknown physiological and behavioural variables in the field. In future studies, recapture rates may be further increased by using underwater receivers carried by divers and also by attaching small magnets to the carapace so that the DSTs attached to crabs that moult in deep water may be retrieved from the surface. Monitoring the conditions experienced by crabs living in estuaries allows for the use of ecologically relevant parameters for behavioural and physiological experiments, as well as providing data that will be valuable in modelling the energetics and distribution of adult *Cancer magister* living in estuaries.

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Figures

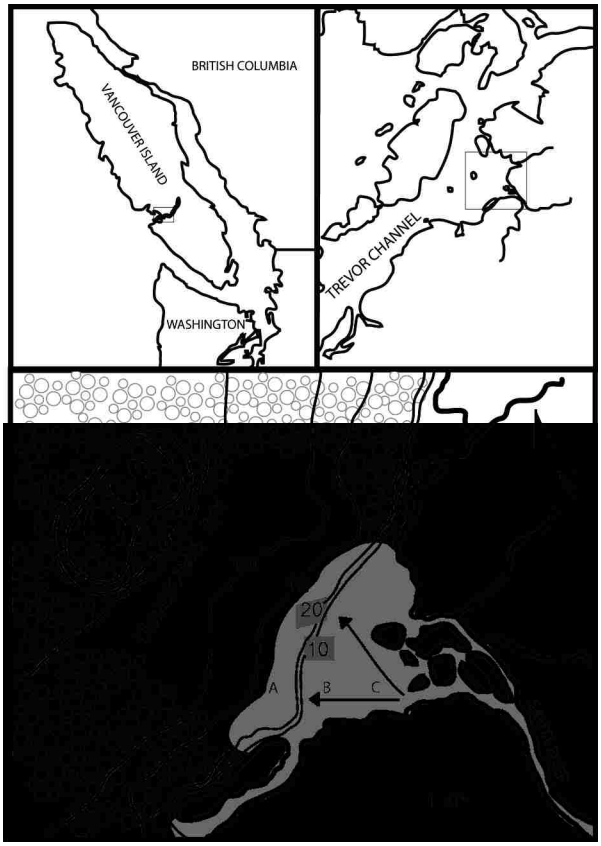

Figure 2.1: The Sarita River estuary, Barkley Sound British Columbia (49º 01.94'N, 125º 18.34' W). Circles represent areas of cobble substrate. Grey area represents the influence of freshwater from the Sarita river within Numukamis bay and the two arrows indicate the location and direction of flow in the main channels of the estuary. Letters indicate the 27m (A), 5m (B) and 2m (C) depth fixed stations where temperature, salinity and depth were recorded. Depth contours are in meters below MLLW.

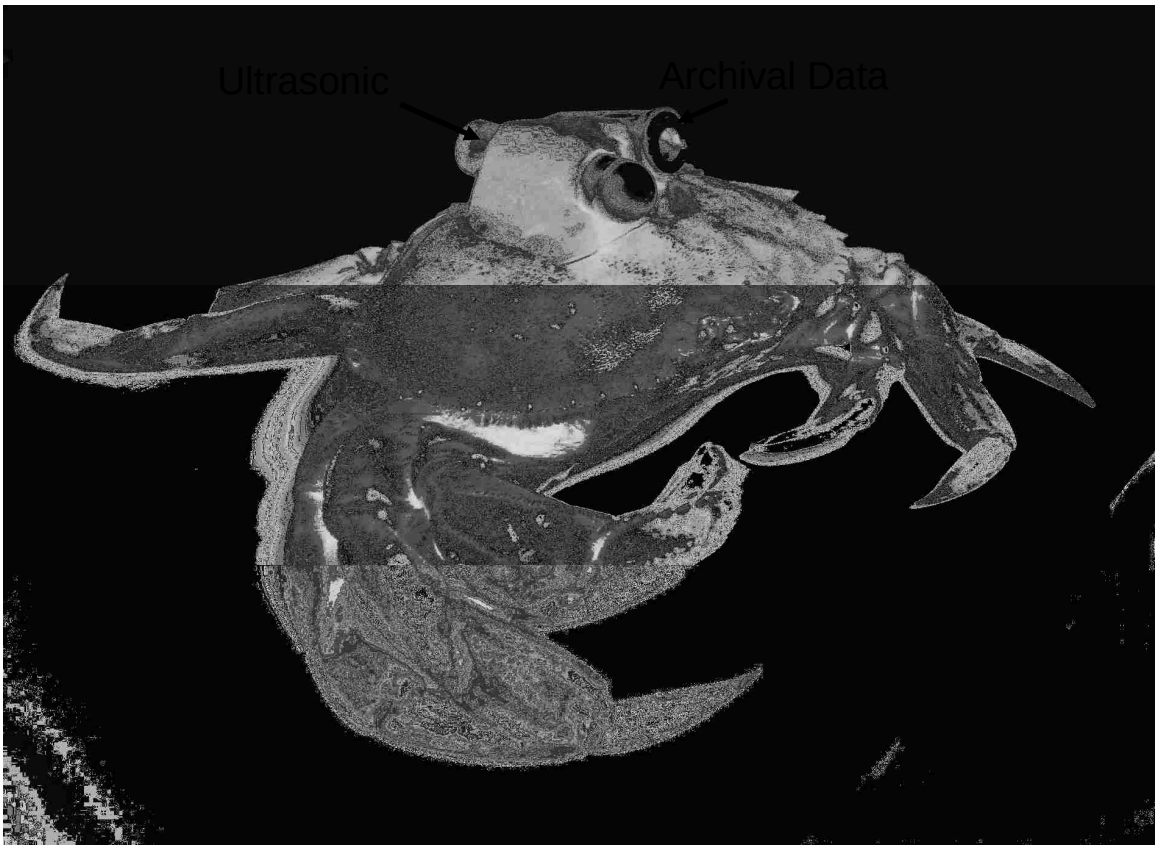

Figure 2.2: Photograph of an adult *Cancer magister* prior to its release, fitted with an archival data storage tag and ultrasonic transmitter for tracking. Both tags were held in place on the carapace with a moulded Z-spar epoxy.

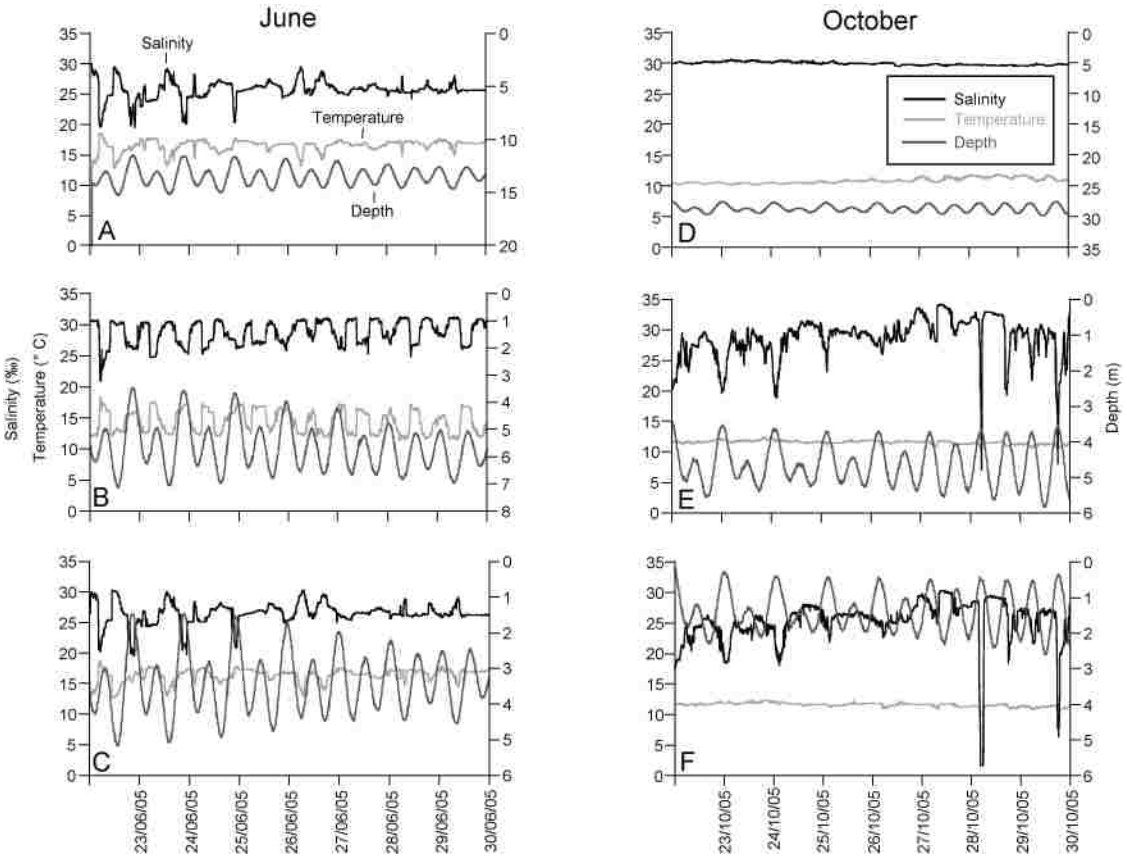

Figure 2.3: Salinity, temperature, and depth recordings measured at 27, 5, and 2m below MLLW within the main channel of the Sarita river estuary over a tidal cycle encompassing both spring and neap tides in June (a-c, respectively) and October (d-e, respectively), 2005.

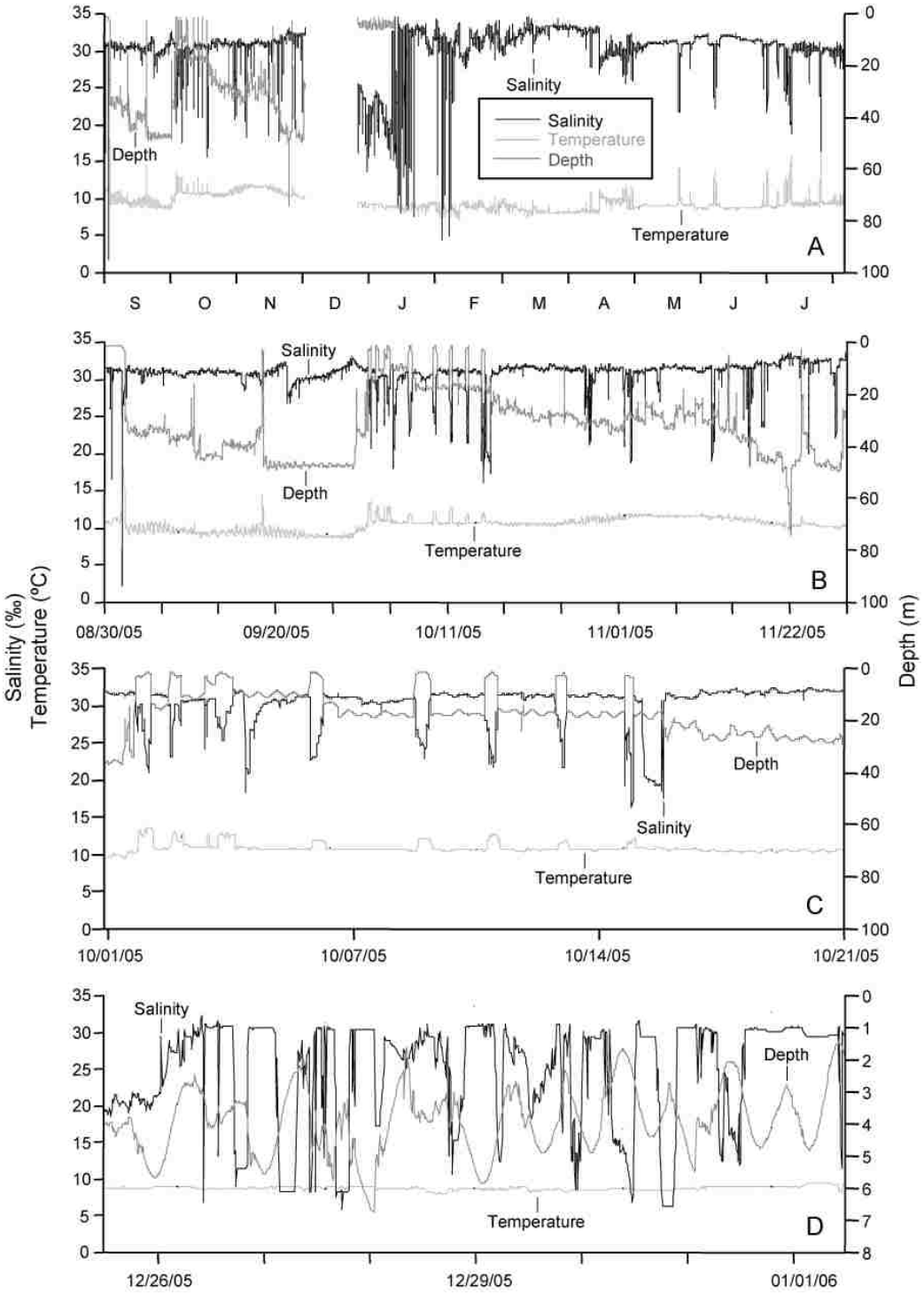

Figure 2.4: Salinity temperature and depth experienced by Crab A during a) an entire year, and subsets: b) first 90d following release in August 2005 c) 3 weeks in October d) 1 week following re-release in winter.

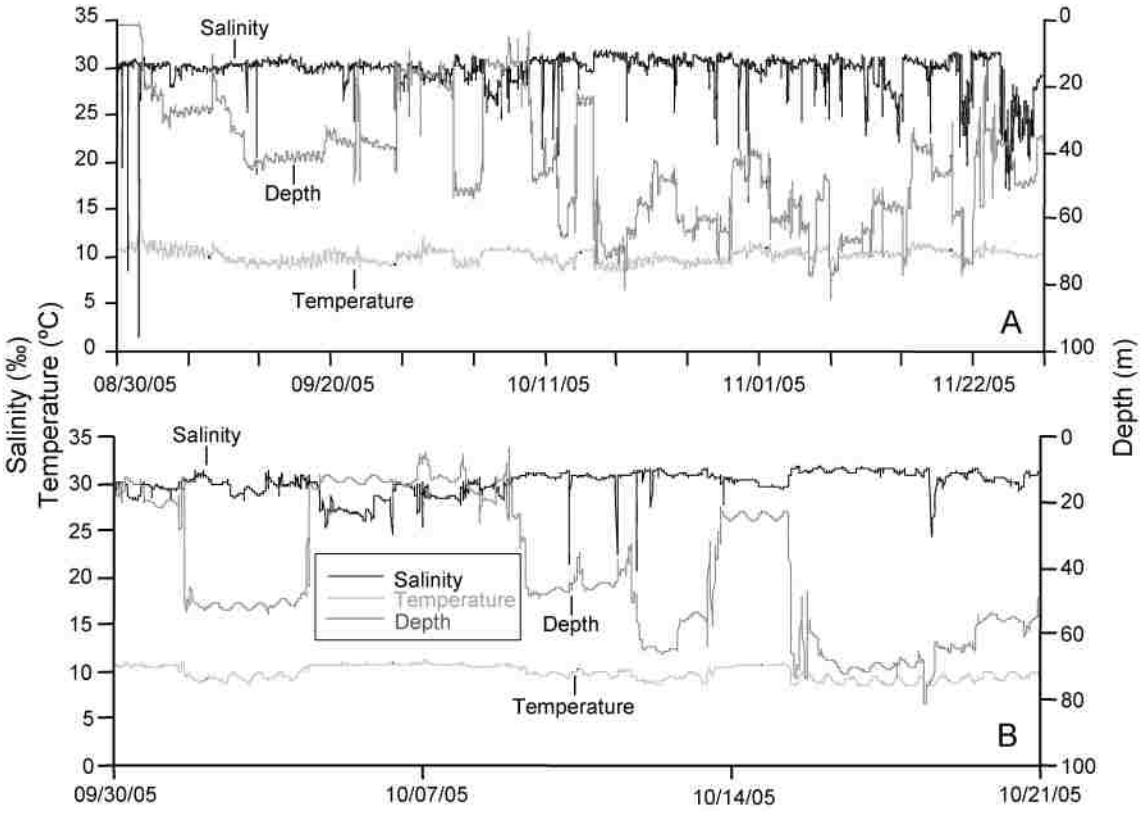

Figure 2.5: Salinity, temperature, and depth experienced by Crab B during a) 90d following release in August 2005 and b) a subset of 3 weeks during October.

CHAPTER 3

SALINITY AND THERMAL PREFERENCE

Abstract

Combined field and laboratory experiments were carried out to investigate the salinity and thermal preference of adult Dungeness crabs, *Cancer magister*. In the field, crabs were fitted with archival CTD data tags to monitor the salinity, temperature and depth experienced at 10 min intervals. Crabs spent the majority of time in conditions that did not present a physiological challenge and at depths below 15 m. However, all crabs that were at large for more than a week made migrations to depths shallower than 15m. Exposure to stressful salinity and temperature conditions showed a distinct pattern and could be divided into short exposures (< 20 min) that were associated with movements to deeper water, and longer exposures where avoidance was not evident. Results obtained from laboratory experiments using salinity (16 to 32‰) and temperature gradients (8 to 22 ºC) confirmed data from tagged crabs in the field. Overall, crabs showed a strong avoidance of low salinity. However, when crabs that had been starved for 21 d were presented with a food stimulus in low salinity, they showed directed movements towards the stimulus and spent more time in low salinity. Likewise when presented with a food stimulus in high temperatures, starved crabs spent more time searching for the stimulus in high temperature than those which had recently fed. The results of this study suggest that crabs are primarily exploiting estuaries during times when conditions are not physiologically stressful, but that exposure may be altered by the presence of food.

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Introduction

Estuaries are important nursery habitats for many species of decapod crustaceans (Armstrong *et al*., 2003). In the NE Pacific, the abundant food resources provided by intertidal flats in estuaries support large populations of juvenile Dungeness crabs (Holsman *et al*., 2003). A number of studies have examined the importance of estuarine habitats for juvenile *Cancer magister* (Gunderson *et al*., 1990; Holsman *et al*., 2006; Stevens and Armstrong, 1984). These reports have suggested that adult crabs are scarce within estuaries, and their presence has generally been attributed to times of increased food abundance (Stevens *et al*., 1984; Sugarman *et al*., 1983).

The environmental conditions within estuaries present physiological challenges for Dungeness crabs. In the summer months, as the tide recedes, a warm low salinity lens travels down the estuary. In the fall, low tides are accompanied by more severe low salinity, but no concurrent change in temperature (Curtis and McGaw, 2008). Although adult *Cancer magister* occur in these highly dynamic habitats, this species had been classed as a weak osmoregulator (Engelhardt and Dehnel, 1973) and cannot tolerate prolonged exposure to salinities below 12‰ (Cleaver, 1949). It is also relatively intolerant of temperatures above 17 ºC (Pauley *et al*., 1989). When exposed to salinities below 24‰, *Cancer magister* must regulate the osmolality of the hemolymph (Hunter and Rudy, 1975) and displays a number of physiological and behavioural responses indicative of stress. Exposure to salinities below 24‰ is associated with an increase in both heart rate (McGaw and McMahon, 1996) and oxygen uptake (Curtis and McGaw,

2009). Low salinity conditions also lead to brief periods of increased locomotion (McGaw *et al*., 1999) that are consistent with avoidance behaviours. Below 16‰ increases in heart rate and oxygen uptake are exacerbated, and crabs cease feeding below 13‰ even after prolonged starvation (Curtis *et al*., In press). The upper lethal thermal tolerance of *Cancer magister* varies with season, ranging between 20 and 25ºC (Pauley *et al.*, 1989). However, increased mortality has been reported at temperatures above 17ºC (Pauley *et al*., 1989), and temperatures above 15ºC result in a pronounced increase in metabolic rate (Gutermuth and Armstrong, 1989).

Increased competition generated by a lack of resources often forces animals to forage in sub-optimum habitat conditions (Hoffman and Parsons, 1993). It then follows that in more stable habitats, unless competition is great or resources are limited, there is no need to forage in areas with challenging conditions. But as energetic reserves are depleted the level of hunger and the necessity for feeding increases (Wang *et al*., 2006). Previous work has shown that benthic marine scavengers are more likely to forage and feed under increased predation risk when they have not recently fed (McKillup and McKillup, 1994; Moore and Howarth, 1996; Stenzler and Atema, 1977). However, few studies have examined the relationship between foraging and challenging environmental conditions (Webster and Dill, 2007). Based on this, we hypothesized that changes in food abundance may lead to an increased use of habitats with challenging temperature or salinity conditions by Dungeness crabs.

The site chosen for this study was the Sarita river estuary, Barkley Sound, British Columbia. Salinities below 24‰ occur at depths of up to 20 m and temperatures above 15ºC regularly occur during low tides in the summer months. In the fall, salinities below

15‰ are more pervasive and occur at depths of up to 40m, while temperatures rarely exceed 12ºC (Curtis and McGaw, 2008). We previously reported the development of methods for use of CTD data tags on adult crabs. The preliminary results showed that *Cancer magister* make forays into the shallows of the estuary that correspond with spent salmon carcasses being washed downstream and thus increased food abundance in the estuary (Curtis and McGaw, 2008).

While these preliminary results have suggested that there is a connection between food abundance and estuary use, they provided little indication of what crabs were actually doing while in the estuary. As such, to date there has not been any investigation of the relationship between nutritional status or food abundance and habitat use. To test the hypothesis that changes in food availability affect habitat use, we examined the effects of nutritional status and a food stimulus on salinity and thermal preference in the laboratory. Because of the contrived nature of laboratory experiments, it is often difficult to extrapolate observations with those occurring in the natural environment (Bell *et al.*, 2003, Bernatis *et al*., 2007). Therefore results obtained from laboratory experiments were compared with the microhabitat conditions experienced by individual free ranging crabs fitted with archival data loggers in the field.

Methods

General

Adult male *C. magister* of 130-180mm CW were trapped in the Sarita river estuary, Barkley Sound, British Columbia (49º01.94' N, 125º 18.34' W) and transferred

to the Bamfield Marine Sciences Centre, where they were held in running seawater (32‰; 12ºC) for 1 week prior to experimentation. Salinity and temperature were measured using an YSI-30 conductivity meter (YSI Inc., Yellow Springs, OH, USA).

Laboratory Experiments

Salinity and temperature preference experiments were conducted in the laboratory to verify field data and to determine how preference may be affected by food availability and nutritional status. Crabs were starved for either 5 or 21 d prior to experimentation. The 5 d starvation period ensured that all animals were in a post-absorptive state, but avoided large scale physiological changes associated with starvation (Wallace, 1973). A 21 d starvation period was used because after this time energy stores have been depleted, forcing crabs to rely on protein catabolism as a primary energy source (Wallace, 1973) and crabs are significantly more likely to feed in low salinity conditions (Curtis and McGaw, *In review*). Salinity and thermal preferences were assessed separately.

The salinity gradient apparatus consisted of a rectangular chamber measuring 3 x 0.4 x 0.3 m, divided into 5 smaller chambers, each with a passageway (15 x 15 cm) that allowed the crabs to move freely between them. Salinity was adjusted and maintained by altering the flow of fresh water and seawater into each chamber. A gradient between 16 and 32‰ (50-100% SW) was maintained and salinity within a chamber never varied by more than 1‰ during the course of a trial. Details of the methodology and apparatus are covered in detail elsewhere (Curtis *et al*., 2007). Movements were recorded using time lapse video (Panasonic AG-RT650). The mean time spent in each salinity, weighted

mean salinity, mean duration of a low salinity event and frequency of movements was determined. Values were compared using two-way ANOVA.

The thermal gradient was constructed using one half of a 0.4 m diameter plastic pipe, measuring 4 m in length. A linear thermal gradient was maintained by heating one end with a pond heater and cooling the other end with a cooling coil attached to a recirculating water bath. An airstone ran the length of the tank to facilitate mixing and maintain a linear gradient from 8 to 22ºC. This range of temperatures is reflective of those seen in the Sarita river estuary (Curtis and McGaw, 2008). Bricks were placed in the gradient 0.3 m apart to provide a surface for the crabs to back against, reducing bias associated with the ends of the tank (Bernatis *et al*., 2007). Temperature within the gradient was constantly monitored using 5 evenly spaced thermocouples. The temperatures experienced by the crab were monitored by attaching a thermocouple to the top of the carapace using cyanoacrylate glue and dental wax. Thermocouples were 2 mm diameter and 2.5m long. Attachment of the thermocouple did not hinder movement of the crab within the gradient. Temperature was measured using a Sable Systems (Las Vegas, NV, USA) temperature box and recorded continuously using an ADInstruments data acquisition system (Colorado Springs, CO, USA). Data was analyzed by examining the temperature conditions that the crab experienced at 10 min intervals. The mean thermal preference and mean time to reach thermal preference were compared using two-way ANOVA.

A total of 20 different individuals were used for each treatment combination. An individual crab was placed in a randomly chosen area of the tank allowed to settle for 15 min prior to the start of the experiment. In the first series of experiments animals were

monitored for 2h. In a second series of experiments, a food stimulus was added to the aversive end of the tank (either low salinity or high temperature). The food stimulus consisted of approximately 200 g of rock sole, wrapped in plastic mesh to prevent the crab from accessing the food. The apparatus was surrounded by black plastic to standardize activity levels and to minimize outside disturbance. To avoid any potential bias, the gradients were reversed for half of the trials in each treatment.

