PHYSIOLOGICAL RESPONSES OF THE CRABS *CARCINUS MAENAS* AND *CANCER IRRORATUS* TO THE COMBINED CHALLENGES OF DIGESTION AND LOW SALINITY

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ABSTRACT

Osmoregulation and digestion are energetically demanding processes that typically result in an increase in metabolic rate. Crabs that move into low salinity environments to feed must be able to balance the demands of both processes. However, achieving this balance may pose greater challenges for weak osmoregulators and conformers than for stronger regulators. In this study, the rate of oxygen consumption (MO_2) of green crabs (*Carcinus maenas,* an efficient osmoregulator) and rock crabs (*Cancer irroratus,* a weak osmoregulator) was examined as a function of feeding and salinity. MO_2 increased over 2-fold in both species after feeding in 32‰ and returned to pre-fed values in 17-20 hours. When fasted crabs were given an acute hyposaline exposure (24 and 16‰ for *C. irroratus*; 16 and 8‰ for *C. maenas*), MO_2 increased and remained elevated with lower salinities resulting in higher peak values for MO_2. When challenged with low salinity after feeding, *C. maenas* responded with an immediate addition of the MO_2 due to feeding upon that due to salinity stress, whereas *C. irroratus* reacted with a delayed increase in MO_2 following the salinity drop. Several aspects of their physiology and behaviour were investigated in an attempt to explain these differences. Although protein synthesis is reported to account for the majority of the postprandial increase in MO_2, this did not appear to be the case in this study. There was no effect of feeding or low salinity on protein synthesis rates in the leg, gills, or hepatopancreas of either species, and this suggests that protein synthesis can continue in low salinity as long as substrates are available. Further, the behaviour (i.e. activity) of the crabs and aspects of their mechanical digestion could not explain the differences in MO_2. *C. irroratus* became more active than *C. maenas* in low salinity, and *C. irroratus* exhibited a trend towards a decrease in gut contraction rate which resulted in longer

transit times of the meal through the gut in this species. This reduction in gastric processing may have stemmed from an inability of *C. irroratus* to regulate osmotic water onload as efficiently as *C. maenas*, and this likely delayed the additive effect of the metabolic demands of digestion and salinity stress.

Keywords: *Cancer irroratus*, *Carcinus maenas*, digestion, osmoregulation, oxygen consumption, protein synthesis, salinity, specific dynamic action.

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LIST OF ABBREVIATIONS

Adenosine triphosphate……ATP

Body mass……BM

Calorie……Cal

Centimeter……cm

Counts per minute……CPM

Degrees Celsius……^{o}C

Degrees of freedom……df

Disintegrations per minute……DPM

Estimated energy expenditure……EEE

F value……F

Gram……g

Hour……h

Kilocalorie……kCal

Kilogram……kg

Kilojoule……kJ

Kilovolt……kV

Litre.....L

Meter.....m

Microamp.....µA

Microgram.....µg

Microliter.....µL

Milligram.....mg

Millimole.....mmol

Minute.....min

Molar.....M

Nanomole.....nmol

Oxygen.....O_2

Oxygen consumption.....MO_2

Parts per thousand.....‰

Percent.....%

Perchloric acid.....PCA

Phenylalanine.....Phe

Resting metabolic rate.....RMR

Repeated measures……RM

Ribonucleic acid……RNA

Room temperature……RT

Seawater……SW

Second……s

Specific dynamic action……SDA

SDA Coefficient……C_{SDA}

Standard error……S. E.

Versus……vs.

INTRODUCTION

Specific Dynamic Action

Specific dynamic action (SDA) describes the increase in metabolic rate that occurs during the postprandial (i.e. after feeding) state of an organism (Kleiber 1961). This complex physiological event has been studied for over 100 years and continues to receive attention in the fields of nutrition, biochemistry and physiology (McCue 2006). During this time, the body of literature regarding SDA has grown to include studies on a variety of taxa including, but not limited to, mammals (e.g. *Rattus norvegicus*) (Forsum et al. 1981), birds (e.g. *Pygoscelis adeliae*) (Janes and Chappell 1995), reptiles (e.g. *Python molurus*) (Secor and Diamond 1997), fish (e.g. *Cyprinus carpio*) (Chakraborty et al. 1992), amphibians (e.g. *Bufo marinus*) (Wang et al. 1995), and various invertebrates (e.g. *Carcinus maenas, Saduria entomon, Nephrops norvegicus, Ligia oceanica*) (Houlihan et al. 1990; Robertson et al. 2001; Mente et al. 2011; Whiteley and Faulkner 2005; respectively). The effect of SDA on metabolic rate is typically observed as an increase in oxygen consumption (MO_2). Oxygen consumption can remain elevated anywhere from a few hours to a number of days, depending on the size and the type of meal consumed, temperature, and other parameters (Andrade et al. 1997; Secor and Diamond 1997; Secor and Faulkner 2002; Zaidan and Beaupre 2003; Pan et al. 2005).

Specific dynamic action is measured as the total amount of energy expended on metabolic processes, and is calculated by multiplying the total O_2 consumed (i.e. area under the MO_2 curve above resting rate) by 0.014 kJ/mg O_2 which is an estimate of the energy expended per mg of O_2 consumed (Secor 2009). A number of characteristics of the SDA are also typically quantified. These include the scope required for SDA, which is calculated by dividing the

maximum post-feeding metabolic rate by the resting metabolic rate. The duration of the SDA is the time that MO_2 remains elevated, and is measured from the time feeding begins until MO_2 returns to pre-feeding values. The SDA coefficient (C_{SDA}) is the percentage of the food energy that is put into the metabolic processes of SDA, and is calculated by dividing the energy of the meal (in kCal) into the SDA response (Guinea and Fernandez 1997). This is a useful measure because it allows for a comparison of the SDA response without having to take into account the differences in experimental conditions such as meal type and meal size, both of which can differ between studies (Secor 2009).

Investigations into the underlying mechanisms of SDA have revealed that it is a composite of many digestive processes which contribute to the overall energetic demand. These processes include the acquisition and mastication of the meal, peristalsis, enzymatic breakdown, nutrient uptake and transport, and the subsequent intracellular protein synthesis (Secor 2009). The amount of energy required for each component has long been debated among scientists and has not been fully determined for any species to date. The amount of energy required for each SDA component likely differs amongst species and is dependent on the taxa, type of meal and the mode of digestion. An initial increase in MO_2 may be associated with acquisition of a meal, and would include the costs of foraging behaviour and the subsequent handling of food. An in-depth assessment of the proportion of the SDA directed towards meal acquisition among different taxa is currently lacking, but a few studies have examined the energy requirement of food handling in snakes (Cruz-Neto et al. 1999; Cruz-Neto et al. 2001; Canjani et al. 2003). For example, the time taken for a *Boa constrictor* to constrict and ingest a rat of 5-40% its body mass ranges from 15 to 43 minutes and the energy used for

these activities is 0.11-0.21% of the energy assimilated from the meal (Canjani et al. 2003). Once a meal is ingested, it is broken down mechanically by the actions of teeth, gizzards and/or the gut itself (Piersma et al. 2003; Secor 2009; McGaw and Curtis 2013). However, mechanical digestion is generally considered to have a minor contribution to the overall SDA budget (Tandler and Beamish 1979; Kiørboe et al. 1985; Carefoot 1990; Campbell et al. 2000). The contribution of mechanical digestion to SDA in Burmese pythons (*Python molurus*) is thought to be very low in comparison to the chemical component (i.e. enzymatic breakdown), because SDA is higher in snakes fed pureed mice compared with those fed whole mice (McCue et al. 2005). In addition, the cost of chewing in manatees (*Trichechus inunguis*) is estimated to be just 3.4 and 5.4% of the energy content of a meal of grass and water hyacinth, respectively (Gallivan and Best 1986). In contrast to these findings, when green crabs (*Carcinus maenas*) are given a meal of homogenized fish, the SDA response is 35% lower than those given a meal of whole fish. This implies that a large portion of energy is required for the mastication of the meal (McGaw and Penney 2014). This is not unexpected as the foregut and gastric mill of crabs are controlled by over 40 muscles which are used for cutting, grinding and movement of ingested food (McGaw and Curtis 2013).

The contribution of the enzymatic breakdown of food, and the subsequent uptake/transport of nutrients, to SDA varies depending on species and diet (Secor 2009). A few studies have related these aspects of digestion to SDA in invertebrates (Rosas et al. 1995; Curtis et al. 2013), however, most of the existing literature has focused on reptiles (Secor and Diamond 1995; Overgaard et al. 2002; Secor 2003). The increased energetic costs associated with enzyme secretion are due mainly to the cost of upregulating the cellular systems

responsible for producing them. *Python molurus* expends 528 kJ/kg to digest a whole rat, but when the cost of breaking down and transporting the meal into the intestine is eliminated by injecting a ground rat directly into the intestine, only 175 kJ/kg are used (Secor 2003). This difference in energy expenditure is due to the secretion of gastric acid and enzymes, and this comprises 55% of their SDA response. In addition to enzyme activity, nutrient uptake/transport may contribute to SDA. The increase in the energetic demand associated with this digestive process is a result of the increased activity of ATPase driven transporters of the intestinal tract required for the uptake of amino acids, carbohydrates and other nutrients (Secor and Diamond 1995).