Field Work

Crabs were released in the Sarita river estuary in July and August 2004, and between August and December 2005. The methodology for data tag attachment and retrieval is covered in detail in Curtis and McGaw (2008). The archival data tags (DST-CTD, Starr-Oddi, Iceland) recorded the temperature, salinity (conductance) and depth (pressure) conditions experienced by free ranging crabs at 10min intervals. Crabs were also fitted with individually coded ultrasonic telemetry tags (CT-82-2E, Sonotronics Inc., Tucson, AZ, USA) to enable tracking using a directional hydrophone and receiver (Sonotronics DH-4 and USR-96). Following recapture, the data was recovered from the CTD tags and the average duration and depth of occurrence for salinity and temperature conditions and 95% confidence intervals were calculated. Significant differences were determined based on 95% confidence intervals. The relatedness of salinity and temperature conditions for each 10 min interval was tested using a Chi-square analysis. In order to clarify the large amount of continuous data gathered, specific ranges of salinity and temperature that the crabs experienced were assigned ranks based on behavioural and physiological parameters. A single temperature or salinity event was defined as any

continuous period of time greater than 10 min where the crab was exposed to conditions within the ranges described below. Salinities were ranked as follows 1) 'Optimal Salinity' was considered to be any salinity above 25‰, above this level there is no change in oxygen consumption (Curtis and McGaw, 2009), heart rate (McGaw and McMahon, 1996), or behavioural parameters (McGaw *et al*., 1999) and hemolymph is iso-osmotic with the surrounding medium (Brown and Terwilliger, 1992) 2) 'Mild Stress' was considered to be salinities between 24 and 17‰; within this range, crabs begin to regulate the osmolality of the hemolymph (Hunter and Rudy, 1975), and subsequently display changes in oxygen uptake (Curtis and McGaw, 2009), heart rate (McGaw and McMahon, 1996), and behaviour (McGaw *et al*., 1999) that are indicative of stress. 3) 'Severe Stress' was considered to be any salinity below16‰, at these levels, crabs are unlikely to feed even after prolonged starvation (Curtis *et al*., In press) and display increased oxygen uptake (Curtis and McGaw, 2009) and an increased halokinesis (McGaw *et al*., 1999). Temperatures were also ranked in a similar manner 1) 'Optimal Temperature' was considered to be between 7 and 15ºC as this is the common range of adult animals and within this range crabs show relatively stable metabolic rates (Gutermuth and Armstrong, 1989). 2) 'Mild Stress' was considered to be between 16 and 18ºC; within this range *Cancer magister* displays a sharp increase in metabolic rate, and crabs are reported to also avoid these temperatures in the field (Gutermuth and Armstrong, 1989). 3) 'Severe Stress' was considered to be temperatures above 18ºC; crabs no longer display compensatory increases in heart rate which may limit oxygen delivery (DeWachter and McMahon, 1996; Florey and Kriebel, 1974) and increased mortality rates are observed above this level (Pauley, 1989).

All depth records were corrected for tidal height based on values recorded at Bamfield, B.C., Canada by the Canadian Hydrographic Service and subsequently are reported as depth below mean low low water. Depths shallower than 15 m were considered to be in the estuary based on the presence of low salinity conditions above this depth in summer and fall at the mouth of the estuary (Curtis and McGaw, 2008). Depths shallower than 2 m were considered to be shallow sublittoral.

Results

Laboratory Experiments

Regardless of the degree of starvation or the presence of food, crabs preferred the highest salinity offered (Fig. 3.1A). However, the behaviour exhibited in each of these conditions differed. The number of movements made between chambers in the gradient was dependant on the presence of a food stimulus (Fig. 3.1B; ANOVA df = 1, F = 10.516, $P < 0.01$). After both 5 and 21 d starvation, crabs made significantly fewer movements between chambers in the gradient when a food stimulus was present. However the behaviour resulting in these values were different. After 5 d starvation, crabs moved away from the food stimulus and remained in the high salinity end of the tank, resulting in a significantly higher weighted mean salinity than in any of the other treatment combinations (Fig. 3.2A; ANOVA, df =1, F=4.634, $P < 0.05$). After 21 d starvation, crabs moved towards the food stimulus and the duration of each low salinity event was increased as crabs searched for food in the low salinity end of the tank (Fig. 3.2B; ANOVA, df = 1, F = 9.240, $P < 0.01$).

When exposed to a thermal gradient, the preferred temperature range of *Cancer magister* was between 13.0 ± 1.1 ºC and 14.6 ± 1.3 ºC. However, the addition of a food stimulus in the high temperature end of the tank significantly altered thermal preference and these changes were dependant on the duration of starvation (Fig. 3.3A; ANOVA, df = 1, F = 4.953, $P < 0.05$). In the presence of a food stimulus, 5 d starved crabs displayed a significantly lower thermal preference than in any other treatment combination ($P < 0.05$). The amount of time required for starved crabs to settle in their preferred temperature was also dependant on the presence of a food stimulus (Fig. 3.3B; ANOVA, df = 1, F = 4.771, $P < 0.05$). After 21 d starvation crabs spent significantly more time exploring the gradient before settling in their preferred temperature ($P < 0.05$).

Field Experiments

Data from a total of 15 crabs that were at large between July and December were analyzed. The data collection periods ranged between 6 and 90 d. For free-ranging *Cancer magister*, there was a significant relationship between the salinity and temperature that crabs experienced (Chi-sq = 529.110, df = 4, P < 0.001). Crabs spent over 95% of their time in salinity and temperature conditions (Fig. 3.4) that were not physiologically stressful. Less than 4% of their time was spent in salinity conditions that were classed as mild or severe stress, while less than 1.5% of their time was spent in temperature conditions that were classed as mild or severe stress events. Exposure to mild salinity or mild temperature stress in isolation was as prevalent as would be expected if these conditions were independent of each other. Whereas exposure to mild salinity stress combined with either mild or severe temperature stress were 2 to 5 times

more prevalent than would be expected if salinity and temperature exposure were independent. Exposure to either severely stressful salinity or temperature conditions were rare, accounting for 11 of 279 (4%) and 23 of 162 (14%) exposure events respectively. In total, *Cancer magister* spent approximately 30 times more time in severely stressful temperature conditions than in severely stressful salinity conditions.

The average duration of exposure to mild or severe salinity conditions was significantly shorter than those in optimal salinity (Fig. 3.5A; $P < 0.05$) and the duration of exposure to severe salinity stress was shorter than that of mildly stressful salinity ($P < 0.05$). There was no difference in the depths at which crabs were exposed to optimal or mildly stressful salinity conditions (Fig. 3.5B), however exposure to severe salinity stress occurred at significantly shallower depths ($P < 0.05$). The duration of exposure to mild and severe temperature conditions was significantly shorter than exposure to optimal temperatures (Fig. 3.6A; $P < 0.05$), however the average duration of an exposure to severely stressful temperature conditions was approximately ten times greater than exposure to mildly stressful conditions ($P < 0.05$). The average depth of exposure to each temperature rank was progressively shallower as the degree of temperature stress increased (Fig. 3.6B; $P < 0.05$).

Further examination of the duration of exposure to stressful salinity or temperature conditions showed a unique pattern in their distribution. When in depths of less than 15m, only about 30 % of the salinity conditions experienced were stressful. Approximately 60% of exposures to mild salinity stress were less than 20 min in duration, and approximately 70% of these exposures resulted in an immediate net vertical movement of greater than 1m that was directed towards deeper water (Fig. 3.7A).

Similarly, all but 2 of 11 exposures to severe salinity stress were less than 10min in duration and resulted in an immediate movement towards deeper water. The duration of exposure to mild temperature stress was also characterized by a large proportion of short exposures. About 50% of exposures to temperatures above 15ºC were less than 20 min in duration, and of these about 70% resulted in an immediate movement towards deeper water (Fig. 3.7B). All exposures to temperatures that were mildly stressful occurred at less than 15m depth. All but one of the temperature exposures that were severely stressful occurred in the shallow sublittoral (< 2m depth), or intertidal zone. All trips into the sublittoral or intertidal zones occurred at night, over high tides and crabs were never in less than 1m of water. Figure 3.7C shows an example of exposure to mild salinity and temperature stress while the crab was in the shallow sublittoral zone at night.

Discussion

Environmental conditions play a key role in determining the distribution of many aquatic organisms. Previous work has shown that both temperature (Lewis and Roer, 1988) and salinity (Jury *et al*., 1995) affect the distribution and habitat use of crustaceans, and these effects have been linked to the animal's physiological tolerance (Barnes, 1967; Stillman, 2002). Therefore, these tolerances may dictate the ability of crustaceans to penetrate and exploit habitats that are prone to challenging environmental conditions. For *Cancer magister*, the scarcity of adults in hyposaline habitats has been attributed to their poor osmoregulatory performance (Stevens *et al*., 1984). The results of the current study suggest however that estuary use by adult *Cancer magister* may be more prevalent

than has previously been reported. In addition, crabs may be using avoidance behaviours to minimize exposure to physiologically challenging conditions when exploiting these habitats.

Adult crabs spent over 90% of their time in deeper waters where they were not exposed to challenging temperature or salinity conditions. On average, crabs entered into the depths shallower than 15m once every 5 d and all crabs at large for longer than a week entered the estuary at some point prior to recapture. The short amount of time spent in shallow water is reflective of previous reports on the scarcity of adults within estuarine habitats (Gunderson *et al*., 1990; Stevens and Armstrong, 1984). However, the sampling methods used in these studies do not reflect the prevalence of use by individual animals (Curtis and McGaw, 2008). It is likely that the estuary is being used by a large percentage of individuals in the population, but that only a small fraction is foraging within the estuary at any one time (Gibson, 2003).

In the field, the pattern of duration and depth of exposure to low salinity and high temperature differed, likely in part due to seasonal and spatial differences in the prevalence of these conditions. Overall, within the Sarita river estuary, low salinity conditions occurred at deeper depths than high temperatures and tended to extend into deeper waters as run-off increased during the fall. Previous work has shown that as run-off increases, *Cancer magister* moves towards deeper higher salinity waters to avoid low salinity conditions (Stone and O'Clair, 2001). Despite a general migration to deeper waters, tagged crabs continued to make forays into shallow waters throughout the fall. Higher temperatures occur at shallower depths but become less prevalent as the fall progresses (Curtis and McGaw, 2009). Tidal differences between low salinity and high

temperature conditions are reflected in the depth of exposure, with crabs being exposed to stressful temperatures at significantly shallower depths than stressful salinities. Seasonal differences in temperature conditions also likely contributed to the large discrepancy in the length of exposure between optimal and mildly or severely stressful temperature conditions as a result of stressful temperature conditions not existing in the late fall.

During the summer, high temperatures and low salinities typically occur in concert with each other due to the input of warm, freshwater into the estuary. The prevalence of these conditions likely contributed to the increases in exposure to mildly stressful temperature and salinity conditions in combination. The physiological responses of decapod crustaceans to low salinity are dependent on both temperature and how long they are exposed to such conditions. In isolation, exposure to salinities below 24‰ for less than 6 h (as observed for crabs in the field), does not lead to an increase in metabolic rate (Curtis and McGaw, 2009), despite an increase in heart rate (McGaw and McMahon, 1996). However, exposure to temperatures above 15 ºC results in a substantial increase in oxygen uptake (Gutermuth and Armstrong, 1989). When stressful salinity and temperature conditions are experienced concurrently, osmoregulatory mechanisms begin to breakdown (Tagatz, 1971). Therefore, the interactive effects of temperature and osmoregulation present a greater challenge than each of these demands in isolation. Accordingly, in *Cancer magister*, the degree to which crabs are able to hyperregulate the osmolality of the hemolymph is reduced at 20ºC (Brown and Terwilliger, 1992). This effectively means that a given salinity may present a greater physiological challenge when the crab is also being exposed to stressful temperatures. However, these exposures

likely have little effect on the overall energy budget of adult *Cancer magister*, since they account for less than 1.5% of all recorded temperature and salinity conditions.

Previous reports suggest that adult *Cancer magister* forage in low salinity regions of estuaries during times of high food abundance (Stevens *et al*., 1984; Sugarman *et al*., 1983). However, these reports have not considered the microhabitat conditions that crabs are actually exposed to and only presumed that crabs were being exposed to low salinity based on their location. Our field results provide evidence that while *Cancer magister* is exploiting resource rich estuarine habitats, animals are also using behaviour to minimize exposure to challenging conditions. Less than one third of the salinity or temperature conditions experienced at depths < 15 m presented a physiological challenge. When crabs were exposed to low salinity or high temperature, a distinct pattern was observed in the duration of exposure. Over 50% of the exposures to stressful temperature or salinity conditions were less than 20 min in duration and the majority of these exposures resulted in directed movements away from the aversive condition and into deeper water. Often following this behaviour, crabs would return to shallower water under more favourable conditions associated with the rising tide (and thus a decrease in temperature and a rise in salinity). During longer exposures, crabs were exposed to challenging salinity and temperature conditions for up to 6 h. Here the crabs did not make movements into deeper water and these exposures likely resulted from crabs being passively exposed to changes in salinity and temperature that occur over low tides. During these longer exposures, immediate net vertical movements indicative of avoidance were rare. These results show that crabs are displaying two distinct behaviours when exposed to challenging temperature or salinity conditions: directed avoidance immediately following exposure,

or no regard for challenging conditions and continuing about their activities. While clear avoidance behaviours have been demonstrated, it is also possible that longer bouts result from an inability to orient to and avoid challenging conditions. By avoiding challenging conditions, crabs are able forage in areas prone to episodes of low salinity and high temperature without incurring additional physiological demands.

Approximately 30% of forays into the depths < 15m resulted in the crabs entering the highly productive shallow sublittoral or intertidal zones that make up the majority of the Sarita river estuary. During these trips crabs also displayed behavioural patterns that minimized the challenges associated with being in these areas. Excursions into the sublittoral and intertidal zones occurred exclusively during nocturnal high tides. Migrating into shallow waters under the cover of darkness minimizes the risk of predation from aerial and terrestrial predators (Dumas and Whitman, 1993; Ellis *et al*., 2005; Gibson, 2003). A similar pattern of nocturnal, tidal migrations has been reported for a number of decapod crustaceans (Gibson, 2003) and correspond to endogenous rhythms in locomotor activity (Naylor, 2005; Taylor and Naylor, 1977). The water temperature is also somewhat cooler and the salinities higher during these nocturnal high tides (Curtis and McGaw, 2009; Rewitz *et al*., 2004), allowing adult *Cancer magister* to exploit shallow the sublittoral and intertidal zones, while minimizing the challenges associated with these habitats.

The novel use of the CTD tags provided fine-scale data showing movements of individual animals in response to salinity, temperature, and depth. While these results have made great advances on the typical benthic trawl or trapping methods to gather data about movements of animals, we can still only surmise why crabs were entering into low

salinity and what they were doing there. The intertidal zone of the Sarita river estuary supports high densities of bivalve clams which are a preferred prey item of *Cancer magister* (Dudas *et al*., 2005). Previous studies have shown that the intertidal zone of estuaries is a critical habitat for maintaining large populations of juvenile Dungeness crabs (Holsman *et al*., 2003), and animals have been shown to make large scale migrations from sub tidal channels to more productive intertidal flats over high tides (Holsman *et al*., 2006). We hypothesised that adult Dungeness crabs were also entering into the estuary to feed; laboratory experiments were used to address these questions.

The patterns of behaviour and preference ranges observed in the laboratory corresponded well with the field experiments. In the salinity gradient, crabs which had recently fed made directed movements away from a food stimulus, spending more time in the high salinity end of the tank. In the thermal gradient, crabs which had recently fed also moved away from a food stimulus, resulting in a lower thermal preference. Crabs that had been starved showed a different response to a food stimulus, spending more time in challenging conditions searching for food. Based on these results, it could be suggested that food may also play an important role in the field. Indeed our recent work shows a large scale movement into the estuary in the fall when spent salmon carcasses were being washed downstream (Curtis and McGaw, 2008). However, without more conclusive proof on actual feeding activity or nutritional status in the field these ideas remain conjecture. Additionally, while results from the field presented here suggest that crabs are making directed movements in relation to sharp salinity and temperature gradients that occur within the Sarita river estuary, if environmental conditions in the field are more diffuse, such orientations may not be possible (Bell *et al*., 2003).

The results of the current study have shown that estuaries represent a valuable resource for adult Dungeness crabs. Previous studies predicting habitat use based on large-scale population sampling or physiological ability may under estimate the prevalence of estuary use. By taking into account the frequency of use by individuals or the modification of foraging behaviour to exploit periods of optimal environmental conditions, the observed pattern is somewhat different. Thus, incorporating data acquired from archival data tags is a significant improvement over previous broad scale sampling methods such as trapping or benthic trawl that may overlook important movement patterns. While estuary use was infrequent, exploitation of these habitats by adult crabs appears to be broad, and foraging within these areas may play an important role during times of increased competition. These findings emphasize the importance of considering the behaviour of individuals when investigating the use of habitats with dynamic environmental conditions.

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Figures

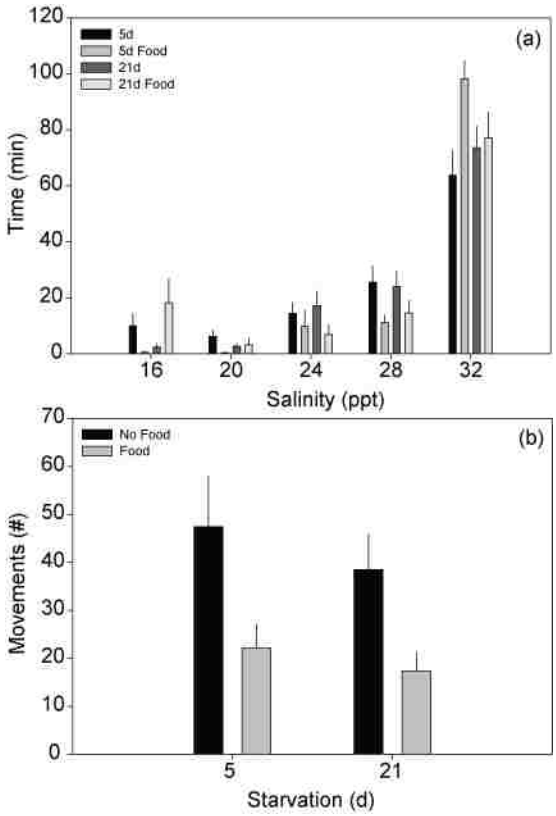

Figure 3.1: The mean amount of time spent in (a) and the number of movements between (b) chambers of a salinity gradient for crabs which had been starved for either 5 or 21d in the presence and absence of a food stimulus in low salinity. Values are mean ± SE for 20 individuals per treatment.

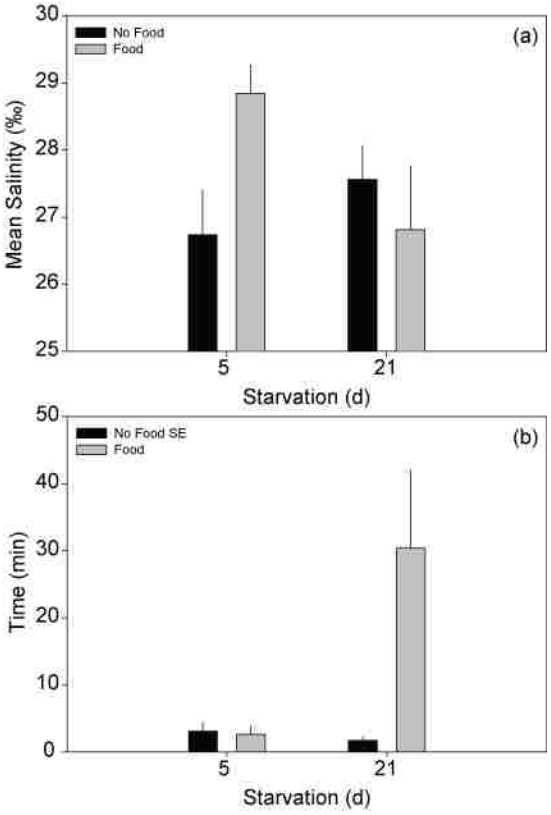

Figure 3.2: The mean salinity experienced (a) and the average duration of a movement into salinity below 24‰ (b), in a salinity gradient for crabs which has been starved for either 5 or 21d in the presence and absence of a food stimulus in low salinity. Values are mean ± SE for 20 individuals per treatment.

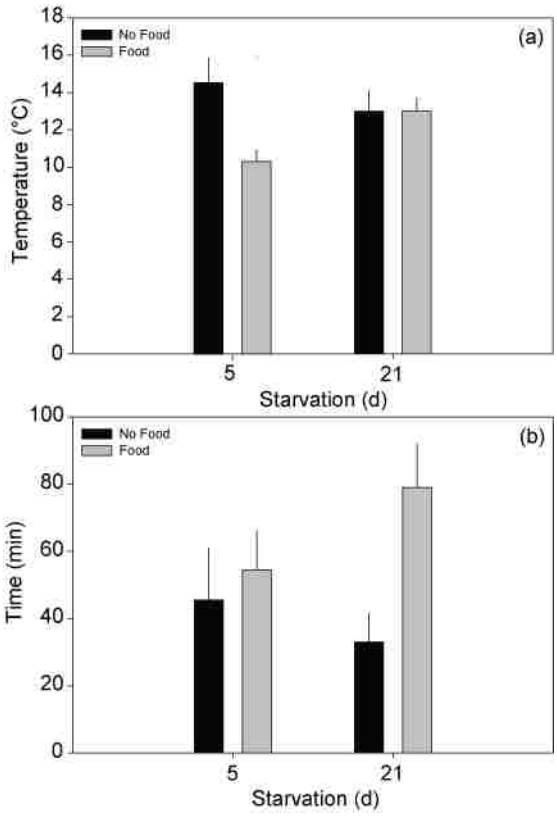

Figure 3.3: The mean thermal preference (a) and the mean time to reach their preferred temperature (b) for crabs in a thermal gradient following 5 or 21d starvation in the presence or absence of a food stimulus in high temperature. Values are mean ± SE for 20 individuals per treatment.

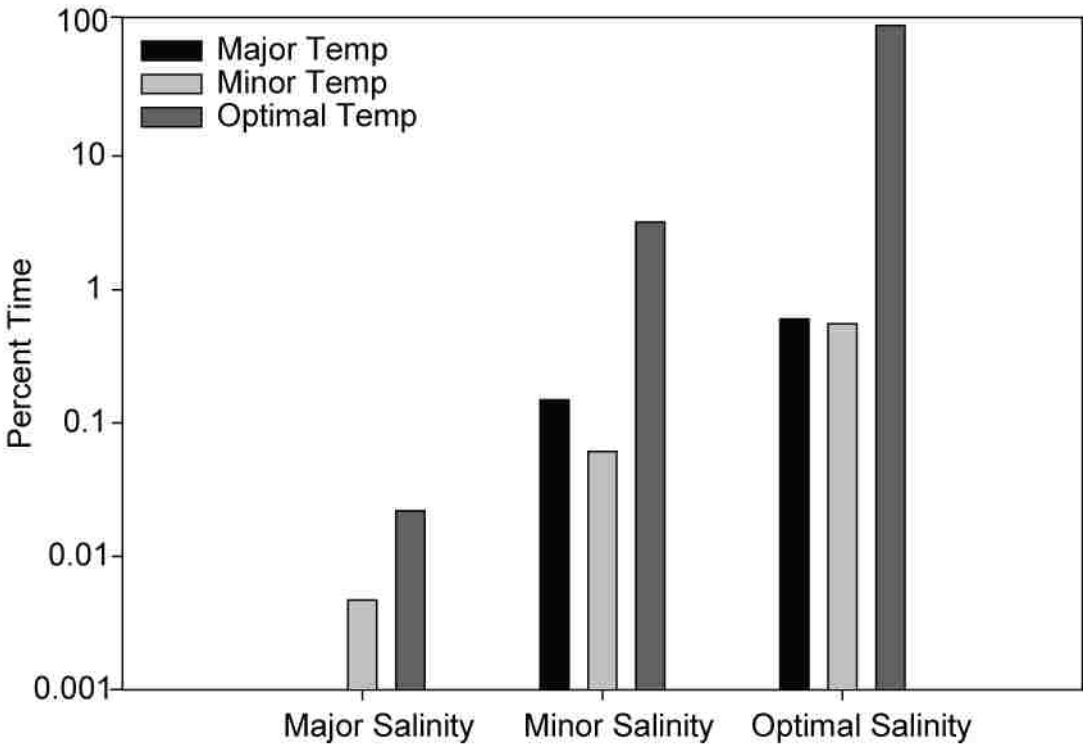

Figure 3.4: The percentage of time spent in optimal, minor, or majorly stressful salinity and temperature conditions for 15 *Cancer magister* fitted with CTD data recorders in the field.

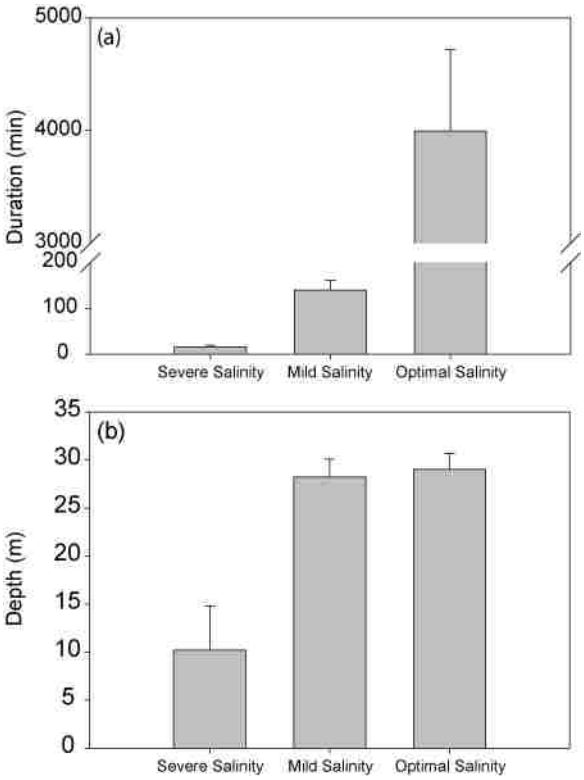

Figure 3.5: The average (a) duration and (b) depth of optimal (n= 142), mild (n = 126) and severely (n = 11) stressful salinity events of crabs in the field. Values are mean ± standard error.

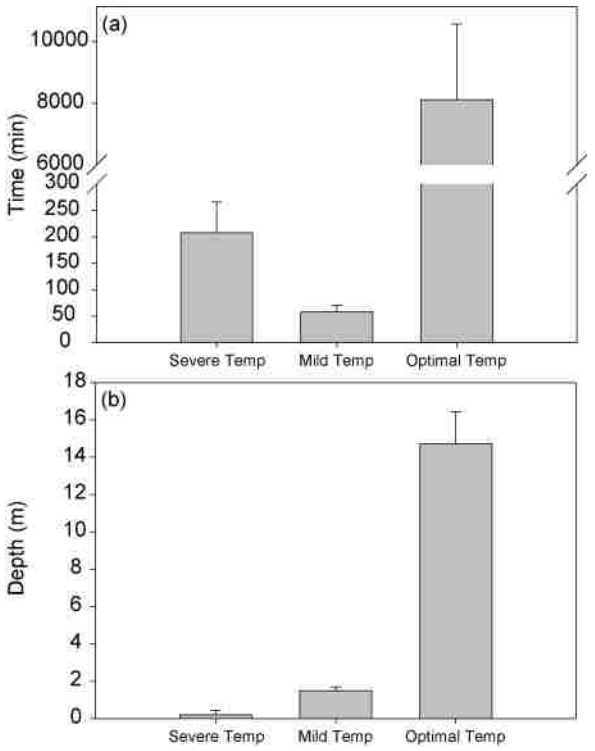

Figure 3.6: The average (a) duration and (b) depth of optimal (n= 142), mild (n = 126) and severely (n = 11) stressful temperature events of crabs in the field. Values are mean ± standard error.

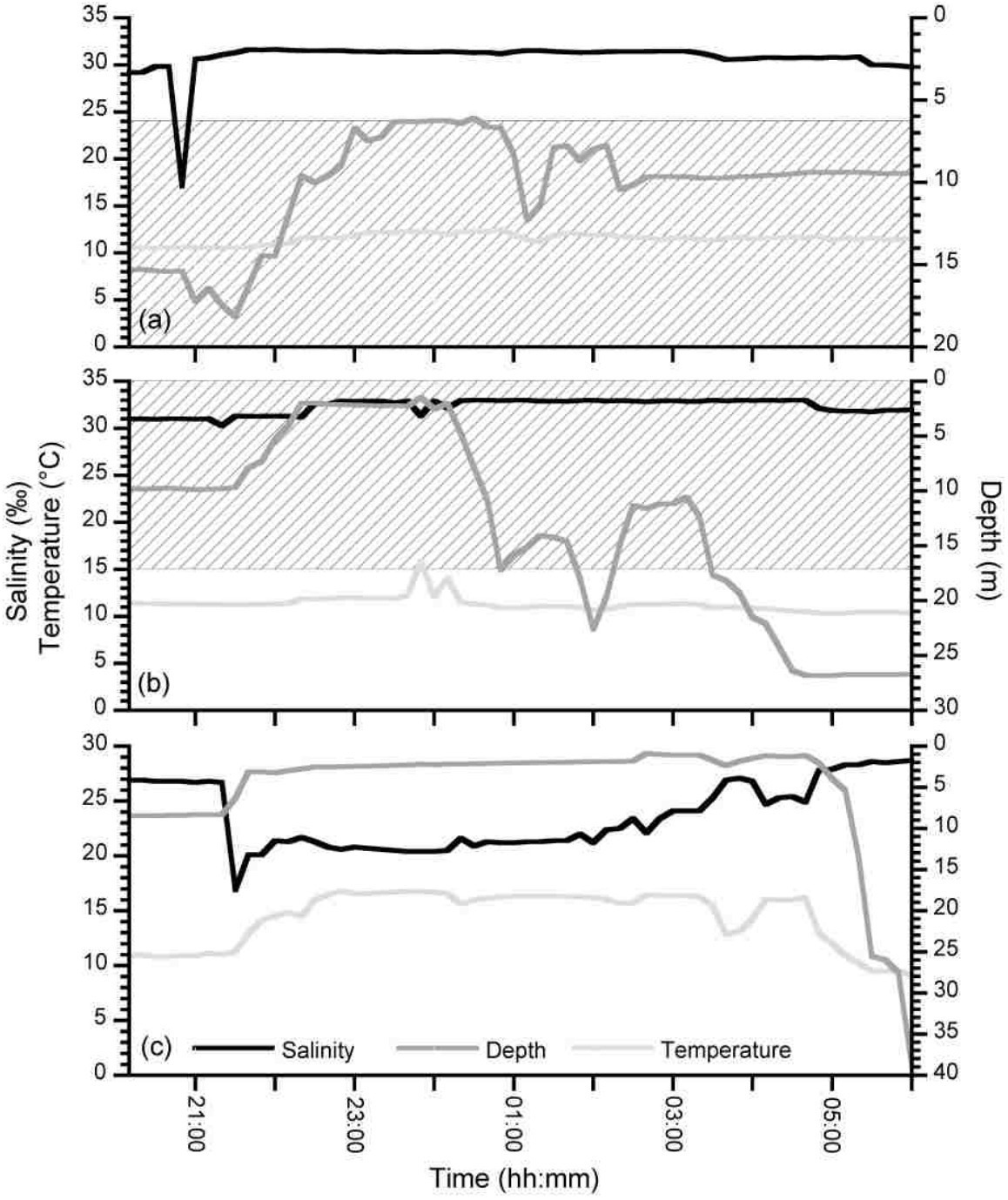

Figure 3.7: Representative salinity, temperature and depth conditions recorded from free ranging crabs in the field. Data shows the avoidance of (a) low salinity, (b) high temperature following short exposures that resulted in movements into deeper water. An example of a longer exposure, where the crab is exposed to both high temperature and low salinity is also shown (c). Diagonal lines represent the range of stressful salinity conditions (a) or temperature conditions (b).

CHAPTER 4

FEEDING BEHAVIOUR

Abstract

Adult *Cancer magister* make forays into hyposaline estuarine habitats during times of high food abundance. However, as weak osmoregulators, they are poorly equipped to deal with the concurrent demands of osmoregulation and digestion. Therefore, the potential interaction between nutritional status and feeding in a physiologically challenging environment was investigated. Changes in the proportion of crabs feeding, the amount of food consumed, the time spent feeding, and the efficiency with which a meal was consumed were examined in response to the length and severity of hyposaline exposure, and the duration of starvation. Reductions in the a) number of animals feeding, b) the amount of food consumed, and c) the time spent feeding were observed in salinities where *C. magister* actively osmoregulates the concentration of its internal fluids. Although this reduction in feeding was likely a stress response, the crabs were able evaluate the level of salinity stress: there was a dose-dependent reduction in feeding and they were able to discriminate between salinities separated by as little as 3.5 ‰. The likelihood that animals would feed in low salinity increased with starvation. Thus, the aversion to food uptake in physiologically stressful conditions may be overridden by the need to procure nutrients. In the natural environment, we suggest that *C. magister* are employing an ‘eat and run’ strategy, moving into the estuary, consuming a meal, and retreating to higher salinities to digest.

This work is currently in press as:

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Introduction

The decision to forage and subsequently feed results from a trade-off between the benefits and risks associated with feeding under a given set of conditions (Stephens and Krebs, 1986). When food sources become scarce animals may endure additional risks in order to obtain food. Much of the work associated with foraging theory has focused on animals foraging under the risk of predation (Lima and Dill, 1990). Using behavioral titrations, some of these studies have even determined an energetic equivalence for predation risk by investigating how much food needs to be available before an individual will enter into an area where predation risk is high (Abrahams and Dil,l 1989; Webster and Dill, 2006). A number of studies have assessed this trade-off for benthic or intertidal marine scavengers feeding under a perceived increased risk of predation, namely dead conspecifics (Stenzler and Atema, 1977; McKillup and McKillup, 1995; Moore and Howarth, 1996). Recently, it has been shown that similar trade-offs may exist between foraging and challenging environmental conditions (Webster and Dill, 2006; Webster and Dill, 2007).

For crustaceans living in estuaries, salinity is a key environmental factor affecting their behavior and distribution (Kinne, 1966). Salinity regimes may change tidally and seasonally (Curtis and McGaw, 2008) and as such, osmoregulatory ability may dictate an animal's capacity to exploit estuarine habitats (Barnes, 1967; Spaargaren, 1973).

Decreases in salinity have profound effects on the physiology and subsequently the growth of many estuarine inhabitants. Typically, for crustaceans that are classed as efficient osmoregulators, low salinity exposure results in an increase in heart rate and oxygen uptake (Taylor *et al.*, 1977). Such responses are thought to aid the additional energetic requirements for active ion uptake and regulation of membrane permeability. For weaker osmoregulators and osmoconformers, low salinity exposures in the field are often acute (Curtis and McGaw, 2008) and the resulting decrease in heart rate and/or cardiac output may minimize the gradient for diffusive ion loss (Cornell, 1974; McGaw and McMahon, 1996; Curtis *et al.* 2007). Recent work has shown that these physiological responses to hyposaline exposure may be compromised when crabs feed and subsequently digest in such conditions (Legeay and Massabuau, 2000; McGaw, 2007). Consequently, for crustaceans unable to balance the demands of several physiological systems, hyposaline exposure may cause an alteration in feeding rate and energetic demands, leading to a reduction in the scope for growth (Guerin and Stickle, 1997; Normant and Lamprecht, 2006).

Despite these physiological effects, crabs that are classed as weak osmoregulators or even osmoconformers can be found in habitats prone to episodes of low salinity (Curtis *et al*,. 2007). It is thought that individuals enter into these areas in response to increased food abundance (Sugarman *et al*., 1983; Stevens and Armstrong ,1984; Curtis and McGaw, 2008). Increased competition generated by a lack of resources may also force animals to feed in sub-optimal habitat conditions (Hoffman and Parsons, 1993). Previous work has shown that benthic marine scavengers are more likely to forage and feed under increased predation risk when they are starved (Stenzler and Atema, 1977;

McKillup and McKillup, 1994; Moore and Howarth, 1996). As energetic reserves are depleted the level of hunger and the necessity for feeding increase (Wang *et al*., 2006). We therefore hypothesized that the necessity for feeding may force decapods of poor osmoregulatory ability to feed in low salinity.

The Dungeness crab, *Cancer magister*, is classified as a weak osmoregulator (Engelhardt and Dehnel, 1973). This species commonly occurs in estuaries during juvenile stages (Gunderson *et al.*, 1990; Holsman *et al*., 2006; Curtis and McGaw, 2008). Adult crabs are less common in estuaries, and only appear to make brief excursions into these habitats (Curtis and McGaw, 2008). While this recent work has empirically shown that crabs are entering into the estuary during times of high food abundance, there is little substantiation as to what crabs are actually doing while in low salinity. Based on these observations, we hypothesized that adult crabs were making short forays into low salinity areas to forage. However, feeding in low salinity can be stressful for adult crabs: *C. magister* is unable to balance the simultaneous physiological costs of feeding and osmoregulation, and postprandial crabs experience higher mortality rates in severe low salinity (McGaw, 2006). We further hypothesized that the necessity of procuring a meal must be great before crabs will endure the additional costs of digestion in low salinity. To investigate these questions, we examined changes in the amount of food ingested, the time spent feeding and the likelihood of feeding in response to 1) the degree of low salinity exposure, 2) the duration of low salinity exposure and 3) the time since the last meal (degree of starvation).

Materials and Methods

Animals

Adult male intermolt Dungeness crabs, *Cancer magister*, of 300 to 750 g were trapped in Barkley Sound British Columbia, Canada, from June to October 2005. Animals were transported to the Bamfield Marine Sciences Centre, and held in running seawater (SW) of 32 ± 1 ‰ and at a temperature of 12 ± 1ºC for a minimum of one week prior to experimentation. Crabs were fed fish (*Lepidopsetta bilineata*) every other day. Salinity and temperature regimes were monitored using an YSI-30 conductivity meter (YSI Inc., Yellow Springs, OH).

Protocol

The interactive effects of the degree of low salinity exposure, the duration of low salinity exposure and the duration of starvation on 1) the proportion of *C. magister* feeding, 2) the amount of food consumed, and 3) the amount of time spend feeding were investigated. Prior to experimentation, the crabs were removed from the holding tanks, transferred to a 300 L tank and allowed to feed *ad-libitum* on rock sole (*L. bilineata*) for 1 h. After ensuring that each individual had fed, the food was removed and crabs were starved for 2, 5, or 21 d. These starvation periods were chosen to correspond with physiological changes. A 2 d starvation period is the minimum time period used in physiological and behavioral studies (Elner and Hughes, 1978); at this point the meal has been cleared from the foregut (McGaw and Reiber, 2000). At 5 d starvation, the physiological effects of the previous meal have passed and metabolism has returned to basal levels (Wallace, 1973; McGaw and Reiber, 2000). Following 21 d starvation, there

is a further decrease in metabolic rate that is thought to be associated with switching of metabolic substrates (Wallace, 1973), and an increased urgency for feeding (Wang *et al*., 2006). Once the animals had been starved for the required period they were transferred to a sea table (150 x 70 x 20 cm) which was divided into 10 equal sized chambers, and allowed to settle for 3h after handling. The salinity was then changed to 25, 50, 75 or 100% SW (8, 16, 24 or 32 ‰, respectively), over a period of 30 min by draining a portion of the tank and replacing it with aerated dilute seawater of the same temperature.

Crabs were exposed to the test salinities for 0.5, 6, or 24 h. A 0.5 h exposure would represent the crab making a brief foray into low salinity to feed (Curtis and McGaw, 2008) or being confronted with an abrupt halocline and showing avoidance behaviors. A 6 h exposure is representative of the salinity conditions that *C. magister* would experience during the tidal cycle at a fixed location in the estuary (Curtis and McGaw, 2008). A 24h exposure is representative of chronic low salinity conditions that persist in the estuary during times of high run-off (Curtis and McGaw 2008); this time period also allows the osmolality of the hemolymph to reach new stable levels (Siebers *et al.*, 1972; McGaw, 2006). Following each exposure time, the crabs were presented with a piece of pre-weighed fish muscle (approximately 10% of their body mass). The fish had been soaked for 6h prior to experimentation in the treatment salinity which minimized changes in wet mass during the course of the experiment. When a crab ceased feeding for more than 15min the feeding bout was considered finished and the amount of time spent feeding was recorded. Crabs that did not feed within 1h of being presented with food were scored as non-feeders. The uneaten food was then removed and weighed, and the amount of food consumed was calculated. This three-way design resulted in a total of 27

separate treatment combinations, in which each of the 3 variables was measured. A minimum of 20 crabs were used for each treatment, and treatments were repeated until at least 5 crabs had fed so that an estimate of variance could be obtained. A total of 570 individuals were tested.

Data Analysis

The effect of salinity, starvation and acclimation on the proportion of crabs feeding for each trial was determined using a generalized linear model with binomial error. The main effects and all two-way interactions were included as fixed effects. The three-way interaction was not included due to sample size limitations on the complexity of the model. The model was estimated using PROC GLIMMIX in SAS v.9.1 (SAS Institute 2002-2003).

The effects of salinity, starvation and acclimation time on 1) the amount of food consumed, 2) time spent feeding and 3) efficiency (the amount of food consumed in a given time) were initially examined with identical mixed model ANOVAs (PROC MIXED; SAS v.9.1). Fixed effects included main effects of salinity, starvation and acclimation and all two- and three-way interactions, in addition to each individual's wet weight as a covariate. Two- and three-way interactions with the covariate were included in the initial model, but these terms were dropped when they had high p-values ($P>0.20$) due to concerns about sample size effects on accuracy of the model. Trial nested within each salinity-starvation-acclimation time combination was included as a random effect. Only animals that fed were included in the models, and all dependent variables were $\log_{10}$-transformed. Significant effects were followed up using Tukey post-hoc tests.

Unless otherwise stated, values presented are the overall least squares means and contain all levels of the other two factors. Results are presented with asymmetrical error terms resulting from back transformation of log-transformed values. Significance was determined at $\alpha = 0.05$ for all analyses.

Results

Likelihood of Feeding

The proportion of *C. magister* that fed varied with the level of salinity exposure and the duration of starvation (Table 4.1; Fig. 4.1). As the salinity decreased, fewer crabs fed in each successively lower salinity (Fig. 4.1a). *C. magister* would not feed below 40% SW even following 21d starvation; therefore, the 25% SW treatment was not considered in the analyses. The duration of exposure to low salinity did not have a significant effect on the proportion of crabs feeding (Table 4.1; Fig. 4.1b). In contrast, the proportion of crabs that fed increased with starvation (Fig. 4.1c).

There were no statistically significant interactions among salinity, starvation, and acclimation; however, trends were observed that may have been masked by the low power and overall effects associated with other salinities (Fig. 4.2). When each salinity was examined separately, differences were observed in the 50% SW treatments; here 21 d starvation increased the proportion of crabs feeding by three-fold over the proportion that fed after 2 d starvation. This difference was significant when a post-hoc test was applied ($t = 5.04$, $df = 38$, Tukey-adjusted $P < 0.0004$). In contrast there was no effect of starvation time on the proportion of crabs feeding in 75% or 100%SW.

Food Consumption

Given that a crab started feeding, the amount of food that it consumed varied with salinity and starvation, but not with the duration of low salinity exposure (Table 4.2). The amount of food consumed by crabs in 50% SW was significantly reduced relative to those in 100% SW (Fig. 4.3a). There were no significant interactions among any of the treatments. Crab mass was positively related to the amount of food consumed (Table 4.2) and varied with the level of starvation (Table 4.3). This relationship resulted from smaller crabs consuming relatively larger meals following 21d starvation (Fig. 4.4). Additionally, this relationship likely contributed to the significant overall effect of starvation on the amount of food consumed, but masked differences between the levels of starvation (Table 4.2, Fig. 4.3a).

Time Spent Feeding

The amount of time crabs spent consuming a meal varied with salinity (Table 4.4; Fig. 4.5). *Cancer magister* spent almost twice as much time feeding in 100% or 75% SW compared to 50% SW (Fig. 4.5a). There was no significant effect of acclimation or starvation on the amount of time spent feeding, nor were there any significant interactions among the effects (Table 4.4; Fig. 4.5b and c). Larger crabs spent significantly more time feeding (Table 4.3; Table 4.4; Fig. 4.6).

Feeding Efficiency

The efficiency (food consumed in a given amount of time) with which crabs consumed a meal varied with salinity (Fig. 4.7, Table 4.5). Crabs consumed a given

amount of food faster in 50% SW than in 75% SW and there was a trend towards crabs being more efficient in 50% SW than in 100% SW, which fell just short of significance ($P = 0.08$). Starvation, acclimation time, and crab mass did not have significant effects on feeding efficiency, nor were there any interactions among the variables (Table 4.5).

Discussion

Estuaries are a habitat rich in prey and sparse in predators that provide a valuable resource for juvenile Dungeness crabs (Stevens *et al.*, 1984; Gunderson *et al.*, 1990). However, much less is known about the use of these habitats by adult animals. Forays into estuarine habitats by adult Dungeness crabs are most prevalent during times of high food abundance (Sugarman *et al*., 1983). During these forays, crabs may either actively enter into areas of low salinity or be passively exposed with the changing tide (Curtis and McGaw, 2008). In either case, salinity plays a key physiological role and it appears that *C. magister* is poorly adapted to deal with the demands of digestion while exposed to hyposaline conditions (McGaw, 2006). Therefore, the benefits of feeding in such conditions must outweigh the physiological challenges (Lima and Dill, 1990). The results of this study suggest that there is an interaction between the aversion to feeding resulting from low salinity exposure and immediacy for procuring a meal.

For such an interaction to exist, animals must be able to integrate associated environmental signals. Decapod crustaceans are able to integrate complex chemosensory signals (Cromarty and Derby, 1997) via input from olfactory sensilla (Hallberg *et al.*, 1997). Changes in environmental salinity are detected by hair-peg organs located on the

legs (Schmidt, 1989) and the antennules (Van Weel and Christofferson, 1966), as well as receptors in the branchial chamber (Hume and Berlind, 1976; Dufort *et al*., 2001). *Cancer magister* was able to discriminate between salinities separated by less than 3.5‰; the crabs would feed in 50% SW, but not in salinities lower than 40% SW. A substantial reduction in the number of crabs feeding in 50% SW compared with 75% SW suggests that crabs are integrating the degree of low salinity exposure (Fig. 4.1a). Immediately after detecting a drop in environmental salinity, *C.magister* displays behavioral (Sugarman *et al*., 1983; McGaw *et al*., 1999) and cardiovascular adjustments (McGaw and McMahon, 1996). These adjustments are sensitive indicators of stress (Florey and Kriebel, 1974) and take place well before any marked decrease in hemolymph osmolality, which can take over 24h to reach new stable levels (Siebers *et al*., 1972; McGaw, 2006). In salinities below 75% SW, *C. magister* showed a similar aversion to feeding regardless of the duration of exposure and subsequent changes in the internal milieu (Fig. 4.1b). Given their ability to detect small changes in external salinity (Sugarman *et al.*, 1983) and to integrate complex chemosensory signals, it seems reasonable that crabs are responding to low salinity as a nociceptive stimulus rather than decreases in hemolymph osmolality.

An interaction between low salinity and starvation also requires that animals are able to integrate nutritional status. Correspondingly, as the time since their last meal increased, so did the proportion of crabs feeding (Fig. 4.1c). This pattern was most apparent in 50% SW and the increase in the proportion of crabs feeding following 21 d starvation appeared, to a large extent, to offset the decrease resulting from low salinity exposure (Fig. 4.2). However, this interaction had a threshold, as crabs would not feed below 40% SW, even after 21d starvation. This strategy would be advantageous given

that postprandial crabs show increased mortality at salinities below this level (McGaw, 2006). An animal's nutritional status may therefore tip the behavioral balance in favor of enduring low salinity exposure in order to obtain urgently needed sustenance. A similar trade-off between nutritional status and feeding while exposed to a nociceptive stimulus has been observed for a number of benthic scavengers when presented with food and the scent of dead conspecifics (Stenzler and Atema, 1977; McKillup and McKillup, 1994; Moore and Howarth, 1996). Thus it appears that some of the concepts applied to animals foraging in the face of predation (Lima and Dill, 1990) may also be applicable to animals foraging when exposed to environmental challenges such as low salinity (Webster and Dill, 2006).

As the time since feeding increases, so too does the urgency for procuring a meal (Wang *et al.*, 2006). When allowed to feed *ad-libitum*, *C. magister* feeds about once per day (Curtis and McGaw, Unpublished obs.), which corresponds closely with the emptying of the foregut (Curtis and McGaw, 2009). In many molluscs, appetite is a graded function based on gut fullness (Elliott and Susswein, 2002) and recovery from the physiological demands associated with digestion may be linked to the return of appetite in dog fish (Sims and Davies, 1994). Accordingly, most work examining the interplay between starvation and feeding motivation in benthic marine scavengers has only looked at a single level of starvation (Stenzler and Atema, 1977; McKillup and McKillup, 1994; Moore and Howarth, 1996), comparing 'hungry' vs. 'not hungry' responses. In the present study, 3 starvation periods (2, 5, and 21 d) were used, each corresponding to a physiological change that alters the degree of urgency for procuring another meal (Wang *et al.*, 2006). At 2 d post-feeding, crabs are no longer satiated (McGaw and Reiber,

2000). At 5d post-feeding, the physiological changes associated with digestion have passed, but no large-scale physiological changes associated with starvation have occurred within this time (Wallace, 1973). By 21 d post-feeding nutritional stores are depleted and crabs are likely relying on protein catabolism as their primary means of energy production (Wallace, 1973; Sanchez-Paz *et al*., 2006). In *C. magister*, it appears that feeding motivation is regulated by a more complex system than satiation alone. Based on a satiation model, the crabs should have been equally likely to feed at all levels of starvation because their gut was no longer full (Elliott and Susswein, 2002). However, it was not until 21 d starvation that a significant increase in the proportion of animals feeding was observed (Fig. 4.1c). The degree of nutritional deprivation, rather than gut fullness, appeared to be of prime importance. While a clear internal mechanism for sensing long term changes in nutritional status is difficult to substantiate, many terrestrial arthropods are able to sense nutritional deficiencies in their diet and make compensatory changes in their feeding behavior by selecting food items or portions of prey items that are rich in the deficient nutrient (Mayntz *et al*., 2005; Pompilio *et al*., 2006). Additionally, it has been shown that these animals are not only able to associate olfactory inputs with a reward, but also with the degree of reward provided (Behmer *et al*., 2005).

A definitive neurological or hormonal basis for the trade-off between nutritional status and low salinity exposure has yet to be shown for *C. magister*, but it appears that both chemosensory stimuli and the degree of nutritional deprivation affect feeding behavior. In the sand fiddler crab, *Uca pugilator*, when the eye stalks are ablated, subsequently removing hormonal control exerted by the sinus gland/X-organ complex, feeding inhibition due to satiation is removed (Sears *et al.*, 1991). This response may be

due to the action of a putative hormone referred to as Feeding Inhibition Factor (FIF). Preliminary results suggest that the anorexic effects resulting from low salinity exposure may also be related to endocrine products originating in the sinus glad (Curtis and McGaw, In prep.). In the current study, even after a brief (30min) exposure to low salinity, crabs that refused to feed in low salinity did not regain their appetite for over 4 h following return to 100% SW (Curtis and McGaw, Unpublished obs.). This timing corresponds to the circulating time of FIF (Sears and Rittschof, 1991), supporting the assumption that the anorexic effects resulting from low salinity exposure are the result of a neurohormonal release, rather than changes in the internal osmolality of the animal.