Protein Synthesis

Once the food has been digested and absorbed across the gut it is transported to the cells and the availability of substrates, including amino acids, stimulates an increase in the rate of protein synthesis. Protein synthesis is reported to be a major component of SDA and its contribution to the postprandial increase in MO_2 is estimated to vary from 20-50% in crustaceans (Houlihan et al. 1990; Mente et al. 2003) to as high as 70% in reptiles (Secor 2009; McCue et al. 2005). Further, experimentally determined values for the cost of protein synthesis are quite variable (50-3,500 mmol ATP per gram of protein synthesised) and depend on the species examined, as well as the specific tissue and environmental/experimental parameters (Brown and Cameron 1991a, b; Pannevis and Houlihan 1992; Whiteley et al. 1996). The actual "cost" of protein synthesis can be difficult to accurately assess given that there are additional

metabolic processes occurring at the time proteins are being synthesised, and these can add to the observed metabolic response (Storch and Pörtner 2003).

Rates of protein synthesis are usually assessed in one of three methods: constant infusion, stochastic endpoint and the flooding dose method (Fraser and Rogers 2007). Constant infusion involves the injection of a solution containing a radiolabelled amino acid into the blood stream of the organism at a constant rate. The amount of radiolabel taken up by the tissues and incorporated into proteins can then be quantified (Haschermeyer and Smith 1979). The stochastic endpoint method measures protein synthesis using an amino acid that is labelled with a stable isotope which has been blended with a meal and fed to the organism. The isotope is incorporated in new proteins and, following their deamination, the resulting nitrogenous waste is collected. The values of total nitrogen and isotope in the sample are used to calculate protein synthesis rates (Fraser and Rogers 2007). Finally, the flooding dose method involves an injection of radiolabelled amino acid in a saturated solution of unlabelled amino acid. This solution floods the tissues so that the radiolabelled amino acid adds to the intracellular free pool of amino acids available for protein synthesis. After an incubation period, the tissue is collected, and the fraction of the radiolabel that has been incorporated into protein and that remaining in the free pool is measured. This information is then used to calculate the protein synthesis rate (Garlick et al. 1980; Fraser and Rogers 2007).

The rate of protein synthesis depends upon environmental conditions. Protein synthesis rates increase with temperature in rainbow trout (*Oncorhynchus mykiss*) (Mathers et al. 1993), Atlantic wolfish (*Anarhichas lupus*) (McCarthy et al. 1999) and postprandial barramundi (*Lates calcarifer*) (Katersky and Carter 2010). Protein synthesis rates also increase in crustaceans with

temperature and are higher in fasted isopods (*Saduria entomon*) at 13°C as compared to animals maintained in 4°C (Robertson et al. 2001).

Changes in environmental O_2 have also been shown to have an effect on the rate of protein synthesis. A reduction in O_2 levels from full saturation (80-100%) to 10% leads to a 50-60% decrease in the rate of protein synthesis in the liver, heart and gill of the cichlid (*Astronotus ocellatus*) (Lewis et al. 2007). The rate of protein synthesis also decreases in the heart, brain and liver during anoxia in crucian carp (*Carrassius carrassius*) (Smith et al. 1996). Further, MO_2 in post-prandial green crabs is lowered during hypoxia in a tissue-specific manner, and this leads to a large reduction in protein synthesis in the hepatopancreas compared to other tissues (Mente et al. 2003).

Changes in salinity also influence the rate of protein synthesis in some invertebrates. When the salinity is lowered from 20-25‰ to 3.5‰, the rate of protein synthesis in the hemocytes of the American oyster (*Crassostrea virginca*) decreases, but the rate of synthesis of osmotic stress proteins increases (Tirard et al. 1997). Intanai et al. (2009) measured protein synthesis rates in freshwater prawns (*Macrobrachium rosenbergii*), and juveniles have lower whole body protein synthesis rates when acclimated to 0 and 30‰ compared to 14‰. However, there is no difference in protein synthesis at these salinities in post-larvae of this species even though MO_2 is significantly higher at 0 and 30‰. The authors speculate that the reason for the difference between these two developmental stages might be how they provide the energy for coping with osmotic stress. This could be either by depressing protein synthesis or increasing metabolic rate.

Osmotic Stress

In addition to the energetic costs associated with SDA, aquatic organisms have to expend energy maintaining homeostasis, especially in near-shore environments that may fluctuate considerably with respect to O_2 levels, temperature and salinity. Maintaining the osmolality of body fluids within set limits is an energetically demanding process and an extensive body of literature exists on osmotic regulation in crustaceans and teleosts (see reviews by Pequeux 1995; Hwang and Lee 2007; McNamara and Faria 2012). The effect of hypo-osmotic stress on the metabolism of animals is typically observed as increase in MO_2, and this has been observed in many aquatic animals including lobsters (*Homarus americanus*) (Jury et al. 1994), Senegalese sole (*Solea senegalensis*) (Herera et al. 2012) and *Crassostrea virginca* (Shumway and Koehn 1982). This increase in metabolic rate is associated with the upregulation of ATPases, active ion uptake at the gills (Piller et al. 1995) and increased locomotor activity (i.e. halokinesis) (Taylor and Naylor 1977; Thomas et al. 1981).

Many crustaceans forage in estuaries and the intertidal zone where they are exposed to low salinity. This presents a challenge to these organisms since digestion and osmoregulation both require energy expenditure. The ability to balance the demands of these processes depends on the osmoregulatory ability of the species. Efficient osmoregulators such as the blue crab (*Callinectes sapidus*) can survive in salinities from 0 to 117‰ and tolerate rapid changes in salinity (Tan and van Engel 1966; Williams 1984; Guerin and Stickle 1992). During exposure to low salinity, postprandial *Callinectes sapidus* add the increased MO_2 due to SDA upon the increased MO_2 associated with osmoregulation (Curtis and McGaw 2010). Weaker osmoregulators such as the Dungeness crab (*Cancer magister*) also inhabit estuaries, but they

are less tolerant of low salinity and can only survive salinities as low as 25% seawater (SW) (McGaw et al. 1999). Postprandial *Cancer magister* exhibit the typical increase in MO_2 following feeding, but when salinity is lowered, the rate of MO_2 decreases returning to resting values, and this suggests a prioritization towards osmoregulation (Curtis and McGaw 2010).

The differences in postprandial MO_2 are reflected in the digestive physiology of the species. The transit rate of food through the digestive tract in *Callinectes sapidus* is not affected by low salinity, suggesting that digestion continues unabated during hyposaline exposure. In contrast, the transit rates of digesta in *Cancer magister* are much longer in low salinity (Curtis and McGaw 2010), which suggests that this species cannot balance the demands of both processes. A reduction in the processing rate of a meal may have downstream effects, such as slowing nutrient absorption, and subsequently limiting the amount of substrates available for protein synthesis which could reduce the effect of SDA on metabolism (Curtis and McGaw 2010; Curtis et al. 2013). Nevertheless, this work only raises more questions as to how crustaceans can modulate digestive processes and how each contributes to the overall SDA.

Objectives

The first aim of my thesis was to determine if the interactive effects of feeding and salinity that have been reported for other crustaceans (e.g. *Cancer magister* and *Callinectes sapidus*) (Curtis and McGaw 2010) extend to other species, namely, the green crab (*Carcinus maenas*) and the rock crab (*Cancer irroratus*). *Carcinus maenas* is native to Western Europe, but has invaded coastal environments in temperate regions around the globe (Klassen and Locke 2007). This species is classified as an efficient hyper-osmoregulator (McGaw et al. 1999) and

can survive a wide range of salinities from 5‰ (Broekhuysen 1936) to 52‰ (Klassen and Locke 2007). These crabs are typically found in estuaries and the intertidal zone where salinity fluctuates in the range of 8 to 35‰ (Siebers et al. 1982). *Cancer irroratus* lives in temperate coastal environments ranging from Labrador to South Carolina, can be found from full-strength seawater (32-34‰) to salinities down to approximately 16‰, and is classified as a weak osmoregulator (Neufield and Pritchard 1979; Charmantier and Charmantier-Daures 1991). If differences in postprandial MO_2 occurred in animals in low salinity, the second aim of the study was to investigate possible reasons for this response. Changes in locomotor activity were measured because efficient osmoregulators tend to become more active when salinity is lowered, whereas weaker regulators and osmoconformers become quiescent (Taylor et al. 1977; McGaw et al. 1999; Curtis et al. 2007). Given that increases in activity are associated with a substantial increase in O_2 demand, differences in MO_2 between weak regulators/conformers and strong regulators may simply reflect differences in behaviour. Mechanical digestion in decapod crustaceans is associated with a significant increase in MO_2 (McGaw and Penney 2014) and if postprandial animals can stop, slow or delay these mechanical processes it could potentially reduce energy expenditure. Finally, intracellular protein synthesis is a major contributor to the postprandial increase in MO_2 (Mente 2003; Secor 2009). However, very few articles (Whiteley et al. 2001; Mente et al. 2003) have investigated how the rate of protein synthesis is influenced by biotic and abiotic factors in postprandial crustaceans. Nonetheless, evidence suggests that postprandial crabs can slow protein synthesis during hypoxia (Mente et al. 2003), and if a similar ability is observed in low salinity, it could be indicative of a diversion of resources to processes associated with survival in low salinity.