After feeding, crabs must subsequently cope with the metabolic demands associated with digestion, referred to as apparent specific dynamic action (SDA; McCue, 2006). The scope (Pan *et al*., 2005) and duration (Ansell, 1973; Beamish, 1974; Houlihan *et al*., 1990) of the SDA response increases with ration size and *C. magister* is no exception (McGaw, Unpublished obs.). Low salinity exposure resulted in a decrease in the amount of food consumed (Fig. 4.3a), which likely resulted in a lower overall SDA (Curtis and McGaw, 2009). This reduction in SDA may be further aided by a reduction in foregut activity that slows food passage through the gut, subsequently minimizing costly downstream processes such as protein synthesis (Curtis and McGaw, 2009).

While a reduction in meal size may help to facilitate foraging in low salinity, reduced caloric intake may have a negative effect on growth rates (Guerin and Stickle, 1992). An alternative explanation for reduced meal sizes is that crabs simply lack the energetic resources to consume larger meals while coping with the physiological demands associated with low salinity exposure. Such a limitation is possible since *C. magister*

prioritizes the cardiovascular and ventilatory responses to low salinity exposure over those associated with digestion (McGaw, 2006). Nevertheless, the fact that the crabs consumed a given amount of food faster in low salinity (Fig. 4.7), and the costs of actually ingesting a meal are low (Rovero et al. 2000), would suggest that this is unlikely.

Following 21 d starvation, smaller *C. magister* consumed larger meals relative to their counterparts which had been starved for 2 or 5 d (Fig. 4.4). Yet, larger crabs consumed similar meal sizes regardless of starvation. The tolerance of starvation among crustaceans appears to be highly variable, with some species such as the Chinese mitten crab, *Eriocheir sinensis*, routinely surviving periods of starvation greater than 70d (Wen *et al*., 2006). Within a species however, smaller crabs seem to be more prone to food deprivation (Moir and Weissburg, 2009). Smaller animals possess relatively fewer energy reserves and have higher mass specific metabolic requirements, meaning that those reserves which they do have will be more rapidly depleted (Clifford and Brick, 1983). Therefore, for smaller *C. magister*, consuming larger meals may be a means of compensating for an additional nutritional deficit despite an increased SDA. An increased susceptibility to starvation may therefore contribute to the prevalence of smaller individuals in hyposaline waters (Stevens *et al*., 1984).

When exposed to low salinity, *Cancer magister* spent less time feeding (Fig. 4.5a) and consumed a given amount of food faster (Fig. 4.7). The metabolic costs associated with the act of ingesting a meal are relatively low, accounting for about 2% of the energy gained from the meal (Rovero *et al*., 2000). It has been postulated that rather than measuring actual energy expenditure, the time spent consuming a meal may be a better metric of the cost of foraging (Juanes, 1992). In the laboratory, both fed and postprandial

crabs show an equal aversion to low salinity exposure, preferring the highest salinity offered (Curtis and McGaw, 2004); however after prolonged starvation crabs spend more time in low salinity searching for food (Curtis and McGaw, In prep). Upon finding a meal, *C. magister* does not move the food to areas of higher salinity, but instead remains in these conditions to feed (Curtis and McGaw, Unpublished Obs.) and therefore they must continue to endure the physiological demands associated with low salinity exposure. The reduced time spent feeding in low salinity (Fig. 4.5) may be the result of a trade-off between the tendency towards avoidance behaviors and the necessity of procuring a meal. Additionally, increased feeding efficiency in low salinity (Fig. 4.7) further suggests that crabs are not energetically restricted, but rather are employing an 'eat and run' strategy to minimize exposure. Such a strategy is supported by field observations (Curtis and McGaw, 2008). During times of high food abundance, adult *C. magister* move up into the shallow, lower salinity regions of the estuary, presumably to feed. However these excursions into low salinity typically only last a few hours before the crabs retreat to deeper, higher salinity waters. This 'eat and run' behavior would minimize the need to use physiological mechanisms to cope with the simultaneous demands of digestion and osmoregulation.

In resource limited habitats, competition may force animals to reside or forage in challenging environmental conditions (Hoffman and Parsons, 1993). We have shown that unless the necessity for feeding is great, inhibitory mechanisms may prevent *C. magister* from feeding in salinities below 75% SW. In crabs that do feed in these hyposaline environments, a reduction in the amount of food consumed may reduce the effects of SDA (Curtis and McGaw, 2009), thus enhancing their ability to prioritize the

physiological responses to low salinity over those of digestion. Additionally, reducing the amount of time spent feeding would minimize exposure to low salinity; while retreating to more favorable salinity conditions would allow them to digest the meal more efficiently (Curtis and McGaw, 2009). Though these strategies would provide an immediate reprieve from the challenge of concurrent osmoregulation and digestion, they may also lead to a substantial reduction in food intake and thus growth if conditions become more ephemeral and low salinity becomes more pervasive.

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Tables

Table 4.1: Effects of starvation, acclimation, and salinity on the proportion of crabs feeding in each trial. The degrees of freedom (df) are provided as numerator, denominator df; bold denotes significant p-values.

Effect	df	F	P-value
Starvation	2, 38	8.00	**0.0013**
Acclimation	2, 38	0.61	0.5483
Salinity	2, 38	50.23	**<0.0001**
Starvation*Acclimation	4, 38	2.18	0.0898
Starvation*Salinity	4, 38	1.03	0.4041
Acclimation*Salinity	4, 38	0.37	0.8263

Table 4.2: Effects of starvation, acclimation, and salinity on the amount of food consumed. The degrees of freedom (df) are provided as numerator, denominator df; bold denotes significant p-values.

Effect	df	F	P-value
Starvation	2, 29	7.59	**0.0022**
Acclimation	2, 29	1.58	0.2224
Salinity	2, 29	4.14	**0.0263**
Starvation*Acclimation	4, 29	0.46	0.7672
Starvation*Salinity	4, 29	1.85	0.1457
Acclimation*Salinity	4, 29	0.38	0.8215
Starv*Acclim*Salin	8, 29	0.71	0.6797
Crab Mass	1, 333	51.06	**0.0000**
Crab Mass* Starvation	2, 333	6.46	**0.0018**

Table 4.3: Intercept and slope for the equation: $\log_{10}$(dependent variable) = intercept + slope*crab mass for each significant effect, where food mass and crab wet mass were expressed in grams, and time spent feeding was expressed in minutes. Significant differences among slopes are denoted by different letters.

Dependent Variable	Duration of Starvation	Intercept (SE)	Slope (SE)		r^2
Food mass	2d	0.2220 (0.1419)	0.0015 (0.0002)	b	0.2167
	5d	0.1348 (0.1311)	0.0016 (0.0002)	b	0.3645
	21d	0.7453 (0.1180)	0.0005 (0.0002)	a	0.0807
Time feeding		0.0011 (0.0002)	0.8739 (0.0956)		0.0724

Table 4.4: Effects of starvation, acclimation, and salinity on the time spent feeding. The degrees of freedom (df) are provided as numerator, denominator df; bold denotes significant p-values.

Effect	df	F	P-value
Starvation	2, 29	0.33	0.7220
Acclimation	2, 29	0.67	0.5193
Salinity	2, 29	8.40	**0.0013**
Starvation*Acclimation	4, 29	0.11	0.9771
Starvation*Salinity	4, 29	1.43	0.2498
Acclimation*Salinity	4, 29	0.38	0.8205
Starv*Acclim*Salin	8, 29	0.33	0.9464
Crab Mass	1, 335	23.58	**0.0000**

Table 4.5: Effects of starvation, acclimation, and salinity on feeding rate (g/min). The degrees of freedom (df) are provided as numerator, denominator df; bold denotes significant p-values.

Effect	df	F	P-value
Starvation	2, 29	0.44	0.6491
Acclimation	2, 29	0.19	0.8290
Salinity	2, 29	4.65	**0.0177**
Starvation*Acclimation	4, 29	0.58	0.6760
Starvation*Salinity	4, 29	1.12	0.3670
Acclimation*Salinity	4, 29	0.25	0.9073
Starv*Acclim*Salin	8, 29	1.22	0.3244
Crab Mass	1, 335	1.21	0.2714

Figures

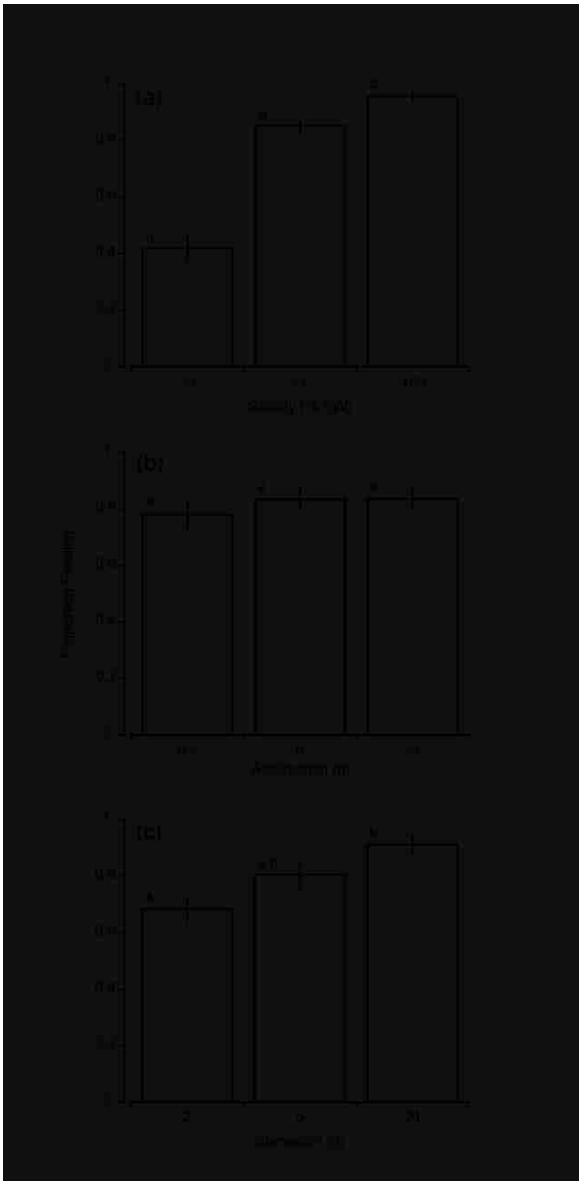

Figure 4.1: The overall mean proportion (± SE) of crabs feeding following exposure to **a** 50, 75 or 100% SW for **b** 0.5, 6, or 24h after **c** 2, 5, or 21d starvation. Different letters indicate significantly different treatments

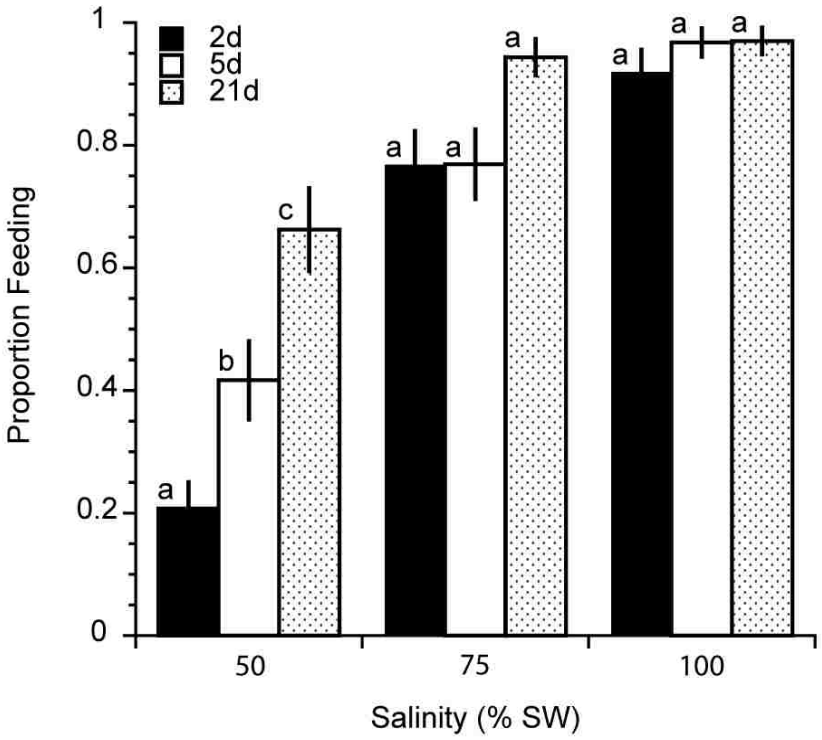

Figure 4.2: The mean proportion (± SE) of crabs feeding following 2, 5, or 21 d starvation within each level of salinity. Within each level of salinity, different letters indicate significantly different values. Values include all levels of exposure time

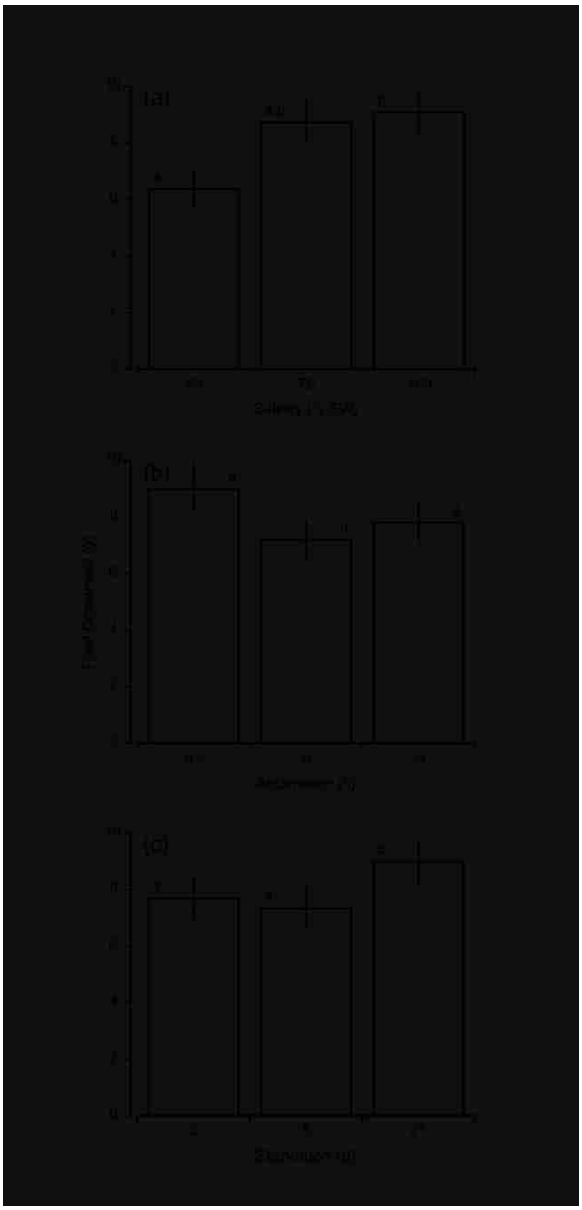

Figure 4.3: The overall mean amount of food consumed (± SE) following exposure to **a** 50, 75 or 100% SW for **b** 0.5, 6, or 24h after **c** 2, 5, or 21d starvation. Different letters indicate significantly different values

Figure 4.4: The relationship between crab mass and the amount of food consumed following 2, 5, or 21 d starvation. Trend lines are shown for significant relationships (see Table 3 for regression details)

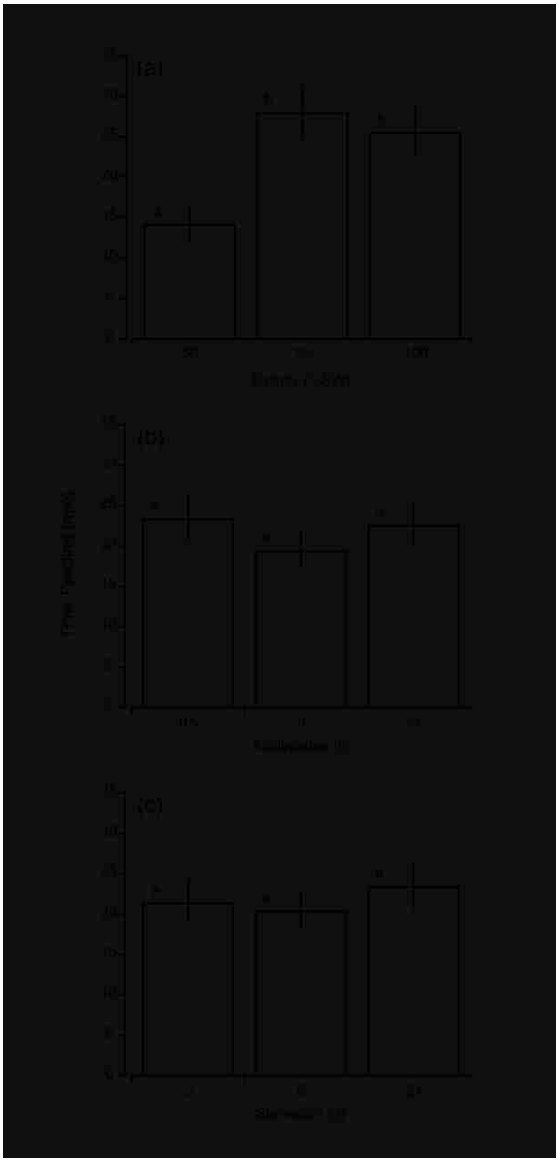

Figure 4.5: The overall mean time spent feeding (± SE) following exposure to **a** 50, 75 or 100% SW for **b** 0.5, 6, or 24h after **c** 2, 5, or 21d starvation. Different letters indicate significantly different values

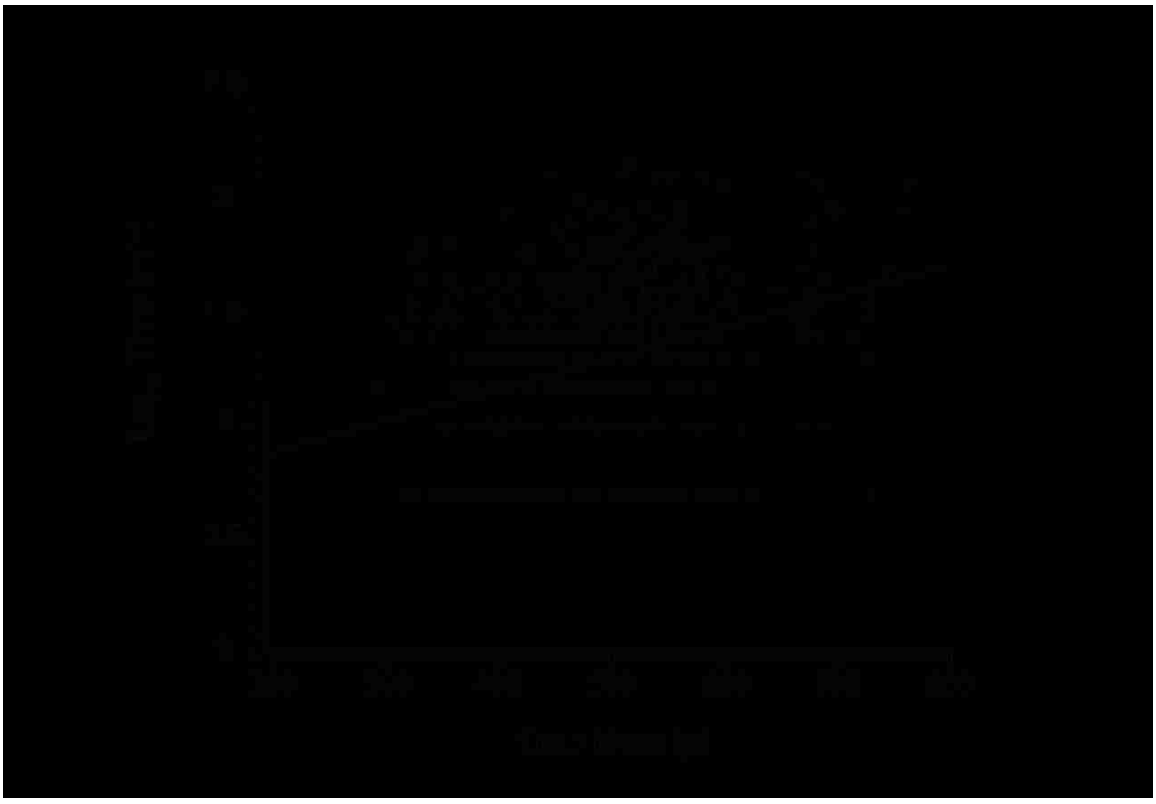

Figure 4.6: The overall relationship between crab mass and the amount of time spent feeding. Trend line shown for significant relationship (see Table 3 for regression details)

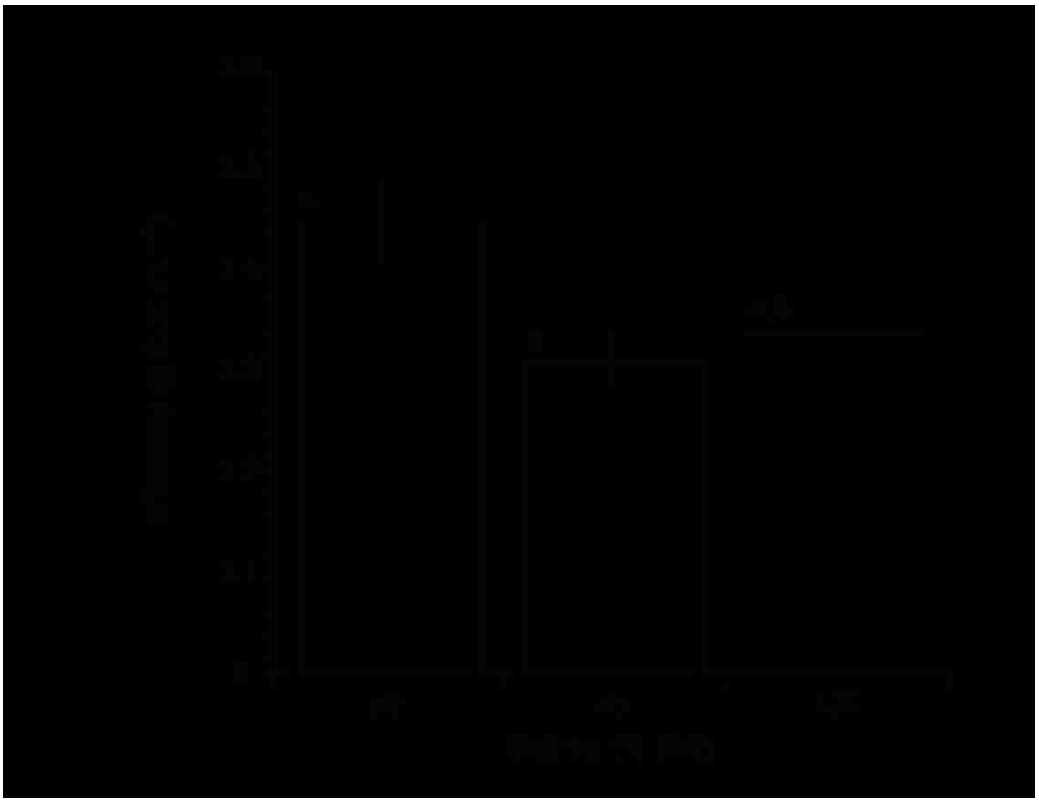

Figure 4.7: The overall mean efficiency (± SE) with which crabs consumed a meal in 50, 75 or 100% SW. Different letters indicate significantly different values

CHAPTER 5

HORMONAL CONTROL OF FEEDING BEHAVIOR

Abstract

The Dungeness crab, *Cancer magister*, is classed as a weak osmoregulator, nevertheless this species will enter into low salinity regimes of estuaries during times of high food abundance. The present study investigated the possible regulatory role of neurohormones on feeding behaviour during acute low salinity exposure. When the crabs eyestalks were ablated, removing the sinus gland (which is the source of a postulated inhibitory hormone), they consumed more food and fed for a longer period of time compared with intact animals. Eyestalk ablated animals would even attempt to feed in freshwater, whereas intact animals would only consume food in salinities above 40%SW. The results suggest that feeding behaviour during low salinity exposure in *C. magister* is regulated by an inhibitory neurohormone originating in the sinus gland. This mechanism may help animals balance the demands of competing physiological processes.

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Introduction

Changes in environmental temperature, oxygen tension or salinity can have marked effects on the feeding behaviour of aquatic organisms (e.g. Arnesen *et al.*, 1993; Brante and Hughes, 2001; Pihl *et al*., 1991). In ambient conditions, the feeding behaviour of many invertebrates is regulated by a satiation response resulting in the animal being either hungry or satiated (Elliott and Susswein, 2002). Sears *et al*. (1991) suggest that in decapod crustaceans this response is regulated by a putative hormone produced in the sinus gland of the eyestalks which they termed Feeding Inhibition Factor (FIF). Our recent work has shown that feeding decisions are regulated by a more complex mechanism than satiation alone. Rather than a simple satiation response, decapod crustaceans appear to be able to integrate the degree of starvation and the physiological challenge presented by their surrounding environmental conditions when making feeding decisions (Curtis *et al*., In press).