MATERIALS AND METHODS

Experimental Animals and Holding Conditions

Adult, intermoult *Cancer irroratus* and male *Carcinus maenas* (both 50-150 g) were collected in North Harbour and Bay Bulls, Newfoundland, using baited crab traps. The crabs were transported to the Department of Ocean Sciences, Memorial University of Newfoundland, where they were held in tanks (32‰) supplied with flow-through SW at ambient temperature. Prior to experimentation, the animals were transferred to SW tables in the lab where they were acclimated to 15°C for at least 2 weeks under constant dim light. The crabs were fed shrimp and/or fish twice a week, but all crabs were fasted for at least 3 days prior to experimentation to ensure that the gut had been cleared and that the physiological effects of the previous meal would not influence experimental results (Wallace 1973).

Experimental Set-up and Protocols

Haemolymph Osmolality

The osmolality of the haemolymph was measured in both species (n = 8 each) to determine whether their osmoregulatory ability is affected by feeding. Individual crabs were held in perforated plastic storage containers (25 x 15 x 7.5 cm) and placed in a SW tank of recirculated, aerated water at 15°C adjusted to the desired salinity of 8, 16, 24 or 32‰. *C. irroratus* were not tested at 8‰ since preliminary experiments determined that this salinity was below the survivable range for this species. The crabs were allowed to acclimate to the

salinity for 2 days, after which they were fed a piece of shrimp of 2% their body mass (BM). Haemolymph (100 µL) was withdrawn from the base of a walking leg using a 1 mL syringe and 21 gauge needle prior to feeding to determine the osmolality (mmol/kg) of the fasted crabs, and then at 2 and 6 h after feeding to determine whether the haemolymph osmolality changes after feeding. This repeated sampling of small amounts of haemolymph does not affect the osmolality of crustaceans (Bamber and Depledge 1997). The osmolality of 10 µL of sample was measured using a vapour pressure osmometer (model Vapro 5520, Wescor Inc., Logan, Utah, USA). These values were compared against the osmolality of the sea water at the time the samples were collected.

Oxygen consumption

Oxygen consumption (mg O_2/kg/h) was measured using an L-DAQ intermittent flow respirometry system (Loligo systems, Tjele, Denmark). This fully automated system is equipped with two pumps connected to a cylindrical plexiglass® chamber (20 cm in diameter x 12 cm deep) that was submerged in a tank containing full strength SW. The first pump continually flushes SW from the tank through the chamber when MO_2 measurements are not being made. During the measurement period, the first pump is shut off and a second pump re-circulates water through the chamber at a rate of 10 L/min to ensure that O_2 gradients do not build up within the chamber. Oxygen consumption was calculated as the decline in O_2 levels during a 35 min period while the chamber was sealed, then the chamber was continuously flushed for 25 min between readings. Data were recorded on a Loligo data acquisition system (Tjele,

Denmark) which calculated MO_2 as mg O_2/kg/h. The experiments were carried out in constant dim light, which helped reduce any diurnal rhythms, and the tank containing the chambers was surrounded by black plastic sheeting to avoid visual disturbance to the animal. The crabs were left to acclimate to the respirometry chambers overnight. The resting metabolic rate (RMR; post-absorptive, minimal activity) was recorded for a 5 h pre-treatment control period, then the animals were subjected to an experimental treatment and MO_2 was recorded for an additional 48 h.

In the first series of experiments, following the 5 h pre-treatment period, the animals (n = 7-10 per species) were fed a piece of shrimp of 2% their BM and MO_2 was monitored until it returned to pre-feeding levels (i.e. for 48 h after feeding). Any animals that did not eat all the food they were offered were not used in the analyses. In a second series of experiments, respiratory responses to hyposaline exposure were measured in fasted individuals of both species (n = 10-11). In these experiments, the salinity was lowered to 24, 16 or 8‰ (75, 50 or 25% SW) by displacing the seawater in the tank with freshwater at 15°C. Salinity was monitored throughout the experiment with a multi-parameter meter (model 5200A, YSI, Yellow Springs, OH, USA) and did not vary by more than 0.5‰. *C. irroratus* were exposed to salinities of 24 and 16‰, while the *C. maenas* were exposed to 16 and 8‰. *C. maenas* were not tested at 24‰ as this salinity is not low enough to elicit a substantial behavioural response from this species (McGaw et al. 1999). In the final series of experiments, the animals (n = 7-10 per treatment) were subjected to low salinity once they had been fed. In these experiments, the change in salinity occurred at two different time points: a) 1 h after feeding was complete, but protein

synthesis would be just starting (Secor 2009); and b) 5 h after feeding when MO_2 had reached a maximum and protein synthesis rate would be increasing (Mente et al. 2003).

Several parameters were calculated using the data obtained from the MO_2 experiments: 1) the time to reach peak MO_2 after feeding; 2) the scope of the SDA response which was calculated as peak MO_2 divided by the resting MO_2; 3) the duration of the SDA response (i.e. until O_2 dropped back to pre-feeding levels); and 4) the SDA (or estimated energy expenditure, EEE) of each animal which is a measure of the total energy spent on digestion (and/or osmoregulation) and was calculated as the total increase in MO_2 above the RMR. This parameter is reported in units of kJ using the conversion factor of 1 mg O_2 = 0.014 kJ (Secor 2009). Each of these parameters were calculated from the MO_2 curve of each individual crab and the average was presented in a table for each experimental treatment.

Gastric Processing

The rate of gut contraction and the transit time of food through the gut was investigated to determine if low salinity exposure altered gastric processing rate in the crabs. Crabs (n = 8 per treatment) were placed in separate perforated plastic containers (15 cm long x 15 cm wide x 5 cm deep) within the holding tank in 32‰, and left overnight to settle. They were then fed a radio-opaque meal that was prepared by combining homogenized shrimp with electrolytic iron powder and gelatin in proportions of 65, 15 and 20%, respectively. The crabs were allowed to feed *ad libitum* for 1 h or until all the food was consumed before being transferred to a separate low salinity test tank.

The rate of contraction (contractions/min) of the pyloric sac and time (h) taken to clear the meal from each region of the gut (foregut, midgut and hindgut) was measured using a LIXI X-ray machine (LIXI PS-500; Carpentersville, IL, USA) and LIXI image processing software. The settings for the fluoroscope were a tube voltage of 35 kV, tube current set to 155 µA and a focal window of 5 cm. For X-ray analysis, the crabs were gently coaxed from the holding container into a water tight plastic container and transferred to the X-ray machine. Measurements were made on both species at 32‰ and in test salinities of 16 and 8‰ for *C. maenas*, and 24 and 16‰ for *C. irroratus*. A still image and a 10 s video were taken at hourly intervals for the first 6 h, every 2 h for the next 6 h, and then every 6 h until the gut had been cleared of the meal.

Digestive Efficiency

The digestive efficiency (DE) of each species of crab was calculated to determine if low salinity affected the absorption of nutrients from the meal. Individual crabs (n = 8 per treatment) were placed in plastic containers (25 cm long x 15 cm wide x 7.5 cm deep) with 1 mm mesh sides, and were offered a piece of shrimp of known mass and allowed to feed for 1 h. Following feeding, the remaining shrimp was removed and weighed to determine how much each individual consumed. The feces of each crab was collected daily over the following 3 days. The shrimp and feces were rinsed twice in distilled water to minimize salt crystallization from the seawater, and then dried in a 60°C oven for 48 h.

The caloric value (Cal/g) of approximately 0.1 g of food and feces was measured by direct calorimetry using a 6725 semi microcalorimeter (Parr Instrument Company, Moline, IL, USA) that had been calibrated with benzoic acid pellets. The apparent DE was calculated as,

$$\text{Digestive efficiency} = \frac{(\text{Ei})(\text{Mi}) - (\text{Ef})(\text{Mf})}{(\text{Ei})(\text{Mi})} \text{ x } 100\%$$

where Ei and Ef are the energy content (Cal/g) of the ingested food and feces, respectively, and Mi and Mf are the dry mass (g) of the ingested food and feces, respectively. The value for DE is considered to be 'apparent', as additional components of the crab's feces, such as mucus and sloughed off gut epithelium, will add to the energetic content of the feces. These will be present in the digested food and cannot be accurately accounted for (Johnson and Lillywhite 1979).

Protein Synthesis

The rate of protein synthesis was determined using the flooding dose method (Garlick et al. 1980) which has been used successfully for measurement of protein synthesis in *C. maenas* (El Haj and Houlihan 1987; Houlihan et al. 1990; Mente et al. 2003). However, since this method has not been used with *C. irroratus*, it was necessary to validate this technique in this species to ensure that: a) the concentration of intracellular phenylalanine (Phe) becomes elevated after injection; b) intracellular tritiated Phe remains elevated and stable over time; and c) there is a linear increase in the amount of the tritiated Phe incorporated into protein over time (Houlihan et al. 1990). Validating this technique was accomplished by measuring the concentration of

tritiated Phe in the leg, gills and hepatopancreas of 5 crabs at 0.5, 1 and 2 h after an injection of a solution containing 150 mmol/L of cold Phe with 100 µCi/mL of tritiated (L-2,3,4,5,6-^{3}H) Phe (Perkin Elmer, Inc., Waltham, MA, USA) in crab saline, and this solution was injected into crabs to deliver 1 µCi per gram of mass (Houlihan et al. 1990). The specific activity of the tritium in the intracellular free pool, as well as that incorporated into the protein of each tissue, was measured. To test criterion (a), the Phe level in the tissues of the 5 injected crabs was compared to that of 5 additional uninjected crabs to ensure that Phe had flooded the tissues (i.e. became elevated). Criteria (b) and (c) were tested by plotting a time course of tritium specific activity in the intracellular free pool vs. that incorporated into protein. The procedure for determining the rate of protein synthesis using the flooding dose method with tritiated Phe is described below.

The day before the experiment, crabs (n = 8 per treatment) were placed into separate perforated plastic containers (14 cm long x 14 cm wide x 7.5 cm deep) within their holding tank, and allowed to adjust to the containers overnight. The next day the containers with the crabs were transferred to another tank (with the same experimental parameters) to prevent contamination of lab equipment with tritium, and the crabs were left to settle in this tank for 1 h prior to experimentation.

In the first experiment, the crabs were given a meal of shrimp of 2% BM in 32‰ seawater and injected 7 h after feeding. In the second experiment, fasted crabs were transferred from 32‰ to 16‰ and injected 2 h after the transfer. For the final experiment, the crabs were fed shrimp of 2% BM in 32‰, then 5 h after feeding, they were transferred to 16‰ and injected 2 h after the transfer. These time points were selected to ensure that the rate of

protein synthesis would be measured at the same time period following the feeding/salinity treatment. At 1 h post injection, the crabs from each experimental group were removed from the tank, euthanized by destruction of the supraesophageal ganglion and then the 4th walking leg, gills and hepatopancreas were removed by dissection. These tissues were of interest because it has been shown that they have high rates of protein turnover in crustaceans (Mente et al. 2003), and there is comparable data for these tissues in other species of crustaceans (Mente et al. 2011). Further, the gills and hepatopancreas are of particular interest given their role in osmoregulation and digestion, respectively.

After collection, the tissues were gently blotted on a piece of paper towel, individually wrapped in a piece of pre-weighed tin foil, immediately frozen in liquid nitrogen and transferred to -80°C freezer until the analysis. The time between dissection of tissues and their subsequent freezing did not exceed 5 min.

To prepare samples for analysis, the tissues were removed from the -80°C freezer and kept on dry ice. They were weighed and ground up in liquid nitrogen with a precooled, sterilized mortar and pestle. A cooled, 1.8 mL Eppendorf tube was filled with the resulting tissue powder to be used for scintillation counting, and Phe and protein assays. Another cooled Eppendorf tube was filled with 0.25 mL of additional powder for measurement of RNA concentration.

For scintillation counting, Phe and protein assays, a subsample of tissue powder was placed into a pre-weighed test tube and weighed to determine the amount of sample. Two mL of 6% perchloric acid (PCA) was added to the tissue powder, which was then vortexed and allowed to sit on ice for 15 min. After a brief remixing, 1 ml of the tissue/PCA mix was

transferred into a separate Eppendorf tube and centrifuged at 5600 *g* for 5 min at room temperature (RT). The supernatant, containing the free pool Phe, was collected and the pellet which contained Phe incorporated into protein was washed twice by resuspending it in 1 mL of 6% PCA and centrifuging again for 5 min. The resulting supernatant was discarded. Finally, 1 mL of 0.3 M sodium hydroxide (NaOH) was added to the pellet and the samples were incubated at 37°C until the protein pellet was completely digested. The free pool and protein fractions were stored at -20°C until the assays were performed.

Scintillation counting was performed by placing 250 µL of the free pool or digested protein samples into scintillation vials with 10 mL of Ecolume scintillation cocktail (MP Biomedicals, Santa Ana, CA, USA) and measuring radioactivity as counts per minute (CPM) using a Packard Tri-carb 2100TR liquid scintillation counter (Perkin Elmer Life Sciences Inc., Boston, MA, USA). Counts per minute were converted to disintegrations per minute (DPM) by dividing the CPM by the counter's efficiency (64%).

The Phe concentration in the free pool was determined by fluorimetric assay using Phe assay kits (Abcam, Cambridge, UK), and the protein concentration in the digested pellets was determined using a Bradford protein assay kit (Thermo Scientific, Waltham, MA, USA) with bovine serum albumin used as a standard. The rate of protein synthesis was expressed as the incorporation rate (in nmol Phe/mg protein/h) of tritiated Phe into the tissues and was calculated as,

$$Incorporation\ rate = \frac{DPM_b}{s_a}\ x\ \frac{1}{protein}\ x\ \frac{1}{t}$$

where DPM_b is the radioactivity of tritiated Phe in the protein fraction, s_a is the specific activity (DPM/nmol) of the free pool fraction, *protein* refers to the total protein (mg) in the entire tissue and *t* is the incubation time in h (Lewis and Driedzic 2007).

Increases in the rate of protein synthesis are thought to be achieved through increases in RNA activity, where an increase in the production of protein can be accomplished through an increase in the amount of RNA available for synthesising that protein (MacMillan and Houlihan 1989; Fraser and Rogers 2007). For this reason, it is important to consider RNA content in studies that measure and anticipate a change in the rate of protein synthesis since a decrease in the rate of protein synthesis may result from a lower RNA content.

RNA was extracted from 0.25 mL of powdered tissue using a modified version (Treberg et al. 2005; Lewis and Driedzic 2007) of the single step method for RNA isolation (Chomczynski and Sacchi 1986) with Trizol reagent (Invitrogen, Life Technologies, Burlington, ON, Canada). Trizol (800 µL) was added to the tissue powder, and this solution was transferred to a QIAshredder (QIAGEN, Venlo, Netherlands) column and centrifuged at 12,000 *g* for 2 min at RT. Following centrifugation, the column was discarded and the tubes containing the samples were centrifuged again to remove any remaining insoluble material. The resulting supernatant was placed into an Eppendorf tube, an additional 200 µL of Trizol was added, and the sample was incubated at RT for 5 min before 200 µL of chloroform was added. Then, the samples were shaken manually, incubated for 3 min at RT and then centrifuged at 12000 *g* for 15 min at 4°C. Following centrifugation, the samples had separated into 3 phases. The aqueous phase (containing RNA) was removed by pipette and put into a new tube, and 500 µL of isopropanol was added to precipitate the RNA. The sample was then mixed, and incubated at RT for 10 min

before being centrifuged at 12000 *g* for 10 min at 4°C. The resulting supernatant was removed and the RNA pellet was washed with 1 mL of 75% ethanol. This was done by mixing the RNA with the ethanol by gentle inversion, and then centrifuging at 7600 *g* for 1 min at 4°C. The supernatant was discarded and the RNA pellet was air dried for 10 min. Afterwards, 100 µL of nuclease free water was added to the pellet and the sample was heated at 55°C for 15 min with the pellet being resuspended by pipetting after the first 5 min. The samples were stored at -80°C until analysis. The concentration of RNA was measured using a NanoDrop spectrophotometer (NanoDrop 1000, Thermo Scientific, Waltham, MA, USA) and the RNA content was expressed as the RNA (µg) per mg of tissue.

Behaviour

The behaviour of crabs was observed to determine whether increases or decreases in locomotor activity may have influenced the metabolic rate of postprandial crabs in a hyposaline environment. Individual crabs (n = 8) of both species were fed a piece of shrimp of 2% BM in the holding tank. Once the meal was consumed, individual animals were placed into a raceway (90 cm x 18 cm) in an experimental tank containing seawater of 32, 24, 16 or 8‰ (*C. maenas* was not tested at 24‰, and *C. irroratus* was not tested at 8‰). The raceway was marked at every 15 cm interval with a piece of black tape. A sheet of clear acrylic was placed on top of the raceway to prevent the crabs from escaping, and a lamp was suspended over the tank so that lighting was equal which reduced the shaded areas in the lanes where the animals could potentially retreat to. A time lapse video system (GoPro® HERO3+; GoPro Inc., CA, USA) was

used to record the activity levels of the crabs. Activity was recorded as movements per h (i.e. the number of times a crab passed over a piece of black tape per h) for a total period of 8 h.

Statistical Analyses

The characteristics of the SDA (i.e. RMR, peak MO_2, time to peak MO_2, duration, scope of SDA, SDA/EEE, C_{SDA}) were compared within species using student's t-tests. These comparisons were as follows: fasted crabs in 16‰ vs. fasted crabs in 8‰ (or 24‰); crabs fed then transferred to 16‰ after 1 h vs. those fed and transferred to 8‰ (or 24‰) after 1 h; and crabs fed then transferred to 16‰ after 5 h vs. those fed and transferred to 8‰ (or 24‰) after 5 h. Student's t-tests were also used to compare each treatment between species.

A 2-way repeated measures (RM) ANOVA was performed on the MO_2, haemolymph osmolality (fed and fasted crabs), gut contraction and behaviour/activity data for each species. In these 2-way RM ANOVAs, the measurement taken over time (i.e. MO_2, osmolality, contractions, or activity) was used as the RM factor with the other factor being salinity. In another series of RM ANOVAs, species was used as the second factor to test for differences between species within a salinity (32‰ or 16‰). The ANOVAs were followed by Fisher's LSD post hoc tests where needed. For the protein synthesis validation measurements, the free pool levels were tested with a 1-way RM ANOVA, and the incorporation of tritiated Phe into protein was tested with a linear regression on $\log_{10}$ transformed data.