For crustaceans inhabiting estuaries, low salinity is the single most important factor governing their distribution (Barnes, 1967). The Dungeness crab, *Cancer magister*, is classed as a weak osmoregulator, nevertheless it makes excursions into hyposaline estuarine habitats, possibly to feed (Barnes, 1967; Curtis and McGaw, 2008; Stevens *et al*., 1984; Sugarman *et al*., 1983). The effects of severe low salinity exposure are exacerbated in animals that have recently fed, resulting in increased mortality rates (McGaw, 2006). This inability to balance the physiological processes of digestion with those associated with low salinity probably accounts for the reduced feeding rate observed in *Cancer magister* in low salinity (Curtis et al., In press). This aversion to feeding in low salinity can be partially overridden; as the time since the last meal

increases, crabs are more likely to feed in low salinity and consume more food (Curtis *et al*., In press). However, there is a threshold to this response, and Dungeness crabs will not feed below 40% SW regardless of the level of starvation, suggesting some sort of regulatory feedback mechanism (Curtis *et al*., In press). There have been no follow-up studies since Sears *et al*. (1991) suggested that appetite is in crustaceans is controlled by sinus gland hormones. The fact that we found low salinity exposure causes a reduction in food intake in Dungeness crabs (Curtis *et al*., In press) provided us with an ideal opportunity to test the hypothesis that inhibitory substances produced in the sinus gland regulate food intake during hyposaline exposure. To test this hypothesis, we examined 1) the likelihood of feeding, 2) the amount of food consumed and 3) the time spent feeding in intact and eyestalk ablated (ESA) *Cancer magister* when exposed to a range of salinities from seawater to freshwater.

Materials and Methods

Adult male intermoult Dungeness crabs, *Cancer magister*, of 300 to 750 g were trapped in Barkley Sound British Columbia, Canada and transported to the Bamfield Marine Sciences Centre. Animals were held in running seawater (SW) of 32 ± 1 ‰ and at a temperature of 12 ± 1ºC for a minimum of one week prior to experimentation. Crabs were fed fish (*Lepidopsetta bilineata*) every other day. Prior to experimentation, animals were removed from the population and starved for 2 d. Salinity and temperature regimes were monitored using an YSI-30 conductivity meter (YSI Inc., Yellow Springs, OH).

In ESA crabs, the sinus gland/X-organ complex was removed 24 h before experiments were started by cutting the eye stalks at their base using a hot scalpel, which subsequently cauterized the wound. This technique resulted in 100% survival after 1 week. Twenty-four hours after the operation, crabs were transferred to a sea table (150 x 70 x 20 cm) that was divided into 10 equal sized chambers, and allowed to settle for 3h. The salinity was then changed to 0, 25, 50, 75 or 100% SW (0, 8, 16, 24 or 32 ‰, respectively), over a period of 30 min by draining a portion of the tank and replacing it with aerated freshwater of the same temperature. Once the test salinity was reached, crabs were exposed for a further 30 min, at which time they were presented with a piece of pre-weighed fish muscle. To minimize changes in mass due to osmotic water exchange with the media, the food was soaked in the test salinity for 6h prior to experimentation. Feeding status was determined at 5 min intervals. If a crab did not feed within 1h, it was scored as a non-feeder. Once an individual began feeding, if it ceased feeding for more than 15min a feeding bout was considered finished. The time spent feeding was recorded and the amount of food consumed was calculated based on the mass of the uneaten food. For intact crabs, a minimum of 20 individuals were tested, if less than 5 individuals fed, the experiment was repeated until a total of 40 individuals had been tested. This method confirmed that intact crabs would not feed below 50% SW and provided a reasonable estimate of variance near this lower threshold. For ESA crabs, 10 individuals were tested at each salinity level. Different individuals were used for each treatment. The amount of food consumed and the time spent feeding were compared for intact and ESA crabs using a two-way ANOVA. Data were $(y + 1)$ transformed due to the large number of zero values for intact individuals. Significant effects were further analysed using Fisher's LSD

post-hoc tests. The effects of salinity and ESA on the number of crabs feeding were determined using a Chi-square test ($P < 0.05$)

Results

There was a significant difference in the percentage of intact (Chi-sq = 24.816, df = 4, $P < 0.001$) and ESA (Chi-sq = 44.514, df = 4, $P < 0.001$) crabs feeding as a function of salinity (Fig. 5.1). Low salinity exposure significantly reduced the likelihood of feeding in intact crabs; 70% of individuals fed in 75% SW, while less than 20% of the crabs fed in 50% SW. Intact crabs would not feed in 25% SW or in freshwater. In contrast all of the ESA crabs fed in salinities as low as 25% SW, and over 40% of individuals attempted to feed in freshwater (Fig. 5.1).

There was a decrease in the amount of food consumed (Fig. 5.2A; ANOVA, df = 4, F = 9.332, $P < 0.001$) and the time spent feeding (Fig. 5.2B; ANOVA, df = 4, F = 5.832, $P < 0.001$) in both intact and ESA crabs as a function of salinity. However the effects of low salinity were less pronounced in ESA crabs. In 100% SW, there was no significant difference in the amount of food consumed, although ESA crabs fed for significantly longer ($P < 0.05$). ESA crabs consumed more food (Fig. 5.2A; $P < 0.05$) and fed for a longer period of time in all salinities below 100% SW (Fig. 5.2B; $P < 0.05$) compared with intact individuals. The amount of food consumed by intact crabs was significantly reduced in 75% SW and further reduced in 50%SW (Fig. 5.2A, $P < 0.05$). In contrast, ESA crabs consumed similar amounts of food in 100, 75, and 50% SW. A significant reduction in food consumption by ESA crabs occurred in 25%SW and

freshwater only ($P < 0.05$). Both intact and ESA crabs fed for a similar amount of time in 75 and 100% SW, however below this level intact crabs showed a greater decline in the amount of time spent feeding ($P < 0.05$).

Discussion

The current study provides evidence that the reduction in food intake observed by *Cancer magister* in low salinity is controlled by inhibitory neurohormones originating in the sinus gland. When intact crabs were exposed to low salinity the percentage of individuals feeding was reduced (Fig. 5.1). The likelihood that an individual will feed in low salinity increases with starvation; however, even with prolonged food deprivation a threshold level of 40%SW is the lowest salinity in which Dungeness crabs will feed (Curtis *et al*., In press). Ablation of the eyestalk and sinus gland removed feeding inhibition due to low salinity exposure (Fig. 5.1), and crabs even attempted to feed in freshwater. There was a discrepancy between the number of ESA individuals attempting to feed in freshwater and the amount of food consumed (Fig.5.1, 5.2A). Although crabs tore off pieces of food with the chelae and attempted to nibble the food with their mandibles for up to 15min, these movements were uncoordinated and when probed with a glass rod, the crabs responded very slowly. It is likely that osmotic water onload restricted their movements preventing them from actually ingesting any food (Davenport, 1972). In 25% SW and 50% SW the crabs did not appear to be inhibited by osmotic water onload. However, they were only exposed to these hyposaline environments for 1h before

they were offered food, which is not a long enough period for a significant change in their internal osmolality at these salinities (McGaw *et al.*, 1999).

The threshold salinity of 40%SW for feeding in intact crabs is just above the lowest salinity (35%SW) that they are reported to survive in indefinitely (Jones, 1941). Data obtained from tagged crabs in the field (Curtis and McGaw, 2008) and salinity preference experiments in the lab (Curtis and McGaw, 2004) confirmed that intact crabs avoid salinities below 35-40%SW. In contrast, when presented with an olfactory stimulus in a hyposaline gradient, ESA crabs will enter into low salinities, and continue to search for food (Curtis, Unpublished obs.). The alteration of actual foraging behaviour also suggests an integration of complex chemical stimuli, rather than a simple inhibitory mechanism of feed or do not feed. While it could also be argued that removal of the eyestalks somehow affected the crab's ability to detect low salinity, when ESA *Cancer magister* were exposed to 50% SW, they immediately displayed an increase in heart rate. The observed increase of approximately 20 beats min^{-1} (Curtis and McGaw, Unpublished obs.) is characteristic of intact animals (McGaw and McMahon, 1996) and such increases in heart rate are sensitive indicators of stress in crustaceans (Florey and Kriebel, 1974)

When interpreting these results, a potentially confounding factor is that removal the sinus gland, could disrupt the release of a number of other hormones, such as Crustacean Hyperglycaemic Hormone (CHH; Fu *et al*., 2005). The primary function of CHH is to regulate circulating levels of glucose in the hemolymph (Fanjul-Moles, 2006). Low salinity exposure results in a release of CHH from the sinus gland and a subsequent increase in hemolymph glucose (Chang *et al*., 1998). Increased blood glucose levels also occur post-feeding, independent of CHH release (Hall and Van Ham, 1998). Therefore it

may be hypothesized that low salinity exposure fools the animal into thinking that it has recently fed and is therefore not hungry. In refute of this, as soon as they had finished eating, ESA crabs would begin feeding again immediately if they were presented with more food. Here, blood glucose was presumably elevated due to feeding (Hall and Van Ham, 1998), suggesting that the increased likelihood of feeding for ESA crabs occurs regardless of satiation, and is not simply a response to decreased blood glucose due to removal of CHH.

While further investigation is necessary to elucidate a definitive mechanism for the inhibition of feeding behaviour by low salinity exposure, the results of this study suggest that it is likely controlled by neuroendocrine products originating in the sinus gland. The rapid inhibitory response and the resulting delayed return in appetite (Curtis *et al*., In Press) may serve as an effective means of preventing *Cancer magister* and other poor osmoregulators from having to cope with the concurrent physiological demands of digestion and osmoregulation.

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Figures

Figure 5.1: The percentage of crabs feeding when exposed to 0, 25, 50, 75, or 100% SW (0, 8, 16, 24 or 32‰, respectively).

Figure 5.2: The amount of food consumed (a) and the amount of time spent feeding (b), for crabs exposed to 0, 25, 50, 75, or 100% SW (0, 8, 16, 24 or 32‰, respectively). Values are mean ± SE

CHAPTER 6

RESPIRATORY AND DIGESTIVE RESPONSES

Abstract

Respiratory responses and gastric processing were examined during hyposaline exposure in two crab species of differing osmoregulatory ability. The efficient osmoregulator, *Callinectes sapidus*, displayed an immediate increase in oxygen uptake when exposed to low salinity in isolation. In contrast, the weak osmoregulator, *Cancer magister*, showed no change in oxygen uptake upon acute exposure (<6 h), but slight increases in oxygen uptake tended to occur over longer time scales (12-24 h). These changes were likely attributable to an increase in avoidance activity after 6 h hyposaline exposure. Following feeding in 100% SW, oxygen uptake doubled for both species and remained elevated for 15 h. When postprandial crabs were exposed to low salinities, *Callinectes sapidus* were able to sum the demands of osmoregulation and digestion. Thus, gastric processes continued unabated in low salinity. Conversely, postprandial *Cancer magister* prioritized responses to low salinity over those of digestion, resulting in a decrease in oxygen uptake when exposed to low salinity. This decrease in oxygen uptake corresponded to a reduction in the rate of contraction of the pyloric stomach and a subsequent doubling of gastric evacuation time. The current study is one of the few to illustrate how summation or prioritization of competing physiological systems is manifested in digestive processes.

This work has been published as:

Curtis, D.L. and McGaw, I.J. (2009) Respiratory and digestive responses of postprandial Dungeness crabs, *Cancer magister*, and blue crabs, *Callinectes sapidus*, during hyposaline exposure. Journal of Comparative Physiology B. DOI 10.1007/s00360-009-0403-z

Introduction

An extensive body of literature exists on the physiological responses of decapod crustaceans to osmoregulatory challenges (Anger, 2003; Charmantier *et al*., 2001; Freire *et al*., 2008; Lucu and Towle, 2003; Pequeux, 1995). Within this framework a number of articles have examined the respiratory and cardiovascular responses to hyposaline exposure. A general pattern is now emerging; efficient osmoregulators tend to display an increase in cardiovascular and metabolic parameters (McGaw and Reiber, 1998, 2000), whereas osmoconformers exhibit a decrease in these variables (Curtis *et al*., 2007). Weaker regulators tend to show mixed responses (Jury *et al*., 1994; McGaw and McMahon, 1996). It is thought that the increase in respiratory and cardiac parameters in efficient osmoregulators compensates for the additional metabolic demands associated with active ion uptake (Findley *et al*., 1978; King, 1965). In poor osmoregulators, decreases in heart rate (and presumably haemolymph flow) may serve to limit diffusive ion loss and increase the offloading of oxygen at the tissues (Cornell, 1974, 1980).

The majority of studies examining the physiological responses to environmental challenges have been carried out on animals that were starved prior to experimentation, in order to avoid the confounding effects of digestion. Apparent specific dynamic action (SDA) refers to the increased metabolic demands of postprandial animals (McCue, 2006)

and encompasses food processing and mechanical digestion, as well as intracellular and extracellular digestion (Beamish, 1974; Jobling, 1983). In crustaceans, SDA is manifested as an increase in oxygen uptake which usually reaches peak values within 4h of feeding and may remain elevated for over 48h (Carefoot, 1990a; Houlihan *et al*., 1990; McGaw and Reiber, 2000; Mente *et al*., 2003; Robertson *et al*., 2002).

Efficient osmoregulators spend extended periods in estuaries, whereas the occurrence of weaker osmoregulators in areas of low salinity is often correlated with times of high food abundance (Curtis and McGaw, 2008; Stevens *et al.*, 1984; Sugarman *et al*., 1983). In either case, it follows that these animals will have to cope with the simultaneous demands of osmoregulation and digestion. It is expected that if animals have a sufficient physiological scope to deal with both demands concurrently, a summation will result. However, an inability to effectively cope with both demands may result in a prioritization towards one of the physiological responses (Bennett and Hicks, 2001).

The inability to concurrently deal with environmental challenges and digestion may restrict digestive processes. Alternatively, slowing or halting gastric processing may spare resources for other processes such as osmoregulation or avoidance behaviours (McGaw, 2006a). Previous reports have shown that evacuation of a meal may be affected by such factors as temperature (Haddon and Wear, 1987), hypoxia (Clemens *et al.*, 1998), and salinity (Roast *et al*. 2000). However, little information exists on the dynamics of digestive parameters or gut activity following exposure to environmental challenges and how they correspond to observed metabolic changes. Recent work suggests that the osmoconformer, *Cancer gracilis*, favours the physiological responses to

digestion over those of low salinity, but is unable to completely prioritize the metabolic responses of one over the other (McGaw, 2006a,b). When postprandial *Cancer gracilis* are exposed to low salinity, they regurgitate food as a possible protective response. However, even 3h after feeding they are likely committed to digestive processes such as protein synthesis, as they still incur some additional metabolic demands (McGaw, 2006b).

It may be expected that osmoconformers are less effective at coping with low salinity and digestion concurrently since they are less likely to be exposed to low salinity than their osmoregulating counterparts (Curtis *et al.*, 2007; Curtis and McGaw, 2008). Therefore in order to gain a full understanding as to how crabs balance the demands of competing physiological systems, the interactive effects of osmoregulatory ability upon digestion were investigated in two species that are classed as osmoregulators. The Dungeness crab, *Cancer magister*, is a weak osmoregulator and inhabits sandy or muddy bays of the northeast Pacific (Pauley *et al.*, 1986). Although it occurs in estuaries, it is unable to tolerate salinities below 12‰ (Cleaver, 1949). In contrast, the blue crab, *Callinectes sapidus*, is a very efficient osmoregulator that occurs in estuaries and coastal waters of the western Atlantic (Hill, 1989). This species is capable of surviving in freshwater and is able to maintain the osmolality of its haemolymph well above that of the medium (Tan and van Engel, 1966).

Callinectes sapidus exhibits an increase both cardiac and respiratory parameters during hyposaline exposure (King, 1965; McGaw and Reiber, 1998). In contrast, oxygen uptake in *Cancer magister* does not change during acute low salinity exposure (Brown and Terwilliger, 1999) and although heart rate increases, a decrease in stroke volume

results in an overall decrease in cardiac output (McGaw and McMahon, 1996). Since these two species show differing physiological responses to low salinity the aims of the present study were to examine the ability of both *Callinectes sapidus* and *Cancer magister* to balance the physiological responses associated with hyposaline exposure and digestion, and to determine the effect that this ability has on the maintenance of digestive processes.

Methods

Adult male, intermolt Dungeness crabs, *Cancer magister* (250-350 g), were trapped in Barkley sound, British Columbia, Canada, between May and August, 2006. They were transferred to the Bamfield Marine Sciences Centre and held in running seawater (SW; 32‰ at 12ºC). Adult female, intermolt blue crabs, *Callinectes sapidus* (150-200 g), were purchased from Gulf Specimens, Florida, USA. Blue crabs were held in a recirculating artificial seawater system (Instant Ocean) at 32‰ salinity and 20ºC at the University of Nevada, Las Vegas. The crabs were fed every other day (fish, *Cancer magister*; shrimp, *Callinectes sapidus*), but were isolated from the general population and starved for 3 d prior to experimentation. This ensured that they were in a post absorptive state, but avoided large scale physiological changes associated with prolonged starvation (Wallace 1973).

Oxygen Consumption

Oxygen uptake was measured using a Qubit D101 intermittent flow respirometry system (Ontario, Canada). Oxygen uptake was calculated for unrestrained crabs at 0.5 h intervals. The chamber was sealed for 10 min and the decline in oxygen tension was recorded. For the remainder of the 0.5 h period, the chamber was continuously flushed. Data was recorded using a Loligo LDAQ data acquisition system (Copenhagen, Denmark). All experiments were carried out in dim light to standardize activity levels and the apparatus was surrounded by black plastic to avoid visual disturbance.

The crabs were held in a cylindrical chamber of 200 mm diameter x 80 mm depth and were allowed to settle overnight prior to the start of the experiment. Following a 2 h control period, the chamber was opened and crabs were provided with a piece of either fish muscle (*Sebastes* sp.; *Cancer magister*) or shrimp muscle (*Panaeus* sp.; *Callinectes sapidus*) totaling 1% of the animals body mass. A 1% ration was used to ensure that all animals consumed the entire meal, since the average meal size for *Cancer magister* is approximately 2% of body mass (Curtis and McGaw, *In Review*). At 1 h post feeding, the salinity was changed to 100, 75, or 50% SW (32, 24, 16‰, respectively; $n = 10$) for *Cancer magister* and 100, 50, or 25% SW (32, 16, 8‰, respectively; $n = 7$) for *Callinectes sapidus*, by draining a portion of the tank and refilling it with aerated freshwater (FW) of ambient temperature (12ºC, *Cancer magister*; 20ºC *Callinectes sapidus*). New stable test salinities were reached after 10 min and did not vary by more than 0.1‰ over the course of the experiment. Oxygen consumption was monitored for an additional 24 h after the salinity changeover. Data for unfed and postprandial animals were compared using two-way repeated measures ANOVA. Different groups were used

for each challenge and the repeated measures were readings from the same individual over time. Total integrated metabolic rate (TIMR) over the 24 h experimental period was used as a convenient method to examine the total metabolic cost associated with digestion under each salinity regime. TIMR was determined by integrating the area under the curve for the rate of oxygen uptake and provided the total amount of oxygen consumed during the treatment period. The mean rate of oxygen uptake during the pre-treatment period for each individual was used as a baseline. The effects of salinity and feeding were compared using two-way ANOVA. Data showing significant effects were further analyzed using Fisher's LSD multiple comparisons test ($P < 0.05$).

Gastric Processing

In a separate series of experiments, the effects of low salinity on gastric processing were determined by following the passage of a radio-opaque meal through the gut system of each species. The radio-opaque meal for *Cancer magister* consisted of 65% pureed fish muscle (*Sebastes* sp.), 25% gelatin and 10% electrolytic iron powder. The radio-opaque meal for *Callinectes sapidus* consisted of: 65% pureed shrimp muscle (*Panaeus* sp.), 20% gelatin, and 15% barium sulphate. Differences in the meals for each species were to optimize palatability (McGaw, 2006a; McGaw and Reiber, 2000; Talbot and Higgins, 1983).

Crabs were held in individual chambers measuring 0.2 m x 0.2 m and allowed to settle for 12 h prior to experimentation. They were then fed approximately 1% of their body mass in 100% SW and allowed to feed for 1 h. The salinity was then changed to 100, 75 or 50% SW for *Cancer magister* ($n = 10$) and 100, 50, or 25% SW for

Callinectes sapidus (n = 7) by addition of freshwater at ambient temperature. The experiments were carried out in constant dim light to minimize variations in activity level and the tank was surrounded by black plastic to avoid visual disturbance.

The passage of the meal through the digestive system was monitored for 72 h using a LIXI PS500 OS X-ray system (Illinois, USA). Only crabs that had consumed the entire meal were included in the analysis. At each time point individual crabs were coaxed into a plastic container without aerial exposure and allowed to settle for 30 s. Images of the gut and a 15 s video of the foregut were recorded. Readings were taken hourly for the first 12 h, every 2 h until 18 h and every 6 h thereafter. Technical specifications for the X-ray were 35 kV tube voltage, 155 µA tube current, and a 5 cm focal window. Emptying time for the fore, mid and hindgut were determined by the voiding of marker from each region of the gut. The times to evacuate each region of the gut were compared using a Kaplan-Meier time to event analysis. This analysis was necessary to generate estimated mean values, since not all animals evacuated their gut during the 72 h experimental period. The rates of contraction for the cardiac and pyloric regions of the foregut were also determined by counting the number of contractions during the 15 s period of video and data was analyzed using a two-way repeated measures ANOVA. Contractions of the cardiac region of both species were sporadic, and it was difficult to discern between movements attributable to contraction of the cardiac stomach and those associated with the gastric mill. In addition, cardiac contractions were not evident in some animals resulting in a large number of zero values. Therefore cardiac contraction rates were not used in the analyses. Different groups were used for each challenge and the repeated measures were readings from the same individual over time.

Data showing significant effect were further analyzed using a Fisher's LSD multiple comparisons test ($P < 0.05$).

Results

Oxygen Consumption

In unfed *Callinectes sapidus*, there was a significant effect of low salinity on oxygen uptake (Fig. 6.1A-C; RM ANOVA, $F = 2.350$, $P < 0.001$). In 100% SW mean values ranged from 34.1 ± 2.3 to 43.9 ± 4.7 mg O_2 kg^{-1} h^{-1} and there was no change in oxygen uptake during the experimental period ($P > 0.05$). When exposed to 25 and 50% SW, the crabs displayed an immediate increase in oxygen uptake, reaching maximum levels of 57.1 ± 5.2 and 66.3 ± 8.9 mg O_2 kg^{-1} h^{-1}, respectively. Accordingly, *Callinectes sapidus* displayed a significant change in TIMR when exposed to 25 and 50% SW (Fig. 6.2A), increasing from pre-treatment levels of -55.4 mg O_2 kg^{-1} to 325.1 ± 151.2 and 415.7 ± 125.0 mg O_2 kg^{-1}, respectively (ANOVA, $F = 4.447$, $P < 0.05$).

When *Callinectes sapidus* were fed they displayed a characteristic increase in oxygen uptake and the magnitude was dependant on the level of salinity exposure (Fig. 6.3A; RM ANOVA, $F = 2.67$, $P < 0.001$). Increases in the rate of oxygen uptake were accompanied by corresponding increases in the TIMR (Fig. 6.2A; ANOVA, $F = 4.447$, $P < 0.05$). In 100% SW, maximal oxygen uptake levels of 94.5 ± 12.9 mg O_2 kg^{-1} h^{-1} were reached 5 h after feeding and were maintained until 7.5 h after feeding. Oxygen uptake decreased slightly thereafter, returning to pretreatment levels at 15 h post-feeding. The mean TIMR over the 24 h experimental period for fed animals was 596.7 ± 143.7 mg O_2

kg^{-1}. When exposed to 50% SW, postprandial *Callinectes sapidus* displayed an additional increase in oxygen uptake reaching maximal levels of 116.4 ± 4.8 mg O_2 kg^{-1} h^{-1}. Oxygen uptake gradually declined thereafter, but remained elevated relative to pretreatment levels for the duration of the experiment ($P < 0.05$). However, this additional increase the rate of oxygen uptake did not result in an increase in TIMR ($P > 0.05$). In fact, the increase in TIMR attributable to digestion for animals in 50% SW was approximately 30% less than that of animals in 100% SW. During exposure to 25% SW maximal levels of oxygen uptake of 131.7 ± 19.2 mg O_2 kg^{-1} h^{-1} were reached 2 h after feeding; these were more than 3-fold greater than pretreatment values ($P < 0.05$). Oxygen uptake for postprandial crabs in 25% SW remained significantly elevated relative to crabs in 100 and 50% SW ($P < 0.05$). The additional demands of concurrent digestion and osmoregulation in 25% SW resulted in a difference in TIMR between unfed and fed animals that was approximately 50% greater than for *Callinectes sapidus* in 100% SW ($P < 0.05$).

In unfed *Cancer magister*, changes in oxygen uptake during low salinity exposure were more variable than those observed for *Callinectes sapidus*. Crabs displayed significant changes in oxygen uptake over time and these changes were different for the two low salinity treatments (Fig. 6.1D-F; RM ANOVA, $F = 2.138$, $P < 0.001$). Changes in the rate of oxygen uptake over time resulted in significant changes in TIMR over 24 h (Fig. 6.2B; ANOVA, $F = 3.233$, $P < 0.05$). In 100% SW, there was no change in oxygen uptake during the experimental period; mean levels ranged between 24.1± 3.3 and 40.8 ± 5.8 mg O_2 kg^{-1} h^{-1}. However, slight declines in the rate of oxygen uptake over 24h resulted in a net decrease in TIMR of 172.7 ± 80.3 mg O_2 kg^{-1}. In 75% SW there was a

slight, but significant decline in oxygen uptake ($P < 0.05$) after 1.5 h, reaching between 22 and 26 mg O_2 kg^{-1} h^{-1}. After 6 h, oxygen uptake increased to pretreatment levels and at 18 h post exposure there was a further significant increase, with oxygen uptake reaching 33.4 ± 3.8 mg O_2 kg^{-1} h^{-1} ($P < 0.05$). When exposed to 50% SW, *Cancer magister* displayed a temporary, but significant increase in oxygen uptake between 6 and 9.5 h post exposure, reaching peak levels of 40.9 ± 7.4 mg O_2 kg^{-1} h^{-1}. A second temporary increase was observed between 18.5 and 22.5 h reaching maximal levels 42.0 ± 6.2 mg O_2 kg^{-1} h^{-1}. This resulted in a significant increase in TIMR of 101.6 ± 71.4 mg O_2 kg^{-1}, ($P < 0.05$).

When postprandial *Cancer magister* were exposed to low salinity they displayed the opposite response to *Callinectes sapidus*, resulting in a reduction rather than in increase in oxygen uptake (Fig. 6.3B; RM ANOVA, $F = 2.22$, $P < 0.001$). In 100% SW, *Cancer magister* displayed a typical SDA response, maximal oxygen uptake rates of 64.6 ± 6.2 mg O_2 kg^{-1} h^{-1} were reached 4 h after feeding ($P < 0.05$) and remained elevated for 16.5 h ($P < 0.05$) resulting in a TIMR of 297.8 ± 69.8 mg O_2 kg^{-1} ($P < 0.05$). After 1 h of exposure to 75% SW, the oxygen uptake of postprandial *Cancer magister* declined slightly ($P < 0.05$) to between 35 and 49 mg O_2 kg^{-1} h^{-1} but remained higher than pretreatment values ($P < 0.05$). In contrast after 2 h exposure to 50% SW oxygen uptake showed a significant decline, reaching levels similar to those measured during pretreatment. While there was no significant difference in TIMR amongst salinities for fed animals, the total integrated metabolic rate attributable to feeding was approximately 90% less for animals in 50% SW than those in 100% SW ($P < 0.05$).

Gastric Processing

In *Callinectes sapidus*, a maximum pyloric contraction rate of 118 ± 9 contractions min^{-1} was observed immediately following ingestion (Fig. 6.4A). Thereafter, rates declined over the following 12 h in all salinities tested (RM ANOVA, $F = 29.31$, $P < 0.001$), and no significant differences were observed as a function of salinity. Likewise, low salinity exposure had no significant effect on gastric evacuation times of *Callinectes sapidus* (Fig. 6.5A; Kaplan-Meier; $P > 0.05$).

Rates of pyloric contraction for *Cancer magister* were also highest immediately after feeding (Fig. 6.4B). However, the response was dependant on the level of salinity exposure (RM ANOVA, $F = 2.23$, $P < 0.001$). In 100% SW, a decline in pyloric contractions occurred during the first 3 h but remained stable thereafter ($P > 0.05$). Pyloric contraction rates also declined during the first 3 h of exposure to 75% SW. There was a temporary increase between 3 and 9 h ($P < 0.05$). At 10 h post feeding, rates declined to levels similar to those recorded for crabs in 100% SW ($P > 0.05$). When *Cancer magister* was exposed to 50% SW, a sharp and immediate decrease in contraction rates occurred during the first hour; a further significant decline occurred at 7 h post feeding, reaching minimum levels of 18.7 ± 7.0 contractions min^{-1}.

For *Cancer magister* in 100% SW the foregut, midgut, and hindgut regions were emptied in 20.9 ± 2.7 h, 27.7 ± 3.9 h, and 33.4 ± 4.1 h, respectively (Fig. 6.5B). The crabs evacuated each region of the gut significantly faster in 75 % SW than in 100% or 50% SW (Kaplan-Meier; $P < 0.05$). The estimated evacuation times of 49.3 ± 6.0 h, 54.0 ± 4.4 h, and 56.0 ± 4.7 h for the fore, mid and hindgut regions respectively in 50% SW

were significantly longer than those observed in 100 or 75% SW (Kaplan-Meier; $P < 0.05$).

Discussion

The increase in metabolic rate due to feeding and digestion, later referred to as the specific dynamic effect of food, has been studied in detail for over a century (Lusk, 1905). In recent years there has been a renewed interest in the effects of feeding on physiological parameters (Bennett and Hicks, 2001; Hicks and Bennett, 2004; McCue, 2006; Secor *et al*., 2000; Wang *et al*., 2001), in particular the effect that digestion has on other systems and how animals balance the concurrent demands of these physiological systems. A number of articles address the ability to sum the effects of digestion, or prioritize digestive or other physiological processes (Andersen and Wang, 2003; Bennett and Hicks, 2001; Hicks and Bennet,t 2004; Jordan and Steffensen, 2007). However, very few of these articles detail how the effects of either summation or prioritization manifest themselves on digestive processes.

For crustaceans living in estuaries, salinity is probably the single most important factor governing their distribution (Barnes, 1967; Spaargaren, 1973). The typical physiological response of efficient osmoregulators to hyposaline exposure is an increase in respiratory and cardiovascular parameters. Blue crabs, which maintain the osmolality of their hemolymph well above that of the medium (Tan and van Engel, 1966) are no exceptions and show this typical response (King, 1965; Taylor *et al*., 1977). In accordance with these previous reports, *Callinectes sapidus* displayed an increase in

oxygen uptake immediately following low salinity exposure (Fig. 6.1A-C). This increase may be due to additional metabolic demands associated with active ion uptake (Findley *et al*., 1978; Piller *et al*., 1995) and/or adjustments associated with increased locomotor activity (McGaw *et al*., 1999).

In contrast, *Cancer magister* which is classed as a weaker osmoregulator (Jones, 1941) displays no change or a slight decline in the rate of oxygen uptake during acute hyposaline exposure (Fig. 6.1D-F; Brown and Terwilliger, 1999), which is coupled with an overall decrease in cardiac output (McGaw and McMahon, 1996). This may be due to behavioural modifications, whereby *Cancer magister* exhibit quiescence during acute low salinity exposure, isolating the branchial chamber and thus minimizing diffusive ion loss (Curtis *et al*., 2007; McGaw *et al*., 1999). However, the reduced ventilation rate observed during the closure response cannot be maintained indefinitely. It is the cessation of this behaviour that is likely responsible for the observed increase in oxygen uptake that occurred during longer periods of hyposaline exposure, resulting in an increase in TIMR (Fig. 6.2B).

The results of the present study add to the extensive literature on the osmoregulatory and ionoregulatory physiology of decapod crustaceans (Lucu and Towle, 2003; Mantle and Farmer, 1983; Pequeux, 1995). However, the practice of ensuring that animals are all at a similar standard metabolic rate has meant that most previous studies have focused on starved animals. In order to thrive, grow and reproduce in a given environment, all animals must feed. Feeding and subsequent digestion are associated with their own set of physiological demands (Beamish, 1974) and these may impair an

organism's ability to cope with environmental stressors (Legeay and Massabuau, 1999; Robertson *et al*., 2002; Wang *et al*., 2001).

In both *Callinectes sapidus* and *Cancer magister*, oxygen consumption doubled following ingestion of a meal and remained elevated for approximately 15 h. While the scope of the SDA response was similar to that reported for other species of crustaceans (McCue, 2006), the duration of this response was shorter. This discrepancy is likely attributable to the smaller, standardized meal size used in the current study (Beamish, 1974; Houlihan *et al*., 1990; McCue, 2006; Pan *et al*., 2005). Feeding and digestion also result in a diversion of hemolymph flow to the digestive structures of these species (McGaw, 2006c; McGaw and Reiber, 2000), presumably to meet the increased oxygen demand associated with ingestion of the meal and its subsequent digestion and assimilation. These changes in haemolymph flow allow crabs to maintain oxygen levels in the face of increased metabolic demand, facilitating oxygen dependant processes (Mente *et al*., 2003).

Whereas the respiratory responses to feeding were congruent with those of osmotic stress in the efficient osmoregulator *Callinectes sapidus*, they appeared to oppose the physiological responses to osmotic stress in the weak osmoregulator, *Cancer magister* (Fig. 6.1 and 6.3). When exposed to low salinity following feeding, *Callinectes sapidus* was able to balance the demands of both digestion and osmoregulation, summing the corresponding increases in oxygen uptake in a dose dependant manner. This summation of responses was evident as an increase in both the scope and duration of the SDA response. Concordant with the summation of respiratory responses, *Callinectes sapidus* was able to maintain digestive processes following low salinity exposure. Thus, they

displayed no change in either pyloric contraction rate or gut evacuation time. However, maintenance of these processes is not without cost, and *Callinectes sapidus* required approximately 50% more oxygen to digest the same sized meal in 25% SW than in 100% SW. In contrast, when *Cancer magister* was exposed to low salinity following feeding, the respiratory responses to low salinity were prioritized over those of digestion (Fig. 6.2 and 6.3). This data is supported by cardiovascular responses, which show a diversion of haemolymph flow away from digestive structures during exposure to 50%SW (McGaw, 2006c). When these postprandial crabs are returned to full SW, there is no overshoot in cardiovascular or ventilatory parameters indicative of an oxygen debt, as would be expected if digestive processes continued unabated (McGaw, 2006c). Accordingly, there was a direct correlation between oxygen consumption and gastric processing when *Cancer magister* was exposed to 50% SW. Within an hour of low salinity exposure, both oxygen uptake and pyloric contraction rate were depressed relative to crabs in 100% SW (Fig. 6.3B and 6.4B) and the TIMR attributable to digestion decreased by 90% relative to crabs in 100% SW. Changes in the rate of pyloric contraction also closely corresponded to changes in gut evacuation time, with crabs displaying reduced pyloric contraction rates and increased evacuation times in 50% SW. In addition, the observed increase in pyloric contraction after 9 h in 75% SW corresponded to a number of animals evacuating the foregut at this time. These results lend further support to previous findings suggesting that pyloric gut contraction regulates the passage of a meal into the midgut and hindgut regions (Heinzel, 1988).

While the close correspondence between changes in oxygen uptake and gastric processing are convincing, the actual energetic costs associated with mechanical

digestion are low (Carefoot, 1990b). Therefore at first glance, a reduction in mechanical digestion may contribute little in reducing energy demands. However, a reduction in mechanical processing of a meal may have downstream effects on extra and intracellular digestion. Intracellular digestion accounts for 24 to 52% of postprandial oxygen consumption (Mente *et al*., 2003). Therefore either passive or active regulation of protein synthesis may prove an effective strategy for sparing resources for other demands and is undertaken by many organisms in times of environmental stress (Hand and Hardewig, 1996). The correspondence between decreased gastric processing rates and reduced TIMR in 50% SW for *Cancer magister* suggest that intracellular digestion is being passively regulated and crabs are favouring the responses to low salinity exposure. Though it is important to note that for *Cancer magister*, despite a reduction in TIMR of approximately 90% in 50% SW, there was no difference in TIMR post feeding amongst the salinity treatments. This discrepancy is likely attributable to 3 individuals that displayed SDA responses in 50% SW that were similar to those in 100% SW. The response that is favoured by *Cancer magister* in 75% SW is less clear: crabs displayed an increased pyloric contraction rate and a corresponding decrease in gut passage time, yet there was also a slight decrease in TIMR attributable to digestion. This juxtaposition suggests that intracellular digestion may be both passively and actively regulated. Work is currently underway to elucidate the effects that low salinity and the subsequent reduction in gastric processing in *Cancer magister* have on downstream digestive processes (enzyme activity and protein synthesis) and digestive efficiency.

An alternative explanation for the observed decrease in oxygen uptake for *Cancer magister* is that this species simply lacks the resources to divert to digestion during low

salinity exposure. Preliminary results suggest that this is not the case: when the eye stalks are ablated (subsequently removing hormonal control mechanisms of the stomatogastric ganglion), the reduced rate of pyloric contraction in low salinity returns to that observed in 100% SW (Curtis and McGaw, Unpublished observation). This suggests that crabs have sufficient resources to carry out digestion in low salinity and that the gastric modulation in low salinity is under control of inhibitory neurohormones.

Since efficient osmoregulators spend extended periods in low salinity, it follows that they should be able to maintain digestive processes in these conditions. By summing the physiological responses to digestion and osmoregulation it appears that *Callinectes sapidus* is well adapted to feeding and digestion in chronic low salinity. In contrast, *Cancer magister* does not appear to be as well adapted for feeding and subsequent digestion in low salinity. This may be echoed in its behavioural responses: our recent work shows that *Cancer magister* only makes short forays into low salinity that are usually associated with times of high food abundance (Curtis and McGaw, 2008). Laboratory experiments show that once they have fed, the animals may retreat to areas of higher salinity in order to digest food more efficiently (Curtis and McGaw, *Unpublished data*). Thus short forays combined with the retreat to higher salinities to digest food may be a means by which weak osmoregulators can effectively exploit estuarine habitats.

The results of this study have shown a direct relationship between summation or prioritization of metabolic responses and the continuation or reduction of gastric processes. In the osmoconformer, *Cancer gracilis*, the cardiovascular and respiratory responses to low salinity exposure and digestion strongly oppose one another, and crabs are unable to balance the demands of these competing processes (McGaw, 2006b). Even

after regurgitating a meal, *Cancer gracilis* must still in endure additional metabolic demands associated with digestion (McGaw, 2006b). In the weak osmoregulator *Cancer magister*, the cardiovascular and respiratory responses to low salinity also oppose those of digestion but are less pronounced (Fig. 6.2; McGaw, 2006c), possibly facilitating the prioritization towards low salinity exposure. This prioritization is manifested as a mitigation of the physiological demands of SDA, in part via a reduction in foregut activity. In contrast, the efficient osmoregulator *Callinectes sapidus*, displays an increase in cardiac and respiratory responses to both low salinity exposure and digestion (McGaw and Reiber, 1998) and the resulting summation of these responses enables digestion in low salinity to continue unabated. This emerging pattern suggests that rather than osmoregulatory ability alone, it may be the ability to cope with the concurrent demands of osmoregulation and digestion that allows crabs to exploit or persist in areas of low salinity. Previous work focusing on the physiological responses to low salinity in isolation may therefore not entirely reflect of the challenges occurring in the natural environment.

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Figures

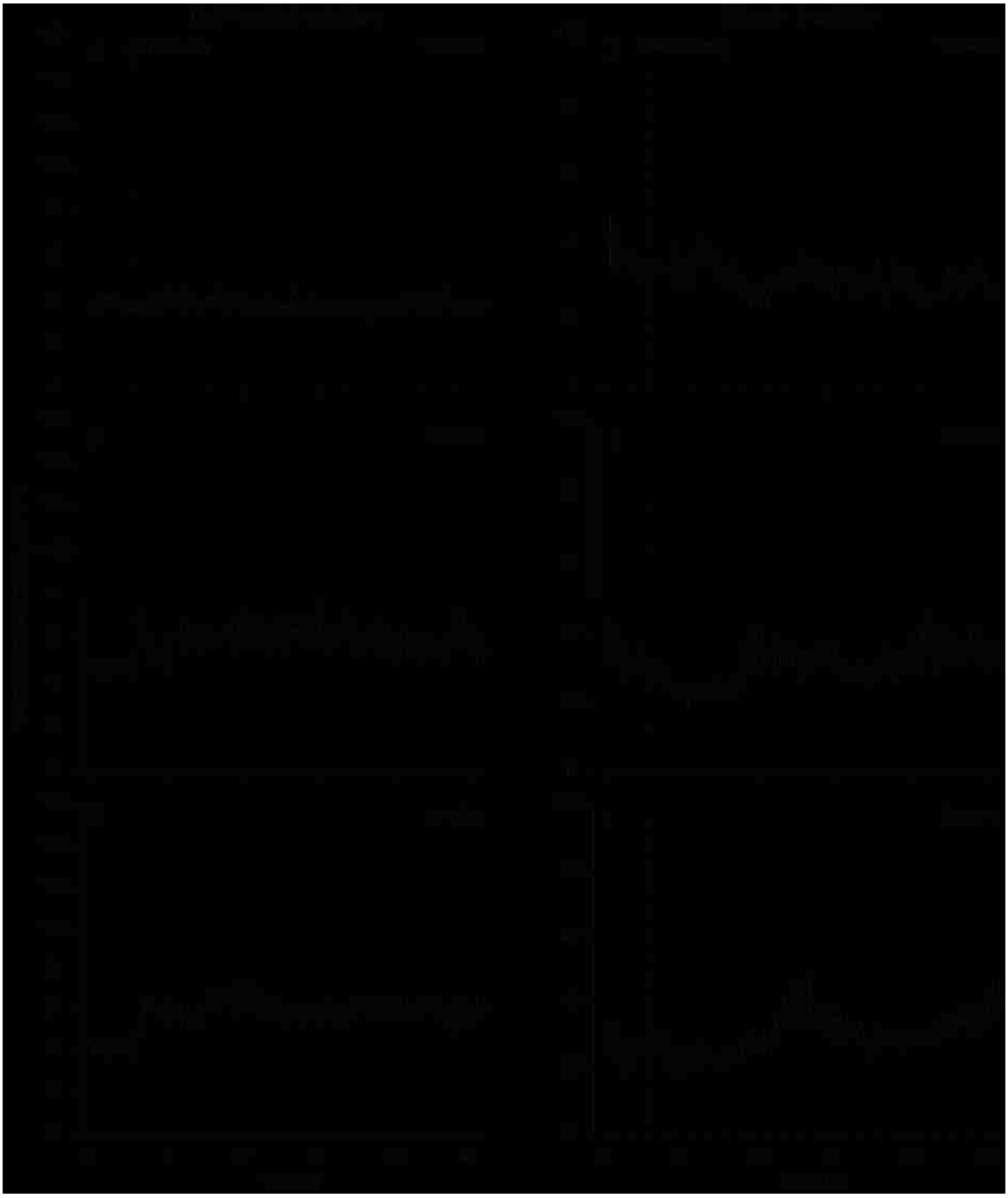

Figure 6.1: Oxygen uptake (mg O_2 kg^{-1} h^{-1}) of 7 *Callinectes sapidus* exposed to a) 100, b) 50 or c) 25% SW and 10 *Cancer magister* exposed to d) 100, e) 75, or f) 50% SW. Values are mean ± SE.

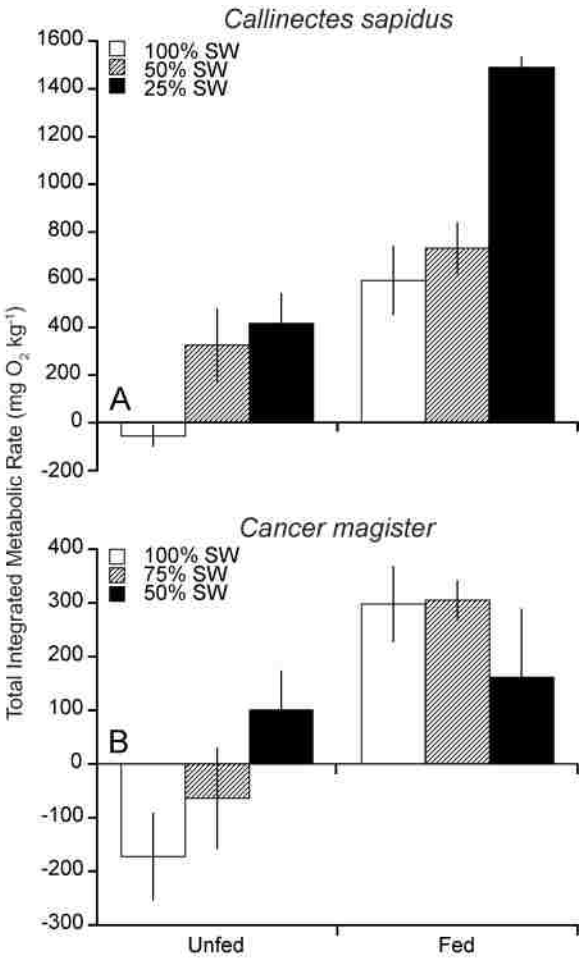

Figure 6.2: Total integrated metabolic rate (mg O_2 kg^{-1}) over 24 h for a) unfed and fed *Callinectes sapidus* exposed to 100, 50, or 25% SW (n = 7) and for b) unfed and fed *Cancer magister* exposed to 100, 75 or 50% SW (n = 10). Values are mean ± SE.

Figure 6.3: Oxygen uptake (mg O_2 kg^{-1} h^{-1}) of a) 7 postprandial *Callinectes sapidus* exposed to 100, 50, or 25% SW, 1h after feeding and b) 10 postprandial *Cancer magister* exposed to 100, 75, or 50% SW, 1h after feeding. Values are mean ± SE.

Figure 6.4: Contraction rate of the pyloric stomach (mean ± SE) for 7 *Callinectes sapidus* exposed to 100, 50, or 25% SW and for 10 *Cancer magister* exposed to 100, 75, or 50% SW.

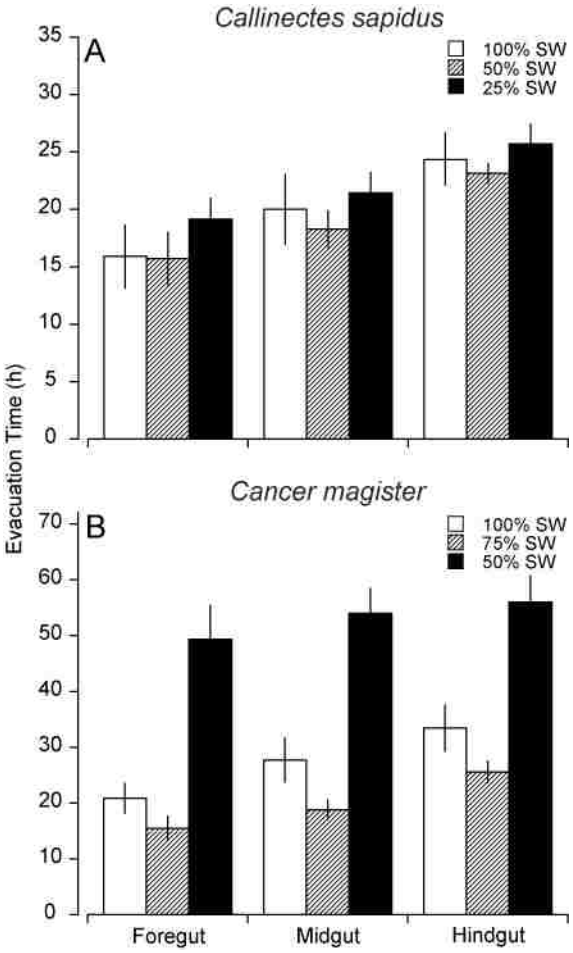

Figure 6.5: Gastric evacuation times (estimated mean ± SE) for the foregut, midgut, and hindgut of a) *Callinectes sapidus* exposed to 100, 50, or 25% SW (n = 7) and b) *Cancer magister* exposed to 100, 75, or 50% SW (n = 10).

CHAPTER 7

EXTRACELLULAR DIGESTION

Abstract

Extracellular digestive processes were examined in the Dungeness crab, *Cancer magister* and the blue crab, *Callinectes sapidus*, during hyposaline exposure. Both species are found in estuaries as adults, but vary in their ability to balance the cardiovascular and respiratory demands of concurrent osmoregulation and digestion. The weak osmoregulator, *Cancer magister*, is unable to balance the demands of osmoregulation and digestion. Concordant with observed decreases in respiration and mechanical digestion, proteolytic digestion within the foregut and hepatopancreas was delayed, resulting in a relative reduction of circulating amino acids post-feeding in low salinity. In contrast, the efficient osmoregulator, *Callinectes sapidus*, sums the demands of osmoregulation and digestion, and mechanical digestion continues unabated in low salinity. Protease activity in the gut fluid and hepatopancreas showed no change or a reduction over time. The transport of amino acids into the cells post-feeding is opposed by an efflux of amino acids at the cellular level, and resulted in a build up of amino acids in the hemolymph. Despite differences in the extracellular responses to low salinity exposure following feeding, both species were able to maintain high digestive efficiencies. When considered in light of the respiratory responses to low salinity exposure, the results of this study suggest a role for passive regulation of energetically costly intracellular protein synthesis by the alteration of amino acid concentration in the hemolymph.

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Introduction

Decapod crustaceans inhabiting estuaries are subjected to environmental conditions that present physiological challenges. In order to grow and reproduce in these habitats, animals must feed. Feeding and digestion presents its own set of physiological demands, which often oppose the responses to environmental challenges. To date, only a handful studies have examined the effects of environmental challenges on the dynamics of digestive processes following the ingestion of a meal (McGaw, 2006b; McGaw, 2007a,b; Curtis and McGaw, 2009).