Digestive efficiency and the gut clearance times were analysed using a 1-way ANOVA with salinity or species used as the factor. A 2-way ANOVA with species and salinity as factors

was not possible with these data sets since each species was not tested at each level of salinity (i.e. *C. maenas* was not tested at 24‰, and *C. irroratus* was not tested at 8‰). The protein synthesis data (i.e. incorporation rate and RNA content) for each tissue was analysed using a 2-way ANOVA with experimental treatment and species used as the factors. These ANOVAs were followed by Fisher's LSD or Dunn's post-hoc tests.

All statistical analyses were performed using Prism (Version 5.0, GraphPad Software Inc., La Jolla, CA, USA) or SigmaPlot (Version 11.0, Systat Software, Inc., San Jose, CA, USA) with the level of significance set at $p < 0.05$. The values presented in the text, figures and tables are means ± 1 standard error (S.E.).

RESULTS

Haemolymph Osmolality

In 32‰, fasted *C. maenas* and *C. irroratus* had a haemolymph osmolality that was similar to that of the SW (approximately 900 mmol/kg). Both species were able to maintain their osmolality above that of the water when in salinities below full strength SW (i.e. iso-osmotic line). *C. maenas* had a significantly higher haemolymph osmolality than *C. irroratus* in 24‰ (822.9 ± 12.1 vs. 724.9 ± 2.8 mmol/kg, respectively) and in 16‰ (674.6 ± 25.0 vs. 524.9 ± 10.5 mmol/kg, respectively) (2-way RM ANOVA: df=1, F=42.9, p<0.001) (Figure 1). The haemolymph osmolality of *C. maenas* in 8‰ was 557.6 ± 20.7 mmol/kg; haemolymph samples were not collected from *C. irroratus* in 8‰ because they did not survive exposure to this salinity. The haemolymph osmolality was also measured in both species at 2 and 6 h after feeding in each salinity to check for any effects of feeding on haemolymph osmolality, but no

significant differences occurred in the osmolality of *C. maenas* (2-way RM ANOVA: df=2, F=3.4, p=0.06) or *C. irroratus* (2-way RM ANOVA: df=3, F=3.5, p=0.05) at any of the time points tested (Figure 2A, B).

Oxygen Consumption

The RMR of both *C. maenas* and *C. irroratus* in the various experiments ranged between 40.1 ± 3.5 and 57.5 ± 5.6 mg O_2/kg/h and did not change significantly during the 5 h pre-treatment period (2-way RM ANOVA *C. maenas:* df=6, F=0.9, p=0.5; *C. irroratus:* df=6, F=1.5, p=0.2). The RMR also did not differ between the species in any of the experimental treatments: fed in 32‰ (t-test: t=0.3, p=0.8), fasted in 16‰ (t-test: t=0.6, p=0.6), fed with 16‰ after 1 h (t-test: t=1.4, p=0.2), or fed with 16‰ after 5 h (t-test: t=0.6, p=0.6) (Table 1 and 2).

The MO_2 response to feeding in 32‰ was similar for the two species (2-way RM ANOVA: df=1, F=0.0006, p=0.9) (Figure 3). When the crabs were fed a piece of shrimp at 2% their BM, MO_2 rose sharply reaching peak values of 123.1 ± 5.4 and 119.0 ± 15.0 mg O_2/kg/h for *C. maenas* and *C. irroratus*, respectively. The MO_2 declined gradually thereafter reaching pre-feeding levels after 17.4 ± 1.2 h for *C. maenas* and 20.3 ± 3.4 h for *C. irroratus* (Table 1 and 2, Figure 3). There was no significant difference between the two species with regard to the timing of peak MO_2 (t-test: t=0.2, p=0.9), peak MO_2 (t-test: t=0.3, p=0.8), scope of SDA (t-test: t=0.8, p=0.4), duration of SDA (t-test: t=0.9, p=0.4), or SDA (t-test: t=0.1, p=0.9) (Table 1 and 2).

When fasted crabs were given a low salinity challenge of 24, 16 or 8‰, MO_2 increased and remained elevated for the duration of the hyposaline exposure period (Figures 4A and 5A). In *C. maenas*, there was no difference in the overall pattern of MO_2 of fasted crabs exposed to

8‰ and 16‰ (2-way RM ANOVA: df=1, F=4.2, p=0.05) (Figure 4A). Further, there were no significant differences in the time to reach peak MO_2 (t-test: t=2.0, p=0.06), the scope of the response (t-test: t=1.2, p=0.2) or EEE (t-test: t=1.3, p=0.2). However, peak MO_2 (t-test: t=2.2, p=0.04) was significantly higher for fasted *C. maenas* in 8‰ compared to those in 16‰ (Table 1). In contrast, the MO_2 of fasted *C. irroratus* exposed to a salinity of 16‰ reached significantly higher levels than it did for 24‰ (2-way RM ANOVA: df=1, F=7.1 p=0.02) (Figure 5A). This resulted in an EEE that was over 3 times higher in 16‰ (t-test: t=3.9, p=0.001) (Table 2). The scope of change in MO_2 (t-test: t=2.3, p=0.03) and peak MO_2 were also higher in fasted individuals in 16‰ (t-test: t=2.2, p=0.04), but there was no significant difference in time to reach peak MO_2 (t-test: t=1.7, p=0.1) (Table 2).

A comparison of the MO_2 of both species in 16‰ while fasting (Figure 6B) showed that the peak MO_2 and the scope of MO_2 response did not differ significantly between species (t-test: t=0.5, p=0.6, and t-test: t=0.5, p=0.6, respectively). However, *C. irroratus* took an additional 14 h to reach peak MO_2 (t-test: t=3.5, p=0.002) and EEE was also higher for this species (t-test: t=2.4, p=0.03) (Table 1 and 2).

When *C. maenas* was exposed to low salinities of 8‰ and 16‰ 1 h after feeding, the increase in MO_2 as a result of the hyposaline exposure was additive to the initial increase in MO_2 associated with feeding (Figure 4B). The pattern of the MO_2 in 8‰ was significantly different when compared to 16‰ (2-way RM ANOVA: df=1, F=5.1, p=0.04) (Figure 4B). However, no differences were found in the peak MO_2 (t-test: t=1.2, p=0.2), time to reach peak MO_2 (t-test: t=0.4, p=0.7), the scope of the MO_2 response (t-test: t=0.3, p=0.8) or EEE (t-test: t=0.7, p=0.5) with this treatment (Table 1). A similar pattern was observed in *C. maenas* when

salinity was lowered to 8‰ and 16‰ 5 h after feeding, but the pattern of MO_2 was comparable for both salinities (2-way RM ANOVA: df=1, F=0.9, p=0.4) (Figure 4C). There was no difference in the peak MO_2 (t-test: t=0.6, p=0.6), time to reach peak MO_2 (t-test: t=0.6, p=0.5), scope of change in MO_2 (t-test: t=0.6, p=0.6) or EEE (t-test: t=1.5, p=0.2) in this species when salinity was lowered 5 h after feeding (Table 1).

A different pattern was observed in the MO_2 of postprandial *C. irroratus* when salinity was lowered 1 h after feeding in that no increase in MO_2 was observed when salinity was lowered to 24‰. Instead, MO_2 decreased gradually following the salinity change, reaching 97.4 ± 11.3 mg O_2/kg/h by the end of the experimental period (Figure 5B). When salinity was lowered to 16‰ 1 h after feeding, *C. irroratus* responded with a slow increase, reaching peak values of 189.6 ± 21.8 mg O_2/kg/h 19.0 ± 4.7 h after feeding (Table 2, Figure 5B). This resulted in significant differences between the two salinity treatments from 27 h onward (2-way RM ANOVA: df=1, F=5.0, p=0.04) (Figure 5B). The scope of the change in MO_2 and EEE in 16‰ were also significantly higher (t-test: t=2.9, p=0.009 and t=5.3, p<0.0001, respectively), but no significant differences were found in the peak MO_2 (t-test: t=1.4, p=0.2) or time to peak MO_2 (t-test: t=1.9, p=0.07) (Table 2). When *C. irroratus* was exposed to 24‰ 5 h after feeding, MO_2 did not change (Figure 5C). However, when the salinity was lowered to 16‰ 5 h after feeding, *C. irroratus* responded with a slow, gradual increase in MO_2 reaching a peak level of 138.5 ± 12.0 mg O_2/kg/h 21 h after the salinity was lowered, and MO_2 remained elevated between 101.0 ± 12.8 to 127.1 ± 11.7 mg O_2/kg/h thereafter (Figure 5C). There were no significant differences in the overall pattern of MO_2 of *C. irroratus* when comparing 24‰ and 16‰ 5 h after feeding (2-way RM ANOVA: df=1, F=1.1, p=0.3) (Figure 5C). Further, there were no differences between

the peak MO_2 (t-test: t=1.2, p=0.3), time to peak MO_2 (t-test: t=1.0, p=0.3) or scope of change in MO_2 (t-test: t=0.5, p=0.6) between these salinities. However, the EEE was significantly higher in 16‰ as compared to 24‰ (t-test: t=2.8, p=0.02) (Table 2).

A comparison of the MO_2 response of *C. maenas* and *C. irroratus* upon exposure to 16‰ at 1 h and 5 h after feeding showed that there was no difference between the species (2-way RM ANOVA: df=1, F=2.9, p=0.1 and df=1, F=0.9, p=0.4, respectively) (Figure 6A, B). However, the interaction between species and time was significant for both treatments (2-way RM ANOVA 1h: df=44, F=26.0, p<0.001 and 5h: df=44, F=3.1, p<0.001). When salinity was lowered to 16‰ 1 h after feeding, differences in MO_2 were detected 27 h after the start of the experiment. From this point onward, the MO_2 of *C. maenas* continued to decline while that of *C. irroratus* remained elevated (Figure 6C). When salinity was lowered 5 h after feeding, significant differences occurred between 11 and 17 h: *C. maenas* responded to the low salinity with an immediate increase in MO_2 followed by a slower decrease over the following 28 h, whereas *C. irroratus* responded with a slower, more gradual increase in MO_2 following the salinity changeover that remained elevated for the entire period (Figure 6D). Despite the differences in the pattern of change in MO_2, there were no differences in the scope of MO_2 change between the two species in either experimental treatment (t-test 1h: t=2.1, p=0.05 and 5h: t=1.3, p=0.2) or EEE when salinity was lowered 5 h after feeding (t-test: t=0.3, p=0.7). However, EEE was significantly different between the species when salinity was lowered 1 h after feeding (t-test: t=3.4, p=0.003), and *C. irroratus* took longer to reach peak levels of MO_2 in both treatments (t-test 1h: t=2.4, p=0.03 and 5h: t=3.1, p=0.008) (Tables 1 and 2).

Gastric Processing

There was no significant difference in the contraction rate of the pyloric sac of *C. maenas* in any of the test salinities (32, 16 and 8‰) (2-way RM ANOVA: df=2, F=0.4, p=0.7). Initial contraction rates in each of the test salinities ranged between 47.8 ± 5.6 to 65.3 ± 4.1 contractions/min, and showed an apparent decrease during the first few h of the experiment. However, this trend was not statistically significant (2-way RM ANOVA: df=9, F=0.08, p=1.0) (Figure 7A).

Pyloric contraction rates were more variable (from 40.5 ± 3.7 to 57.0 ± 4.7 contractions/min) in *C. irroratus,* but there were no significant differences in contraction rate as a function of salinity (2-way RM ANOVA: df=2, F=2.2, p=0.1) (Figure 7B). When the rate of pyloric contraction was compared between the two species, no significant difference occurred between them at salinities of 32‰ or 16‰ (2-way RM ANOVA: df=1, F=0.7, p=0.5; and df=1, F=1.0, p=0.4; respectively) (Figure 7C and 7D).

Salinity did have a significant effect on the transit time of the meal through the gut of *C. maenas* (1-way ANOVA: df=2, F=5.6, p=0.01). The foregut was cleared in 24.2 ± 3.1 h in 16‰ and this was significantly longer than the 12.5 ± 1.8 h taken to clear the foregut in 32‰. In 8‰, the foregut was cleared in 19.4 ± 2.1 h but this was not significantly different from times recorded in 32‰ or 16‰. Low salinity did not affect the transit time through the midgut of *C. maenas*. This region was cleared between 26.3 ± 11.9 and 50.1 ± 6.9 h (1-way ANOVA: df=2, F=2.6, p=0.1). Finally, low salinity did have a significant effect on the transit time through the hindgut (1-way ANOVA: df=2, F=3.8, p=0.04). In 16‰, the hindgut was cleared in 56.3 ± 5.3 h and this was significantly longer than for crabs in 32‰ (29.8 ± 11.4 h). In a salinity of 8‰, the

hindgut was cleared in 34.2 ± 4.7 h, but this was not statistically different than the times recorded in 32‰ and 16‰ (Figure 8A).

In *C. irroratus*, the foregut was cleared in 15.4 ± 2.8 h in 32‰ and 22.9 ± 3.3 h in 24‰ but these values were not significantly different from one another. In 16‰, the foregut was cleared in 45.2 ± 9.0 h and this was significantly longer than the time taken in both 32‰ and 24‰ (1-way ANOVA: df=2, F=8.7, p=0.002). The clearance time for the midgut was 27.5 ± 5.3 h in 32‰ and 35.1 ± 2.4 h in 24‰. These values were not significantly different, but the clearance time for the midgut in 16‰ was over twice as long (1-way ANOVA: df=2, F=18.7, p<0.001). Lastly, *C. irroratus* cleared the hindgut in 32‰ and 24‰ in a similar amount of time (36.8 ± 6.1 and 37.4 ± 2.4 h, respectively), but it took significantly longer (over 2-fold) to pass the meal through the hindgut in 16‰ (79.2 ± 10.0 h) (1-way ANOVA: df=2, F=13.6, p<0.001) (Figure 8B).

There were no significant differences in transit times between the two species in full strength SW (1-way ANOVA: foregut: df=1, F=0.8, p=0.4; midgut: df=1, F=0.009, p=0.9; hindgut: df=1, F=0.3, p=0.6) (Figure 8C). However, *C. irroratus* took longer than *C. maenas* to pass the meal through each region of the gut in 16‰ (1-way ANOVA foregut: df=1, F=6.6, p=0.02; midgut: df=1, F=5.6, p=0.03; hindgut: df=1, F=4.9, p=0.04) (Figure 8D).

Digestive Efficiency

Digestive efficiency was greater than 90% for both species in all salinities. Low salinity did not have a significant effect on DE in *C. maenas* (1-way ANOVA: df=2, F=3.1, p=0.07) (Figure 9A), but it did have a significant effect on the DE in *C. irroratus* (1-way ANOVA: df=2, F=6.2,

p=0.007). The DE for *C. irroratus* in 32‰ and 24‰ was similar (95.3 ± 0.4% and 95.6 ± 0.8%, respectively), while the DE in 16‰ (98.1 ± 0.4%) was significantly higher than that measured in the two other salinities (Figure 9B). The DE of *C. maenas* and *C. irroratus* in 32‰ was similar (1-way ANOVA: df=1, H=2.1, p=0.2), while in 16‰ DE was significantly higher for *C. irroratus* (94.7 ± 1.0% vs. 98.1 ± 0.4%, respectively) (1-way ANOVA: df=1, H=6.8, p= 0.009) (Figure 9C).

Protein Synthesis

The flooding dose method has been validated for *C. maenas* in earlier studies (El Haj and Houlihan 1987; Houlihan et al. 1990; Mente et al. 2003). The flooding dose method was validated for *C. irroratus* in the present study to ensure that: a) the concentration of intracellular Phe becomes elevated after injection; b) intracellular tritiated Phe remains elevated and stable over time, and c) there is a linear increase in the amount of the tritiated Phe incorporated into protein over time (Houlihan et al. 1990). The concentration of Phe (nmol/µL) in the leg, gill and hepatopancreas rose approximately 8-13 fold by 0.5 h as compared to the Phe levels of uninjected crabs, and the specific activity of the Phe in the extracellular free pool maintained a stable level and did not change over the test period (1-way RM ANOVA leg: df=2, F=0.3, p=0.8; gill: df=2, F=1.1, p=0.4; hepatopancreas: df=2, F=0.02, p=0.9) (Figure 10A). In addition, the amount of Phe that was incorporated into the protein of each tissue increased linearly with time in the leg and hepatopancreas (p=0.03 and p=0.01, respectively). The increase in the incorporation of tritium was not significant in the gill (p=0.09) but this is likely due to variation in the data (Figure 10B).

The experimental treatment (i.e. fed in 32‰, fed in 16‰ and fasted in 16‰) did not have a significant effect on the rate of protein synthesis in the leg, gill or hepatopancreas of the crabs (2-way ANOVA leg: df=2, F=0.7, p=0.5; gill: df=2, F=1.9, p=0.2; hepatopancreas: df=2, F=0.2, p=0.8). However, species did have a significant effect on the rate of protein synthesis in the leg (2-way ANOVA: df=1, F=8.8, p=0.005) where fed *C. maenas* in 32‰ had higher rates of protein synthesis as compared to *C. irroratus* (0.08 ± 0.01 vs. 0.01 ± 0.003 nmol Phe/mg protein/h) (Figure 11A). In contrast, *C. irroratus* had higher rates of protein synthesis in the gill (2-way ANOVA: df=1, F=4.4, p=0.04) with a significant difference occurring between fed crabs in 16‰ (0.02 ± 0.005 vs. 0.007 ± 0.001 nmol Phe/mg protein/h) (Figure 11B). A similar pattern of protein synthesis was observed in the hepatopancreas (2-way ANOVA: df=1, F=6.6, p=0.01), again, with the greatest difference occurring between fed crabs in 16‰ (0.09 ± 0.01 vs. 0.2 ± 0.09 nmol Phe/mg protein/h) (Figure 11C).