Once a meal has been procured and ingested, digestion in decapod crustaceans begins in the foregut (see Ceccaldi, 1989; Barker and Gibson, 1977; Muhlia-Almazan and Garcia-Carreno, 2003 for reviews of crustacean digestive processes and anatomy). The foregut is divided into two chambers by the gastric mill, the anterior cardiac region and the posterior pyloric region. The gastric mill is made up of calcified ossicles that grind up the food. During this process, the food is mixed with digestive enzymes and hydrolysis of macromolecules begins. Non-digestable material is filtered out in the ventral portion of the stomach and is passed out of the body via the midgut and hindgut. Digestable materials consisting of fluid and fine particles pass into the hepatopancreas, (digestive gland) via the midgut caeca. The hepatopancreas has specialized cells that serve many functions, including the storage and absorption of nutrients, and the production, storage and secretion of digestive enzymes. Crustaceans produce a wide array of digestive enzymes including amylases, lipases, chitinases, cellulases, and

proteases (Brown, 1995; Glass *et al*., 1989). While the relative activity of each class of enzyme varies by species, proteases are dominant within carnivorous species (Dendinger, 1987; Johnston and Freeman, 2005). Factors reported to affect the secretion and activity of digestive enzymes include ontogeny (Lovett and Felder, 1990), moulting (Ceccaldi, 1989), diet (Muhlia-Almazan *et al*., 2003), feeding (Ong and Johnston, 2006), and salinity (Gaxiola *et al*., 2005; Rosas *et al*., 2002). Following the breakdown of peptides into their precursors, free amino acids (FAAs) are transported into the hemolymph where they are circulated throughout the body. FAAs from the hemolymph subsequently become part of the intracellular FAA pool, contributing to the 40% increase in cellular protein synthesis that occurs post feeding (Mente *et al*., 2001). Increased protein synthesis occurs within 2h of feeding, and accounts for up to 60% of the doubling of oxygen uptake that occurs post-feeding (Curtis and McGaw, 2009; McGaw and Reiber, 2000; Mente *et al*., 2001). At the cellular level the influx of amino acids that occurs post-feeding appears to be in opposition to the cell volume regulatory responses that occur during hyposaline exposure. Amino acids are the major contributor to intracellular volume regulation in crustaceans (Pequeux, 1995), and most species maintain relatively high FAA pools within the cells (Gilles and Pequeux, 1981). When presented with a hypo-osmotic challenge, most crustaceans display a transient efflux of inorganic ions from the cells, which is followed by a compensatory decrease in the influx and increase in the efflux of amino acids (Moran and Pierce, 1984; Moran and Pierce, 1985). Previous studies have shown that the major FAAs involved in cell volume regulation in crustaceans include glycine, alanine, lysine, leucine, serine, and proline, though the contribution of each amino acid (both essential and non-essential) appears to vary by

species and tissue type (Daniello, 1980; Marangos *et al*., 1989). While cells are able to tolerate short term disruptions, maintenance of cell volume and ion composition are critical in maintaining homeostasis. The use of amino acids as compatible osmolytes allows for the maintenance of cellular processes in euryhaline species that vary in their ability to regulate the osmolality of the extracellular fluid (Yancey, 2005).

Crustaceans vary widely in their ability to regulate the osmolality of their extracellular fluid, a process which is carried out primarily at the gill epithelium by the regulation of Na+ (Lucu and Towle, 2003). Recent work has shown that the ability to regulate the concentration of the extracellular fluid may dictate an animal's ability to balance the concurrent physiological demands of osmoregulation and digestion (McGaw, 2006a; McGaw, 2006c). The Dungeness crab, *Cancer magister*, is classed as a weak osmoregulator (Jones, 1941) and inhabits sandy bays and estuaries of the NE Pacific (Cleaver, 1949). As adults, this species enters into areas of low salinity during times of high food abundance (Curtis and McGaw, 2008; Stevens and Armstrong, 1984; Sugarman *et al*., 1983). However, when challenged with low salinity exposure following feeding, this species is unable to balance the concurrent physiological demands, favouring the ventilatory, cardiac, and respiratory responses associated with osmoregulation (Curtis and McGaw, 2009; McGaw, 2006c). This prioritization results in a reduction in gastric processing and is accompanied by reduced food intake (Curtis and McGaw, In press). As a result, *Cancer magister* is able to mitigate the increased oxygen uptake associated with digestion when exposed to low salinity (Curtis and McGaw, 2009). In contrast to *Cancer magister*, the blue crab, *Callinectes sapidus*, is classed as an efficient osmoregulator and inhabits large hyposaline estuaries of the Western Atlantic

and Gulf of Mexico (Hill *et al*., 1989; Tan and van Engel, 1966). When challenged with low salinity exposure following feeding, *Callinectes sapidus* is able balance the respiratory responses to digestion and low salinity exposure, and digestion continues unabated (Curtis and McGaw, 2009).

While recent work has examined the whole organism responses to the physiological demands associated with low salinity exposure following feeding, little is known about how these responses are manifested at the extracellular or intracellular level. It was hypothesized that since *Cancer magister* is unable to balance the demands of osmoregulation and digestion, this species will show additional reductions in digestive processes at the extracellular level and that these reductions may have a passive effect on intracellular processes. It was further hypothesized that since *Callinectes sapidus* is able to balance the demands of osmoregulation and digestion, with digestion continuing unabated, this species will show no change in extracellular or intracellular digestive processes. To investigate these hypotheses, the effects of low salinity exposure on (1) digestive enzyme activity in the gut fluid and hepatopancreas, (2) Circulating FAAs, and (3) digestive efficiency, were examined.

Methods

General

Adult male *Cancer magister* (350 to 500 g) were trapped in Barkley Sound, British Columbia and transferred to the Bamfield Marine Sciences Centre, where they were held in running seawater (32‰; 12ºC). Adult female *Callinectes sapidus* (150 to 250 g) were

purchased from local seafood market and held in a recirculating artificial seawater system (32‰, 20ºC; Instant Ocean) at the University of Nevada, Las Vegas. All animals were held for 1 week prior to experimentation and were fed either fish (*Cancer magister*) or shrimp (*Callinectes sapidus*) every other day.

Similar methodology was used for each set of experiments. Prior to experimentation, crabs were separated from the general population and starved for 3d. Crabs were then placed in a tank measuring 150 x 70 x 20 cm that was divided into 10 equal sized chambers. The experimental tank was surrounded by black plastic to minimize disturbance. In treatments examining the effects of feeding, crabs were presented with a piece of either fish (*Cancer magister*) or shrimp (*Callinectes sapidus*) muscle and allowed to feed for 30 min. In treatments examining the effects of low salinity, immediately following feeding, the salinity was changed to either 50% SW (16‰; *Cancer magister*), or 25% SW (8‰; *Callinectes sapidus*) by the addition of aerated freshwater of the same temperature. For all experiments, temperature was maintained within 1ºC of the holding conditions and once changed salinity did not vary by more than 0.1‰. The level of low salinity for used for each species was above their lethal physiological limit, but within a range at which they show changes in cardiac parameters indicative of physiological stress (Florey and Kriebel, 1974; McGaw and McMahon, 1996; McGaw and Reiber, 1998)

Protease Activity

Changes in proteolytic activity following feeding were estimated for both the gut fluid and hepatopancreas lysates by monitoring the release of the fluorogenic compound

7-amido-4-methylcoumarin (AMC). Succinyl-leu-tyr-AMC (suc-LY-AMC, Boston Biochemical), was used as a substrate for general peptidase activity (Velickovska et al., 2005). At 1 and 6h post feeding (n = 5) in low salinity or full seawater, a sample of gut fluid was removed through the esophagus using a syringe. Crabs were then sacrificed and hepatopancreas tissue was removed. Samples were immediately frozen and stored at -80ºC until use. Hepatopancreas samples were pulverized in liquid nitrogen and homogenized in 4 volumes of 50mM PIPES, pH 6.3; 1mM 2-mercaptoethanol; and 0.1mM EDTA and centrifuged at 10 000 x g and 4ºC for 10min to remove cellular debris. Typical assay conditions in a 100µl reaction volume were 15 µl of either gut fluid (diluted 1:500) or hepatopancreas lysate (diluted 1:100), 100mM PIPES (pH 6.3), 0.0475% SDS and 40 µM substrate. Sample dilutions were adjusted to maintain linearity. Reactions were incubated for 15 min at 25ºC and were ended by the addition of 11 µl of concentrated HCl. The mixture was then centrifuged at 10 000 x g and 4ºC for 10min. Of the resulting supernatant, 80ul was added to 1ml of 0.5M Tris (pH 8.0) and the fluorescence was measured at 365/440 nm. For the hepatopancreas, protein concentrations were determined using a modified Lowry assay (Peterson, 1977).

Amino Acid Concentration

Reverse-phased high performance liquid chromatography (HPLC) was used to quantify amino acid concentrations in the hemolymph for fed and starved crabs (n = 5) following 6h exposure to low salinity. Hemolymph samples were taken from the arthrodial membrane of the walking leg using a chilled needle and syringe. Samples were immediately frozen for later analysis. Prior to analysis, samples were thawed on ice

and proteins were precipitated by the addition of 7% perchloric acid (PCA; v/v) and centrifuged at 10 000 x g at 4ºC for 10 min. The resulting supernatant was then neutralized by the addition of 10% 5M K_2CO_3 (v/v), and centrifuged again. Of the resulting supernatant, 40 µl was added to 80 µl of o-phtalaldehyde reagent (Flouraldehyde reagent, Pierce, Rockford, Illinois) to derivitize free amino acids prior to HPLC analysis.

Amino acid quantification was achieved by HPLC (Shimadzu, Columbia, MD), using methods described by Podrabsky and Hand (2000). Free amino acids were separated using an APEX I ODS column (250 x 4.6 mm, 5µm pore size; Grace, Baltimore, MD) and a mobile phase gradient. Solvent A consisted of 0.1M sodium acetate (pH 7.7), methanol, and tetrahydrofuran (90: 9.5: 0.5; v/v). Solvent B was methanol. The solvent gradient used was as follows (percentages indicate concentration of solvent A, with the balance being solvent B): 0-5min 95%-90%; 5-15min 90%; 15-24 min 90% -60%; 24-35 min 60%-40%; 35 – 39 min 40%, 39-42 min 40-0%; 42-48 min 0%; at a flow rate of 0.7 ml min^{-1}. The column was flushed with water for 5 min between samples and starting conditions were maintained for 10 min prior to the start of a new run. Amino acid peaks were detected at 330 nm, and quantified by integrating the area of each peak using EZ-Start software (Shimadzu, Columbia, MD). This method allowed for the quantification of 12 distinct amino acids, and 18 amino acids in total. Peaks representing gln/his, gly/thr, and ala/tyr could not be separated. As such, data are presented as mOsm, rather than molarity. Total amino acid concentration was determined as the sum of the concentrations of the 18 amino acids quantified.

Digestive Efficiency

Changes in digestive efficiency with hyposaline exposure were determined for 5d starved crabs that were fed a meal of fish muscle (*Cancer magister*) or shrimp (*Callinectes sapidus*). Crabs were fed a meal of known mass and allowed to digest in either full seawater or low salinity. Feces were collected daily and washed with distilled water before drying to constant mass at 60ºC. Caloric content for both the meal and pooled feces samples were determined using bomb calorimetry (Washington State University Wildlife and Habitat lab). Digestive efficiency was calculated using the formula:

$$Digestive\,efficiency\,(\%) = \frac{(dry\,mass\,meal * caloric\,content) - (dry\,mass\,feces * caloric\,content)}{(dry\,mass\,meal * caloric\,content) * 100}$$

Statistical Analysis

The effects of low salinity and feeding on protease activity, amino acid concentration, and digestive efficiency were compared using two-way ANOVA. Fisher's LSD test was used for post-hoc comparisons. Values which were more than 2 standard deviations from the mean were considered outliers and not included in the analysis (Field, 2005).

Results

Protease Activity

In the hepatopancreas of both *Cancer magister* (ANOVA, df = 1, $P < 0.001$) and *Callinectes sapidus* (ANOVA, df = 1; F = 10.431; P < 0.01), salinity dependant changes in total protease activity were observed between 1 h and 6 h post-feeding (Fig. 7.1). In 100% SW, *Cancer magister* displayed no change in total protease activity over time in the digestive gland. In contrast, when exposed to 50% SW, after 6 h total protease activity was nearly double that observed at 1h (Fig. 7.1A; $P < 0.05$). *Callinectes sapidus* showed the opposite response. In 100% SW, there was a significant decline in protease activity within the digestive gland over time (Fig. 7.1B; $P < 0.05$). However in 25% SW, there was no change over time but total protease activity was less than half that observed in 100% SW.

The hepatopancreas of both *Cancer magister* and *Callinectes sapidus* showed salinity dependant changes in protease activity over time, that fell just short of the $P < 0.05$ significance level (Fig. 7.2A; ANOVA, df = 1, F = 4.330, $P = 0.056$). Because important patterns could have been obscured in the statistical analysis, each salinity was analysed separately using a T-test. In 100% SW, there was no significant change over time. However, there was a significant increase in total protease activity between 1 h and 6 h post-feeding in 50% SW (T-test, t = -3.231, P = 0.018). *Callinectes sapidus* displayed a significant reduction in total protease activity with salinity in the gut fluid (ANOVA, df = 1; F = 5.452; $P < 0.05$), but there was no difference between 1 h and 6 h post-feeding for either salinity.

Amino Acid Concentration

In 100% SW, *Cancer magister* displayed a post-feeding increase in the concentration of all of the 18 amino acids measured (Table 7.1). However, when exposed to low salinity alone or low salinity post-feeding mixed responses were observed, with the concentrations of individual amino acids increasing, decreasing or displaying no change. When the overall contribution to hemolymph osmolality is considered, the pattern is much clearer (Fig. 7.3A). There was a significant increase in total amino acid concentration post-feeding in both 50% and 100% SW (ANOVA, df = 1, F = 22.657, $P < 0.001$). Increases in total FAA concentration with salinity fell short of significance at the $P = 0.05$ level (ANOVA, df = 1, F = 3.696, P = 0.07). Upon further investigation, unfed animals displayed a 45% increase in total FAA concentration when exposed to 50% SW, though this was also not significant ($P = 0.06$). There was no significant difference post feeding between 100% and 50% SW.

As with *Cancer magister*, *Callinectes sapidus* displayed a post-feeding increase in all of the individual amino acids measured in 100%SW (Table 7.2). In 25% SW, there a post feeding increase in all amino acids measured, except for glu. Significant changes in total circulating amino acid concentration with feeding (Fig. 7.3B; ANOVA, df = 1, F = 39.830, $P < 0.001$) and salinity (ANOVA, df = 1, F = 6.588, $P < 0.05$) were also observed. In both 100% and 25% SW, there was a significant increase in total amino acid concentration post feeding ($P < 0.05$). When exposed to 25% SW, the post feeding increase in amino acid concentration was over double that of animals in 100% SW ($P < 0.05$).

Digestive Efficiency

When exposed to low salinity, *Cancer magister* displayed a significant increase in digestive efficiency (T-test, df = 9, t = -2.987, $P < 0.05$), increasing from 98.9 ± 0.1 % in 100% SW to 99.4 ± 0.1% in 50% (Fig. 7.4). In contrast, when exposed to low salinity, *Callinectes sapidus*, did not show a significant change in digestive efficiency and was 97.1 ± 1.2 % and 99.0 ± 0.2 % efficient in 100% and 25% SW, respectively.

Discussion

When faced with a dilution the internal milieu, as occurs when animals enter an estuarine environment, they must regulate their cell volume. This is achieved by the movement of compatible osmolytes out of the cell and into the extracellular fluid (Yancey, 2005). In crustaceans, these osmolytes are often amino acids (Pequeux, 1995). The movement of amino acids into the cell following a meal is in opposition to this response. Following feeding amino acids are transported across the hepatopancreas and into the haemolymph where they circulate throughout the body. Amino acids are subsequently transported into the cells, where they are incorporated into proteins during a period of increased protein synthesis (Mente *et al*., 2001). The results of the current study showed that when exposed to low salinity following feeding, both *Cancer magister* and *Callinectes sapidus* display a reduction in the flux of amino acids into the cell concordant with reduced influx and increased efflux observed in response to low salinity alone (Moran and Pierce, 1985), but that these processes are regulated at different levels, resulting in a different metabolic response.

When digesting a meal in 100% SW, protease activity in the gut fluid of both species remained stable over time. However, protease activity within the hepatopancreas of *Callinectes sapidus* decreased during the 6h experimental period (Fig. 7.1). The shorter foregut evacuation time for *Callinectes sapidus* (Curtis and McGaw, 2009), suggests that extracellular digestion has progressed further and additional enzyme production after 6h may not be necessary. When exposed to low salinity, changes in protease activity within the gut fluid and hepatopancreas corresponded to changes in the rate of gastric evacuation and mechanical breakdown for both species. *Cancer magister* displayed an increase in protease activity in the gut fluid and the hepatopancreas over the first 6h of digestion. This delay corresponded to a reduction of contraction rate and an increase in evacuation time for the foregut in low salinity (Curtis and McGaw, 2009), suggesting an overall delaying of digestive processes at the extracellular level. In contrast, *Callinectes sapidus* did not show a change in protease activity over time in either the hepatopancreas or gut fluid in low salinity. This again points to a correlation with mechanical digestion and foregut evacuation, which continue unabated in low salinity (Curtis and McGaw, 2009). In support of the idea that *Cancer magister* delays digestion, digestive enzyme activity in the hepatopancreas was approximately double that of *Callinectes sapidus*, whereas gut fluid protease activity in *Callinectes sapidus* was approximately four times that of *Cancer magister*. This suggests that *Callinectes sapidus* is starting digestion as soon as a meal is ingested, whereas *Cancer magister* may be selectively secreting digestive enzymes into the foregut as a means of further delaying digestion.

Previous work examining digestive enzymes in crustaceans has focussed on enzyme activity and compliment as indicators of trophic resource utilization (Johnston and Freeman, 2005; McClintock *et al*., 1991), digestive capability for the formulation of diets in aquaculture (Lopez-Lopez *et al*., 2005) or the characterization of isozymes (Dendinger and O'Connor, 1990; Perera *et al*., 2005). It has generally been accepted that digestive enzymes are stored as zymogens in specialized B-cells within the digestive gland and their contents are expelled following ingestion of a meal (Muhlia-Almazan and Garcia-Carreno, 2003). A number of studies examining the effects of salinity on digestive enzyme activity in crustaceans have focussed on long term exposures and have been inconclusive, reporting increases, decreases or no change in activity (Gaxiola *et al*., 2005; Marangos *et al*., 1989; Rosas *et al*., 2002). The results of the current study show that the synthesis and release of digestive enzymes in crustaceans are dynamic processes. Assays to determine enzyme activity likely activate any zymogens within hepatopancreas lysates (Muhlia-Almazan and Garcia-Carreno, 2003) and a decrease in protease activity over time would be expected if only stored enzymes were being utilized. However, increases over time in *Cancer magister*, and an overall reduction in activity for *Callinectes sapidus* in low salinity suggest that digestive proteases are being synthesized in response to the ingestion of a meal. This finding is in accordance with previous studies on insects that also show an induction of enzyme synthesis in response to the ingestion of a meal (Kalhok *et al*., 1993; Lehane *et al*., 1995). The discrepancy in protease activity between the two species and as a function of time, underscores the importance of thorough investigation when making inferences about the digestive capabilities of crustaceans.

As extracellular digestion progresses, amino acids and other nutrients are absorbed by specialized cells in the hepatopancreas and begin appearing in the hemolymph, where they subsequently become part of the intracellular pool. Most articles on crustacean osmoregulation have concentrated on efficient osmoregulators and the ionic regulation of extracellular fluid at the level of the gill epithelium (Lucu and Towle, 2003) despite the fact that crustaceans maintain large intracellular FAA pools that are important for cell volume regulation particularly for euryhaline osmoconformers (Gilles and Gerard, 1974; Gilles and Pequeux, 1981; Pequeux, 1995). When confronted with a hyposmotic challenge, crustacean cells respond with a transient efflux of inorganic ions, typically K^+, followed by a reduced influx and an increased efflux of FAAs (Moran and Pierce, 1984). *Cancer magister* was no exception displaying a 45% increase in total FAA concentration within the hemolymph after 6h low salinity exposure. This increase was primarily attributable to increased circulating levels of glutamine/histidine, glycine/threonine, and alanine/tyrosine. This corresponds with previous reports, where glycine and alanine appear to be major osmotic constituents (Gilles and Pequeux, 1981); however, the relative contribution of individual FAAs varies with species (Daniello, 1980). In *Callinectes sapidus*, there was no significant increase in circulating FAAs following 6h of low salinity exposure. This contradicts previous reports on isolated cells, where increases in alanine efflux were observed (Gerard, 1975; Gerard and Gilles, 1972). While there were no overall changes in FAA levels, there were changes in the relative contribution of each FAA (Table 7.2). A possible explanation for this occurrence is that either increased biosynthesis of hemolymph proteins, namely hemocyanin (Boone and Schoffeniels, 1979), or in increased association of FAAs with existing hemolymph

proteins (Zatta, 1987) may be masking the increased FAA efflux associated with low salinity exposure. In species such as *Callinectes sapidus* that display an increase in oxygen uptake following low salinity exposure (Curtis and McGaw, 2009; King, 1965), the storage of FAAs as hemocyanin may provide a convenient means of efficiently meeting this increased oxygen demand. It is also noteworthy that the total FAA concentration in the hemolymph of *Cancer magister* was more than 3 times that of *Callinectes sapidus*. While these measures are not directly comparable, this discrepancy suggests a greater contribution of FAAs to osmoregulation and is possibly a reflection of the reduced ionoregulatory ability of *Cancer magister* (Hunter and Rudy, 1975).

For both species, the doubling in circulating FAAs 6h after the ingestion of a meal is in direct opposition to the cellular level responses necessary to maintain cell volume when exposed to low salinity. If digestion were continuing unabated in low salinity, it would be expected that this increase should equal the sum of the responses to digestion and osmoregulation in isolation. However, when exposed to low salinity following feeding, neither species showed this response. *Cancer magister* displayed an increase in circulating FAAs following feeding in low salinity, but the increase was not greater than would be expected from feeding in 100% SW. If cell volume regulatory processes are continuing, the increase in circulating FAAs attributable to feeding is only half of that of animals in full seawater. In direct contrast, *Callinectes sapidus* showed a five-fold increase in circulating FAAs when exposed to low salinity post feeding. This increase was nearly double that expected from an additive response. These results suggest that when exposed to low salinity, both *Cancer magister* and *Callinectes sapidus* are delaying digestive processes, but that the delay is occurring at different levels. *Cancer magister* is

delaying digestion within the foregut and digestive gland, and in *Callinectes sapidus* digestion is being delayed through reduced flux of amino acids into the cells.

Differences in where the post-feeding flux of amino acids is delayed may be influencing the metabolic demands associated with digestion in low salinity. When exposed to low salinity following feeding, *Cancer magister* displays a reduction in oxygen uptake relative to crabs digesting in ambient conditions (Curtis and McGaw, 2009). While reductions in gastric processing (Carefoot, 1990b) and enzyme secretion themselves likely contribute little to the overall costs of digestion, their effect on circulating FAAs displayed here may serve to reduce downstream processes such as intracellular protein synthesis which are energetically costly (Mente et al., 2001). In contrast to *Cancer magister*, postprandial *Callinectes sapidus* display an oxygen uptake rate in low salinity that is greater than would be expected based on the sum of low salinity exposure and digestion (Curtis and McGaw, 2009). A plausible explanation for this response is that the observed build up of amino acids in the hemolymph is resulting in increased rates of protein synthesis. While reports on invertebrates are scarce, previous work had shown that extracellular increases in the concentration of FAAs can increase protein synthesis rates of vertebrate cells (Bohe *et al*., 2003; Iresjo *et al*., 2005). A possible caveat to this argument is that since FAA transport in crustacean cells is a selective process (Moran and Pierce, 1985), it is unlikely that FAA flux into the cells is increasing to meet increased protein synthesis demands during hyposaline exposure. However, intracellular FAAs are unlikely to be limiting since many marine invertebrates maintain intracellular FAA concentrations of 700-800 mM (Pierce, 1982). In further support of the hypothesis that increased hemolymph FAA concentrations increase more

costly protein synthesis rates, larger meal sizes, which presumably also result in increased circulating amino acids, have an increased cost of digestion (Carefoot, 1990a; McGaw, Unpublished Obs.). While further investigation is clearly needed to substantiate these claims, the results of the current study suggest a role for changes in the level of circulating amino acids in passively regulating intracellular protein synthesis and subsequently altering metabolic demands post feeding.

Despite the observed delays in digestive processes, and the associated decreased metabolic demands for *Cancer magister* and increased metabolic demands for *Callinectes sapidus*, both species maintained high digestive efficiencies in low salinity. In the present study, crabs were offered a set meal size and it is likely that alterations in feeding behaviour have a greater impact on their overall energetic balance during low salinity exposure (Curtis and McGaw, 2009). *Cancer magister* consumes smaller meals and feeds less frequently in low salinity. If low salinity exposures are frequent, the resulting reduction in caloric intake may contribute to decreased growth and reproduction (Mente *et al*., 2001). In contrast to *Cancer magsiter*, *Callinectes sapidus* appears well adapted to the additional demands associated with low salinity and digestion: gastric processing continues unabated (Curtis and McGaw, 2009) and food consumption increases (Guerin and Stickle, 1992). This response is manifested as increased growth rates in low salinity (Guerin and Stickle, 1992), indicating that animals are clearly able to overcome any additional energetic penalty.

While *Cancer magister* and *Callinectes sapidus* display differences in their ability to balance the physiological demands of digesting food in low salinity, when combined with behavioural responses, *Cancer magister* is likely able to exploit hyposaline habitats

with little ill effect. *Cancer magister* make short term forays into estuaries (Curtis and McGaw, 2008) and only enter into low salinity during times of high food abundance (Sugarman, *et al*., 1983; Stevens and Armstrong, 1984) or when animals are starved (Curtis and McGaw, In prep). This transient use of low salinity habitats may allow animals to mitigate the demands of digestion by reducing mechanical digestion and enzyme secretion while retreating to areas of higher salinity where they can digest a meal more effectively. In contrast, *Callinectes sapidus* can reside permanently in large estuaries, often only migrating to high salinity areas to release larvae (Hill, 1989); likely as a result of their ability to maintain feeding rates and extracellular digestion in low salinity.

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Tables

Table 7.1: Concentrations of free amino acids in the hemolymph following 6h of exposure to 100 or 50% SW for unfed and fed *Cancer magister*. Concentrations (n =5) are shown as mean values, expressed in mOsm. Standard errors are shown in parentheses. ND indicates concentrations that were below the level of detection.