RNA content in the leg ranged between 11.6 ± 2.1 and 18.2 ± 2.4 µg/mg tissue (Figure 12A) and between 28.1 ± 4.6 and 63.8 ± 23.7 µg/mg tissue in the gills (Figure 12B). Tissue RNA content was highest in the hepatopancreas of both species, with values ranging from 376.8 ± 158.0 to 929.1 ± 350.3 µg/mg tissue (Figure 12C). No significant difference was found between the RNA content of the legs, gills or hepatopancreas as a function of experimental treatment (2-way ANOVA leg: df=2, F=0.8, p=0.4; gill: df=2, F=1.3, p=0.3; hepatopancreas: df=2, F=1.3, p=0.3) (Figure 12). Finally, there were no significant differences in RNA content for any of the tissues between the two species (2-way ANOVA leg: df=1, F=0.05, p=0.8; gill: df=1, F=1.8, p=0.2; hepatopancreas: df=1, F=0.3, p=0.6) (Figure 12).

Behaviour

The activity levels of *C. maenas* in 32‰ and 16‰ were similar to one another, whereas activity in 8‰ was, for the most part, 2-3 fold higher than in 32‰ and 16‰ (2-way RM ANOVA: df=6, F=6.1, p<0.001) (Figure 13A). Low salinity also had a significant effect on the activity of *C. irroratus* (2-way RM ANOVA: df=3, F=11.3, p<0.001) (Figure 13B). In this species, activity levels were similar in 32‰ and 24‰, reaching maximal values of 92.8 ± 32.1 and 126.3 ± 28.2 movements/h, respectively, whereas activity rates were higher during the first 5-6 h of exposure to 16‰. The maximal level of activity reached 243.1 ± 52.2 movements/h before it declined to levels similar to those measured in 24‰ and 32‰. Like *C. maenas*, *C. irroratus* was most active at the beginning of the experiment and activity declined with time (2-way RM ANOVA: df=6, F=8.2, p<0.001) (Figure 13B).

Activity levels over time were similar between the two species in 32‰ (2-way RM ANOVA: df=1, F=3.6, p=0.08) with the exception of 2 h and 3 h where *C. irroratus* was more active than *C. maenas* (Figure 13C). In contrast, the activity of *C. irroratus* in 16‰ was twice as high during the first 5 h of exposure to 16‰ (2-way RM ANOVA: df=1, F=70.3, p<0.001). Thereafter, the activity rate of *C. irroratus* declined to a level similar to that of *C. maenas* (67.1 ± 20.9 and 35.8 ± 12.7 movements/h, respectively) (Figure 13D).

DISCUSSION

In order to take advantage of the abundance of food often found in the intertidal zone and estuaries, crabs must be able to withstand the changes in salinity that are typical of these habitats. *Carcinus maenas* is classified as an efficient osmoregulator and is capable of

withstanding a wide range of salinities (from 5-52‰; Broekhuysen 1936; Klassen and Locke 2007). In contrast, *Cancer irroratus* has been classified as a weak regulator (Thurberg et al. 1973; Charmantier and Charmantier-Daures 1991), and although they are reported to survive salinities as low as 8.5‰ (Charmantier and Charmantier-Daures 1991), high mortality rates are observed in salinities of 16‰ (Cantelmo et al. 1975). Acute exposure to low salinity resulted in an increased O_2 demand and this was reflected by a rapid and sustained elevation of MO_2 in both species. This rise in MO_2 represents the increased energy demand of processes associated with maintaining the osmotic pressure of their internal milieu, such as the upregulation and synthesis of ion transporting ATPases and amino acid catabolism (Gilles 1973; Pequeux 1995; Piller et al. 1995). Energy demand also rises due to the increased locomotor activity of the crabs in an attempt to escape unfavourable salinities; this is termed halokinesis (Taylor et al. 1977; Taylor and Naylor 1977; Thomas et al. 1981).

Consuming and digesting a meal is another energetically demanding process and the postprandial metabolic increase associated with digestion is termed 'specific dynamic action'. In crustaceans, this metabolic response results in a 2-4 fold increase in MO_2 which can remain elevated for up to 3 days (Whiteley et al. 2001). This increase in MO_2 arises from the energy demands associated with acquisition and mastication of a meal, enzymatic breakdown, nutrient uptake and transport, and the subsequent intracellular protein synthesis (Secor 2009). The patterns of MO_2 following ingestion of the meal as well as exposure to low salinity were similar for both species. However, the pattern of MO_2 differed between the two species when they encountered episodes of low salinity after feeding. *C. maenas* reacted with an immediate addition of the effects of both processes. In contrast, there appeared to be a delay in the

additive effect of digestion and salinity stress in *C. irroratus* resulting in a more gradual increase in MO_2 after hyposaline exposure. Differences in postprandial patterns of MO_2 have also been observed in other species of crabs when encountering low salinity (Curtis and McGaw 2010): *Callinectes sapidus* (an efficient osmoregulator) will add the increase in MO_2 associated with low salinity upon those associated with digestion. In contrast, postprandial *Cancer magister* (a weaker regulator) will exhibit a sustained decrease in MO_2 when exposed to low salinity, suggesting a prioritization of the low salinity response over digestion (Curtis and McGaw 2010). The inability of *C. irroratus* to immediately combine the MO_2 of both digestion and salinity stress is not due to a limitation in aerobic scope because decapods can increase their MO_2 by 5-14 fold during vigorous exercise (Houlihan et al. 1984; McGaw 2007). This suggests that some aspect of osmoregulatory physiology influences the crabs' ability to balance the demands of both physiological processes. The ingestion and the subsequent digestion of the meal did not have an effect on the osmoregulatory ability of either *C. maenas* or *C. irroratus* in dilute seawater. These data support the idea that the differences in MO_2 between the two species likely stem from a change in some aspect of their digestion in low salinity rather than a compromise to their ability to maintain the osmolality of their extracellular fluids.

Once decapod crustaceans ingest food, it is passed into the foregut where the gastric mill cuts and grinds the material. Food can be processed in the foregut for up to 18 hours before it is passed into the midgut region (McGaw and Curtis 2013). Previous work on isopods suggests that this mechanical digestion only comprises a low percentage (<5%) of the overall SDA budget (Carefoot 1990). However, recent work shows that, for decapods, the contribution of mechanical processes to the overall SDA is much more substantial (McGaw and Penney

2014). This is not unexpected since the gastric mill is controlled by over 40 muscles which are innervated by the complex stomatogastric ganglion (McGaw and Curtis 2013). Although there were no statistically significant differences in the rate of pyloric sac contraction between *C. maenas* and *C. irroratus*, there was a trend of higher contraction rates in *C. maenas* that slowed over time, and reduced levels of pyloric contraction in *C. irroratus* that increased over time. The contraction of the pyloric sac moves food along the midgut and into the hindgut, and so directly influences the transit rate of food through the digestive tract (McGaw and Curtis 2013). *C. irroratus* took longer to move the meal through their digestive tract in low salinity compared to *C. maenas* which agrees with the trends of pyloric sac contraction rates. In addition, this difference in transit rate is reflected in the MO_2 of both species in that MO_2 increased rapidly in *C. maenas*, but was delayed for several hours in *C. irroratus*. Links between increases in MO_2 and mechanical digestion in decapods have also been suggested by Mente et al. (2003).

The difference in mechanical digestion between the two species of crabs may potentially be explained through the differences in cell volume regulation. During exposure to low salinity there is usually a rapid increase in body volume caused by the osmotic uptake of water (Willmer et al. 2004). Some of this extracellular water onload is accommodated in the coelom or gut (Gnanamuthu 1966; Ellington and Lawrence 1974; Pequeux 1995) and may compromise gut contraction and function. Also, efficient regulators, like *C. maenas*, can respond rapidly to low salinities with a decrease in water permeability (Smith 1970; Spaargaren 1975) and an increase in urine production (Binns 1969; Norfolk 1978; Harris and Andrews 1982). At the cellular level, crabs will regulate their cell volume by adjusting their intracellular osmolality through the use of organic osmolytes, like amino acids, and this has been seen in

previous studies on both *C. maenas* (Seibers et al. 1972; Henry et al. 2002) and *C. irroratus* (Moran and Pierce 1984). However, osmoconformers and weak regulators are less efficient at regulating cell volume (Freire et al. 2008) and there are differences in the time taken to re-establish the water balance (Pierce 1982). For example, a rapid increase in the concentration of amino acids occurs in the blood of *C. maenas* when salinity is lowered to 10‰ (Henry et al. 2002). In contrast, when *C. irroratus* is transferred from full strength seawater to 60% seawater, the efflux of free amino acids from the cell into the blood does not occur until 6 hours after transfer (Moran and Pierce 1984). This prolonged efflux of amino acids agrees with the lag in the increase of MO_2 (which was approximately 10 hours) observed in *C. irroratus* in this study. This suggests that the delay in gastric processing may be related to the time taken for this species to adjust its intracellular volume before gastric processing can resume. Indeed, the fact that haemolymph osmolality of *C. irrorratus* dropped to a greater extent suggests a compromised mechanism of coping with osmotic stress, and the challenge of cell volume regulation may have compromised the digestive system of *C. irroratus*, thus slowing mechanical digestion and reducing MO_2.