	100% SW		**50% SW**	
[AA]	**Unfed**	**Fed**	**Unfed**	**Fed**
asp	0.85 (0.19)	1.66 (0.46)	0.93 (0.21)	1.06 (0.15)
glu	1.43 (0.54)	1.83 (0.49)	1.47 (0.29)	1.14 (0.29)
asn	2.75 (0.36)	3.40 (0.35)	2.62 (0.54)	4.96 (0.41)
ser	0.94 (0.12)	1.71 (0.24)	1.05 (0.08)	3.81 (0.14)
gln/his	5.28 (0.42)	6.72 (0.51)	8.09 (0.70)	7.95 (0.74)
gly/thr	5.45 (0.65)	8.50 (0.91)	7.85 (1.30)	6.90 (0.77)
ala/tyr	3.23 (0.61)	5.52 (0.95)	4.32 (0.75)	5.64 (0.45)
arg	0.75 (0.15)	1.77 (0.18)	1.63 (0.41)	1.61 (0.12)
lys	ND	ND	ND	ND
trp	0.06 (0.03)	0.81 (0.30)	ND	0.44 (0.05)
met	0.42 (0.32)	0.68 (0.22)	ND	0.71 (0.10)
val	0.74 (0.05)	3.22 (0.48)	1.45 (0.07)	4.14 (0.24)
phe	0.43 (0.32)	0.99 (0.36)	0.04 (0.04)	0.80 (0.08)
iso	0.25 (0.02)	1.28 (0.35)	0.39 (0.03)	1.33 (0.08)
leu	0.25 (0.02)	1.37 (0.21)	0.43 (0.04)	1.96 (0.16)

Table 7.2: Concentrations of free amino acids (mOsm) in the hemolymph following 6h of exposure to 100 or 25% SW for unfed and fed *Callinectes sapidus*. Concentrations (n = 5) are shown as mean values, expressed in mOsm. Standard errors are shown in parentheses. ND indicates concentrations that were below the level of detection.

	100% SW		**25% SW**	
[AA]	**Unfed**	**Fed**	**Unfed**	**Fed**
asp	0.09 (0.05)	ND	ND	ND
glu	0.13 (0.07)	0.19 (0.08)	0.52 (0.20)	0.36 (0.16)
asn	0.22 (0.12)	1.93 (0.59)	0.50 (0.28)	4.18 (0.73)
ser	0.16 (0.04)	1.17 (0.37)	0.28 (0.10)	2.86 (0.90)
gln/his	2.50 (0.23)	4.49 (0.99)	5.69 (1.72)	14.82 (3.36)
gly/thr	1.69 (0.13)	3.66 (0.47)	2.66 (0.66)	5.23 (0.99)
ala/tyr	1.81 (0.17)	3.91 (0.55)	1.56 (0.29)	5.45 (0.90)
arg	0.51 (0.06)	1.32 (0.25)	0.65 (0.06)	1.76 (0.37)
lys	ND	ND	ND	1.40 (1.40)
trp	0.03 (0.03)	0.10 (0.10)	ND	0.21 (0.13)
met	ND	0.19 (0.19)	ND	1.06 (0.45)
val	0.06 (0.06)	1.56 (0.60)	0.35 (0.35)	3.85 (0.71)
phe	0.08 (0.05)	0.78 (0.36)	ND	1.68 (0.41)
iso	0.06 (0.04)	0.32 (0.21)	ND	1.41 (0.28)
leu	0.09 (0.06)	0.84 (0.36)	0.09 (0.09)	3.16 (1.53)

Figures

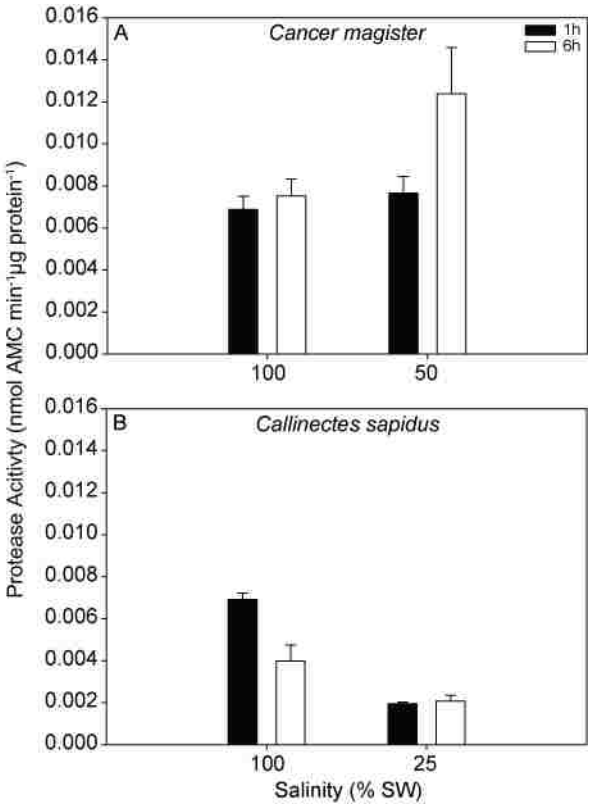

Figure 7.1: Total protease activity in hepatopancreas lysates (n = 5)of (a) *Cancer magister* and (b) *Callinectes sapidus*, 1 and 6h after the ingestion of a meal in 100% SW or low salinity (50% SW and 25% SW for *Cancer magister* and *Callinectes sapidus*, respectively). Low salinity levels were chosen to be within the range that each species shows show cardiovascular responses, but above their lower lethal limit. Protease activity was measured as the release of the fluorophore from a suc-LY-AMC substrate during a 15 min incubation. Values are mean ± standard error, *n* = 5 animals per treatment.

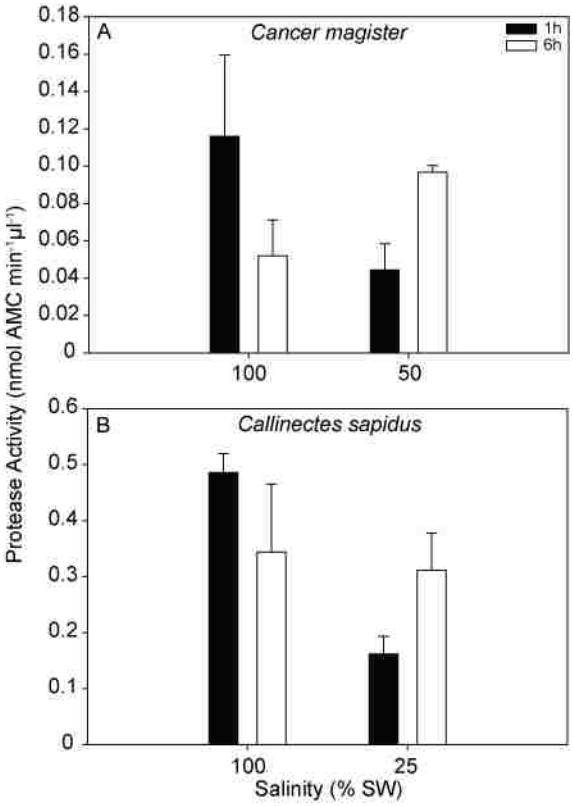

Figure 7.2: Total protease activity in the gut fluid of (a) *Cancer magister* and (b) *Callinectes sapidus*, 1 and 6h after the ingestion of a meal in 100% SW or low salinity (50% SW and 25% SW for *Cancer magister* and *Callinectes sapidus*, respectively). Protease activity was measured as the release of the fluorophore from a suc-LY-AMC substrate during a15 min incubation. Values are mean ± standard error, n = 5 animals per treatment.

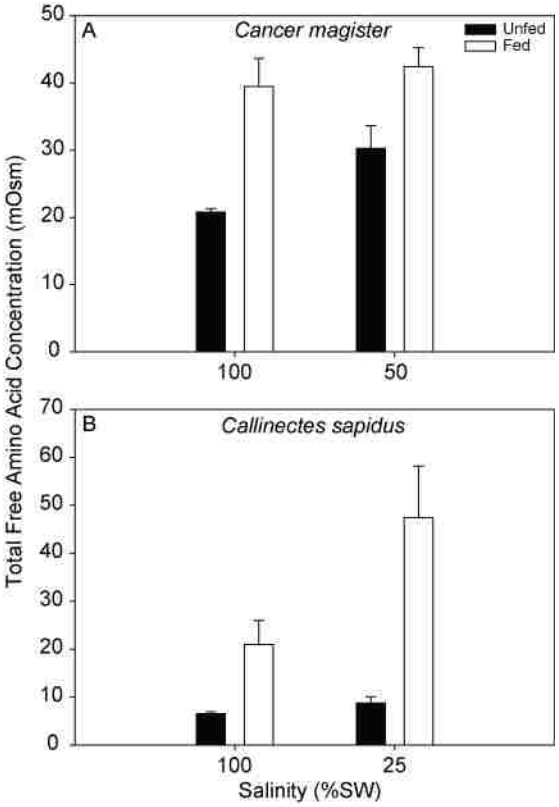

Figure 7.3: Total free amino acid concentration in the hemolymph of (a) *Cancer magister* and (b) *Callinectes sapidus* following 6h in 100% SW or low salinity (50% SW and 25% SW for *Cancer magister* and *Callinectes sapidus*, respectively). Total concentration was determined as the sum of the 18 amino acids quanitified. Values are mean ± standard error, n = 5 animals per treatment.

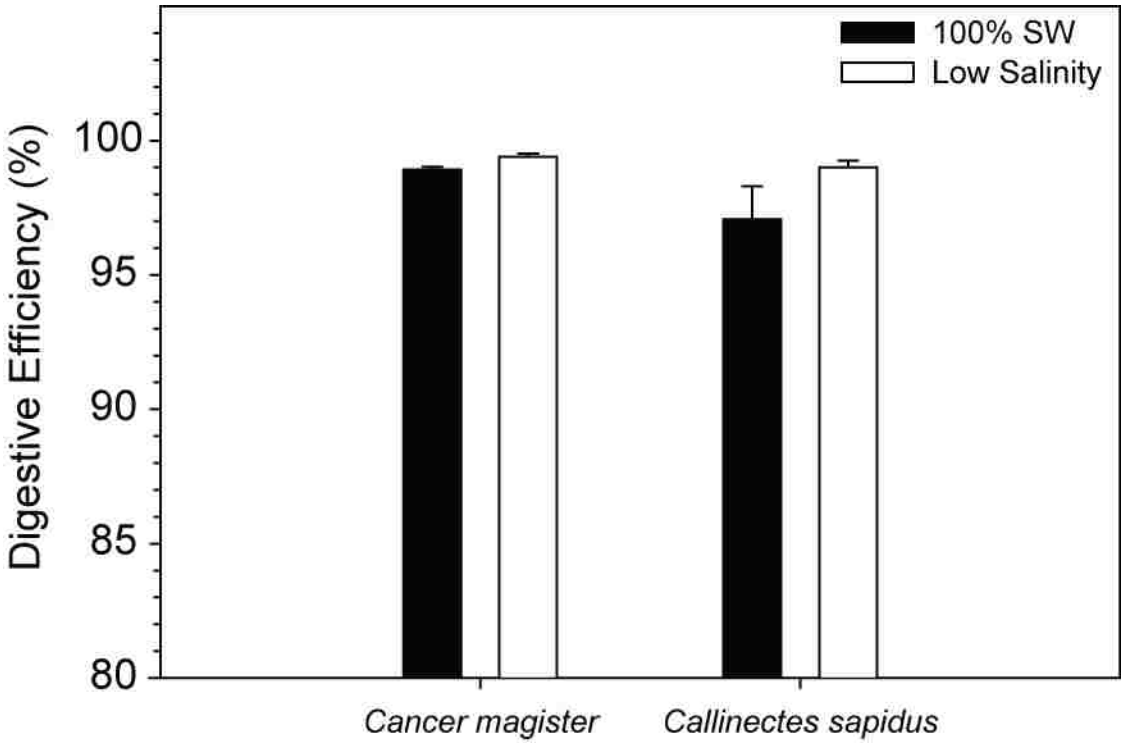

Figure 7.4: Digestive efficiency of *Cancer magister* and *Callinectes sapidus,* digesting a meal in 100% SW or low salinity (50% SW and 25% SW for *Cancer magister* and *Callinectes sapidus*, respectively). Values are mean ± standard error, $n = 5$ animals per treatment.

CHAPTER 8

GENERAL DISCUSSION

Summary of Findings

Physiological tolerances often dictate the distribution of animals along environmental gradients. This is particularly true for species inhabiting estuaries, where temperature and salinity have been shown to affect distribution (Barnes, 1967; Gonzalez-Ortegon *et a*l., 2006; Jury *et al*., 1995; Lewis and Roer, 1988; Rewitz *et al*., 2004). However, when faced with increased competition or limited resources, animals may be forced to forage or persist in suboptimal conditions in order to survive (Hoffman and Parsons, 1993). The ability to exploit suboptimal habitats may therefore be dictated by the mechanisms an animal uses to mitigate environmental challenges, rather than physiological tolerances alone. The results of this dissertation have shown that the Dungeness crab, *Cancer magister*, employs both behavioural and physiological mechanisms in order to exploit areas that are prone to hyposaline conditions.

The first line of defence employed by animals encountering sub-optimal environmental conditions is to avoid them altogether. In the field, adult *Cancer magister* spent the majority of their time in salinity and temperature conditions that do not present a physiological challenge. When crabs entered into the estuary, these forays were usually during times of optimal temperature and salinity conditions, allowing them to exploit the rich resources of the estuary and then retreat to deeper more stable habitats. In addition, when exposed to low salinity or high temperature, crabs usually displayed immediate avoidance behaviours, retreating to deeper waters. These avoidance behaviours are in

contrast to more prolonged exposures to stressful temperature and salinity conditions, where it appears that crabs were continuing to forage without regard for challenging conditions and are likely being passively exposed with the changing of the tide. Previous work has suggested that adult crabs are scarce within estuaries (Stevens *et al*., 1984). The work presented here shows that while only a small fraction of the population is present within the estuary at any one time, it is likely that all adults within the population are exploiting these habitats. This points out the limitations of studies based on benthic trawls or trapping, and underscores the importance of considering individual behaviour when determining the extent of habitat use. While this work has shown that crabs are likely foraging in the estuary, there is still little direct evidence as to what drives the more prolonged exposures to low salinity or high temperature that are observed. Laboratory experiments suggested that both food availability and nutritional status may play a role in altering salinity and thermal preference, and in future studies it will be informative to investigate the relationship between nutritional status and the conditions that crabs are exposed to while feeding in the field.

Foraging decisions result from the trade-off between the challenges presented and the benefits gained. This trade-off has mostly been considered for animals foraging under the risk of increased predation (Lima and Dill, 1990). The work presented here is one of only a handful of studies considering the role of environmental conditions when making foraging and feeding decisions (Grubb and Greenwald, 1982; Webster and Dill, 2006, 2007). The current work has shown that food availability alters the thermal and salinity preference of *Cancer magister* in the laboratory and that these effects are dependant on the time since the last feeding episode. Crabs that have not recently fed

spent more time in challenging conditions searching for a food stimulus. In addition, the likelihood that a crab would feed decreased with salinity, but increased with time since feeding. This suggests that crabs are not only integrating environmental conditions, but also their nutritional status when making foraging and feeding decisions. By not feeding crabs avoid coping with the concurrent physiological demands of osmoregulation and digestion unless the benefits gained from food acquisition are great. These observations reinforce the idea that challenging habitat conditions may play a similar role to predation when animals are making foraging decisions and add essential new data to the concept of the "foraging theory of physiology".

Either avoiding or not feeding in challenging environmental conditions represents the simplest way for animals to avoid having to cope with the concurrent demands of digestion. However in many cases behavioural avoidance is not possible and animals may still need to procure nutrients. In these cases, alterations in both the amount of time spent feeding and the amount of food consumed may help to mitigate the need prioritize the responses to low salinity. By consuming a given amount of food faster in low salinity, *Cancer magister* minimizes the duration of exposure to low salinity, and can retreat to areas of higher salinity where they can digest more effectively. Since the costs of digestion are proportional to the size of a meal (Secor and Faulkner, 2002), the observed reduction in meal size for *Cancer magister* exposed to low salinity may be an effective means of mitigating the costs associated with digestion while in challenging conditions. These responses show an effective behavioural mechanism for minimizing the concurrent challenges of osmoregulation and digestion. In future studies it will be important for investigators to also consider changes in feeding behaviour when

determining the ability of animals to balance the demands of digestion and competing physiological processes.

Both avoidance behaviours and changes in feeding habits require the animal to either sense its surrounding environment, or possibly anticipate changes in the environment. In many cases though, these behavioural responses are not possible and animals may be exposed to changes in environmental conditions at any point during the digestive cycle. In such cases, animals are forced to resort to physiological mechanisms. The experiments here showed that crabs of different osmoregulatory ability also differed in their physiological responses to low salinity exposure post-feeding. Examination of changes in oxygen uptake post-feeding revealed that *Cancer magister*, a weak osmoregulator, is unable to balance the simultaneous demands of osmoregulation and digestion. As a result, crabs displayed no subsequent increase in oxygen uptake while digesting in low salinity suggesting a prioritization towards the physiological responses associated with low salinity. In contrast, *Callinectes sapidus*, an efficient osmoregulator, was able to balance the metabolic demands of both processes, as evidenced by a summation of responses.

Given that the two species exhibited different metabolic responses, I further investigated how prioritization or summation of metabolic responses manifested themselves as digestive responses. This work is the first to examine the metabolic consequences of changes in digestive processes at the mechanical, extracellular and intracellular level for crustaceans. By reducing mechanical digestion and enzymatic breakdown, and the subsequent flux of free amino acids into the hemolymph, *Cancer magister* may be passively reducing the more costly intracellular process of protein

synthesis (Mente *et al*., 2001). In *Callinectes sapidus*, increases in oxygen uptake resulting from low salinity exposure post-feeding were actually greater than would be expected from the responses to digestion and low salinity exposure in isolation. Cell volume regulation during low salinity exposure is in part achieved by a reduction in the flux of amino acids into the cells (Pequeux, 1995). Since extracellular digestion continues unabated when *Callinectes sapidus* is exposed to low salinity, amino acids were still being transported into the hemolymph. These conflicting responses led to a build up of free amino acids in the extracellular fluid and increases in the extracellular concentration of free amino acids have been shown to stimulate protein synthesis (Iresjo *et al*., 2005). Therefore it is likely that the additional metabolic demands observed for *Callinectes sapidus* while digesting in low salinity result from the increased stimulation of protein synthesis. Future studies examining the effects of environmental conditions on the post-feeding increase in protein synthesis should therefore consider both meal size and digestive performance when making inferences about regulatory mechanisms.

Broader Implications of This Work

There has recently been a great deal of interest in the interaction between physiology and behaviour, and the influence of individual behaviour on physiological systems. This has resulted in a number of prominent symposia at international meetings such as the Society for Experimental Biology (2004, 2007). The findings presented at these symposia highlight the influence of behaviour at all levels of organization. For example: King penguins focus their foraging behaviour within a temperature range

spanning just a few degrees, and as a result are able to maximize their foraging efficiency and minimize energy expenditure (Bishop *et al*., 2007). In salmon, a fish's social status as a juvenile may affect their susceptibility to toxins in later life stages (Sloman, 2007). At the cellular level, the timing of when juvenile Atlantic salmon migrate to sea can affect ion uptake kinetics at the cellular level in adults (Murua, 2007). The importance of links between behaviour and physiology are only now becoming recognized by the broader scientific community and are therefore on the leading edge of discovery. My work has shown that through avoidance behaviours and changes in feeding, animals are able to reduce their reliance on physiological mechanisms while foraging and feeding in stressful environmental conditions. These results provide a valuable contribution to the growing awareness of the influence of behaviour on physiology and reinforce the importance of the close interactions and trade-offs between behaviour and physiological function.

Historically environmental choice/preference of aquatic organisms was assessed using choice chambers or small gradient apparatus. While these apparatus were convenient to use and produced replicable results the static environments and sharp gradients used may not accurately reproduce conditions in the field (Bell *et al*., 2003, Bernatis *et al*., 2007). This was partially compensated for by collection of animals in the field (by trawling or trapping) or following animals using directional telemetry. However, the logistics of both methods are still time limited; human researchers must gather the data and therefore these methods only produce a "snapshot" of how an animal interacts with its environment. The introduction of data loggers to examine the microhabitat conditions experienced by animals in the field was initially slow to gain acceptance within the ecological community (Cooke *et al*., 2004). Following the advent of a number

of prominent articles, researchers are beginning to appreciate the value of this technology for investigating the habitat conditions of species where direct observation is not possible (Rutz and Hays, 2007). In the last 10 years, technological advances leading to the miniaturization and a reduction in cost, has garnered increasing interest in their potential for use (Rutz and Hays, 2007). Nevertheless the majority of these studies have, and continue to, focus on larger organisms such as pelagic marine fishes, turtles and marine mammals (see Cooke *et al*., 2004 for review). As such, this work is one of the few studies to employ this technology on such a small animal. The data presented here makes a valuable contribution, moving beyond previous studies as the first to monitor changes in salinity, temperature, and depth, over extended periods, in a highly dynamic environment. These results showed that animals minimize exposure to challenging conditions by exploiting the spatial heterogeneity of environmental conditions. Monitoring the conditions that animals are actually being exposed to paints an entirely new picture of how animals interact with their environment, essentially providing a window into the unknown. The use of this technology to monitor microhabitat conditions provides valuable and exciting new data that were not previously available, and represents an essential new direction in researching how animals interact with their environment.

There is a large body of literature examining the risks associated with foraging under the threat of predation (see Lima and Dill, 1990 for review). This work has consistently shown animals weigh the risks associated with predation against those of dying from starvation; basically a hungry animal is more likely to enter into an area with increased predation risk. However, few studies have examined how physiological ability influences foraging decisions when animals are faced with challenging environmental

conditions (Webster and Dill, 2007). The work presented here makes an excellent parallel to these studies by showing that animals are integrating information about the stresses associated with challenging environmental conditions and weighing them against the benefits gained from feeding. If the environmental conditions in a particular habitat impart a great deal of physiological stress, the balance will be tipped in favour of not foraging or feeding in those conditions. The work presented here paves the way and presents a 'foraging theory of physiology', in which physiological challenges may play a similar role to predation when animals are making foraging decisions.

When examining the responses to changing environmental conditions it is important to take into account the circumstances that occur in nature. Much of the physiological literature has examined the responses to environmental stresses that are far beyond those experienced by animals in the field. While this research had provided essential insight into the mechanisms responsible for the performance of physiological systems, it may not reflect the conditions experienced in nature. Furthermore, it has long been known that digestion may affect other physiological systems, and researchers have traditionally accounted for this by performing experiments on starved animals to ensure they are all in similar basal metabolic state (Secor, 2009). However, since all animals must feed to survive, it is unlikely that they are able to wait until they have finished digesting a meal before being faced with additional physiological challenges. Therefore, previous work focussing on starved animals likely does not reflect the circumstances that occur in nature. In recent years, there has been a call to action for researchers to consider that the increased metabolic demands associated with digestion may impinge on the responses of others systems and that animals may not be able to balance competing

demands (Wang, 2001). The current work has shown physiological ability and digestive state may dictate an animal's capacity to balance concurrent physiological demands. This work has reinforced the idea that the physiological responses to environmental challenges are intertwined with those of digestion. However, it has also shown that the relationship between digestion and competing physiological demands may not exclusively be the result of limited physiological resources, and the alteration of digestive processes may help to facilitate the prioritization of responses towards more pressing environmental challenges.

Within the field of ecophysiology there has been a drive towards understanding how species will respond to climate change, and how these responses will affect distribution on a macro scale (Osovitz and Hoffman, 2007). However, before we can begin to understand how physiological capabilities affect an animal's ability to thrive or subsist under changing environmental conditions, we must first consider the conditions an animal is actually exposed to, the circumstances under which they are exposed, and the behavioural and physiological mechanisms used to cope with these exposures. The results of the current work have shown that each one of these factors can drastically alter an animal's ability to thrive in challenging environments. As such, in order for researchers to truly realize the intricacies of the effects that changing environmental conditions have on an organism, it is vital that they examine each of these factors when making predictions on the effects of climate change.

As a whole, the studies comprising this dissertation have reinforced the importance of considering the responses to environmental challenges at all levels and highlights some important new trends in biology. Biologists are beginning to appreciate

the key role that interactions between ecology, behaviour and physiology play in determining how changes in environmental conditions affect animals in nature. As a part of this, investigators are also beginning to realize the importance of using ecologically relevant parameters in experiments. If an animal never experiences the experimental conditions in nature, the results probably don't mean much. As a result of these trends, a new era of collaborative research is beginning, where researchers can no longer carry out their work in isolation. By using an integrative approach similar to that demonstrated here, biologist will be able to continue to shed new light on how organisms interact with their environment and the influence of changing environmental conditions.

Future Work

As in any good PhD my thesis has raised more questions than it has answered. The most pressing questions that will need to be addressed by future work are:

1. Under what temperature and salinity conditions are crabs actually feeding in the field and how is this affected by nutritional status?

By using archival data tags to log movements of the mouthparts associated with feeding along with the temperature and salinity conditions experienced, it will be possible to determine the true extent of feeding and foraging in low salinity. The data generated will prove extremely valuable in modelling the energetics of *Cancer magister* in estuaries.

2. What is the actual neurohormonal mechanism by which changes in external salinity elicit the release of inhibitory hormones from the sinus gland?

While my current work has shown that eyestalk ablation removes feeding inhibition, the neuronal pathway and hormones involved in this process remain a mystery. In future studies, it will be necessary to characterize the hormone responsible for this inhibition and its targets.

3. Do changes in extracellular digestion translate to actual changes in the rate of intracellular protein synthesis post-feeding?

The current work has shown correlative evidence that changes in digestive process and the subsequent flux of free amino acids into the hemolymph likely alters the energetic costs associated with digestion. Since changes in extracellular amino acid concentration are likely altering protein synthesis, current *in vivo* methods for measuring protein synthesis rates, such as the flooding dose method, confound any changes attributable to digestion. In the future it will be beneficial to develop methodology for determining rates of protein synthesis that do not involve large alterations in extracellular amino acid concentrations.

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