Digestive efficiency was high (>90%) for both species, which is typical for crustaceans (Romero et al. 2006; Urbina et al. 2010; Curtis et al. 2013). This supports the hypothesis that digestive function was merely delayed in *C. irroratus* rather than a more prolonged shutdown of gastric processes as occurs in the osmoconformer, *Cancer gracilis*, during hyposaline exposure (McGaw 2006a). The digestive efficiency of *C. irroratus* in 16‰ was slightly higher than *C. maenas* and this is probably due to the higher residence time of the meal in the gut allowing more time for nutrients to be absorbed (Le Vay et al. 2001; Mitra and Flynn 2007). However,

this difference was very small (<2%), and in biological terms was unlikely to have contributed much, if anything, to overall metabolic differences.

Once the meal reaches the midgut, the liquid portion of the digesta enters the hepatopancreas where it continues to be broken down by digestive enzymes before the nutrients are absorbed by the R cells (Hopkin and Nott 1980; McGaw and Curtis 2013). These substrates are then transported to the body's cells, and the rate of protein synthesis increases and reaches peak levels within 3-5 hours (Houlihan et al. 1990; Mente et al. 2003). It has been widely suggested that this increase in intracellular protein synthesis accounts for a significant portion of the postprandial increase in MO_2 (Houlihan et al. 1990; Mente et al. 2003; McCue et al. 2005; Secor 2009). The delay in gastric processing in *C. irroratus* in 16‰ was expected to have downstream effects, reducing the availability of substrates for protein synthesis, and thus, accounting for the decrease in MO_2. However, the rate of protein synthesis did not differ in *C. irroratus* when fed in 32‰ and 16‰. After feeding, it took 2-2.8 hours for the meal to pass from the foregut of *C. irroratus* to the midgut (*unpublished observation*). Nutrient absorption, and an increase in the rate of protein synthesis, would have already begun in these crabs within the 5 hour period between feeding and the lowering of salinity (Houlihan et al. 1990; Mente et al. 2003). An increase in the rate of protein synthesis would have been well underway before gastric processing began to slow down, and these results suggest that once the rate of protein synthesis has increased, it may not be affected by hyposaline exposure.

Overall, protein synthesis rates were higher in *C. irroratus* compared to *C. maenas* with the exception of the leg. This might be explained by the use of organic osmolytes (i.e. amino acids) to aid in maintaining the osmotic pressure of their internal body fluids (Moran and Pierce

1984; Pequeux 1995; Henry et al. 2002). The efflux of amino acids from the cell during hyposaline stress can reduce the rate of protein synthesis (Pierce 1982). *C. maenas* appears to be more efficient at cell volume regulation compared with *C. irroratus* (Moran and Pierce 1984; Henry et al. 2002), and if *C. maenas* responds to low salinity with a more rapid cellular efflux of osmolytes, then this would quickly reduce the amount of available amino acids (including phenylalanine) and subsequently lower the rate of protein synthesis. Nevertheless, protein synthesis rate might be expected to be higher in the gills of *C. maenas* since this species is a more efficient osmoregulator and ATPase activity increases substantially in osmoregulators upon hyposaline stress (Seibers et al. 1982; Piller et al. 1995). However, there was no significant difference in the rate of protein synthesis in the gills of *C. maenas* between treatments. Studies on the ATPase activity in the gills of crabs, including *C. maenas* (Lucu and Flik 1999; Henry et al. 2002), revealed that short-term exposure to low salinity results in increased activity of existing ATPases. This precedes the increase in the production of new ATPases which occurs if exposure to low salinity is prolonged (Neufeld and Pritchard 1979; Towle and Weihrauch 2001; Lovett et al. 2006). In this experiment, the crabs were exposed to low salinity for only 3 hours, and the tissues samples would have been taken before the production of new ion transporting proteins occurred.

The rate of protein synthesis was highest in the hepatopancreas of both species as compared to the gill and leg. However, the rate of protein synthesis in the leg was higher than expected, considering that the rate of protein synthesis in skeletal muscle is typically lower than that of the gills (Houlihan et al. 1990; Brown and Cameron 1991a, b; Lewis and Driedzic 2007; Mente et al. 2003; Mente et al. 2011). A study on *C. maenas* showed that a decrease in the rate

of protein synthesis towards pre-fed levels occurs in the gill and hepatopancreas 8.5 hours after feeding, whereas that of the leg is still elevated (Houlihan et al. 1990). The underlying cause(s) of this phenomenon has not been explained. Since the readings were taken 8 hours after feeding in this study (i.e. where the greatest difference in MO_2 occurred), it could be that the rate of protein synthesis in the gill and hepatopancreas of the crabs was already decreasing to pre-fed levels, but protein synthesis rate in the leg was still elevated at the time the samples were collected.

The RNA content differed between the leg, gill and hepatopancreas of the crabs and this is similar to previous reports of RNA content in tissues; the hepatopancreas/liver typically has higher levels of RNA than the gill, and the gill has more RNA than the leg/skeletal muscle (Houlihan et al. 1990; Smith et al. 1966; Lewis and Driedzic 2007). RNA content did not vary in *C. maenas* or *C. irroratus* when fasted in 16‰, fed in 32‰ or fed in 16‰. These results are in agreement with those of Intanai et al. (2009) who did not find any significant difference in the RNA content of shrimp (*Macrobrachium rosenbergii*) with varying salinity. In contrast, Houlihan et al. (1990) observed an increase in the concentration of RNA in *C. maenas*, but this increase did not occur until 8.5 hours after feeding. Given that the tissues were sampled 8 hours after feeding in this experiment, the RNA concentration may not have been elevated enough to detect differences between the treatments in either species.

The results of the present study do not support the hypothesis that protein synthesis was a major contributor to difference in the MO_2 of postprandial *C. maenas* and *C. irroratus* in low salinity. Mente et al. (2003) observed a transient peak in MO_2 and arterial PO_2 of *C. maenas* 5 hours after feeding that was not related to protein synthesis. These authors suggest that this

was due to the increase in masticatory and filtering activity. Given that both mastication (McGaw and Penney 2014) and protein synthesis (Houlihan et al. 1990; Mente et al. 2003) are contributors to SDA in crabs, and that the rate of protein synthesis was not affected by low salinity, the delay in the rise of MO_2 in *C. irroratus* was most likely the result of a delay in gastric processing rather than a result of changes in the rate of protein synthesis.

Differences in activity levels between the two species may have also influenced patterns of MO_2 during low salinity exposure. During the MO_2 experiments, it was noticed by periodic inspection of the animals in the experimental apparatus that *C. maenas* tended to become more active upon the drop in salinity whereas *C. irroratus* appeared much less active. This halokinesis has previously been reported for *C. maenas* (Taylor and Naylor 1977; Thomas et al. 1981). The reduced activity *C. irroratus* is consistent with previous findings which show that osmoconforming crabs tend to display a closure response when salinity is lowered. This response involves the crab tucking its legs and eyes inwards, closing their branchial chambers and becoming quiescent (McGaw et al. 1999; Curtis et al. 2007). The closing of the branchial chambers reduces the amount of water flowing over the gills which subsequently reduces O_2 uptake and would be reflected as a reduction in MO_2 (Sugarman et al. 1983; Brown and Terwilliger 1999; McGaw 2006b). The restricted space of the experimental chambers and dim lighting of the respirometry set-up did not allow for direct quantification of these behavioural responses, therefore, a separate experiment was carried out in a well-lit raceway which allowed ample room for the crabs to move back and forth.

Rather than the behaviour observed in the respiratory chambers, both species showed a significant increase in activity when they experienced low salinity in the raceway, with *C.*

irroratus being more active than *C. maenas*. Why the activity rates increased is unclear, but it could be due in part to differences in the animals' perception of their environment. Crabs often seek refuge from predators after feeding and reduce their activity, and both *C. maenas* and *C. gracilis* reduce their activity following feeding in order to divert resources to digestion (Mente et al. 2003; McGaw 2007). In addition, many crabs are highly thigmotactic (i.e. preference for a touch stimulus), for example, *Hemigrapsus nudus* will reduce their activity when shelter is present even when they experience lower salinities (McGaw 2001). The open raceway and the well-lit environment may explain why both *C. maenas* and *C. irroratus,* were more active in the raceway compared to the smaller, enclosed experimental chambers which were held under dim light. Both species likely increased their activity in order to seek shelter or escape the raceway, and this response may have been more pronounced in *C. irroratus* under the conditions of the behaviour experiment.

In conclusion, the results from this study showed that both *C. maenas* and *C. irroratus* can supply energy for digestion and salinity stress when both of these processes occur together, however, this occurs to a much lesser extent in *C. irroratus* as compared to *C. maenas*. The response to feeding and salinity stress was similar between the species when these processes occurred separately, but the pattern of MO_2 was different between them when digestion and salinity stress occurred simultaneously. *C. maenas* responded to low salinity with an immediate summation of the effects of digestion and low salinity, whereas *C. irroratus* responded with a slow, gradual increase in MO_2 over time. These results are different from what has been reported previously. For example, the postprandial MO_2 of *Cancer magister* (a weak regulator) decreases to pre-fed levels when salinity is lowered (Curtis and McGaw 2010). Likewise,

postprandial *C. gracilis* exhibit a permanent decline in MO_2 following low salinity exposure (McGaw 2006a). The weak regulator used in this study shows another pattern, namely a delay in the MO_2 when digestion and salinity stress occur simultaneously. This may be due to the fact that the gastric processing of *C. irroratus* is delayed upon hyposaline exposure whereas digestive processes continue unabated in *C. maenas*. The energy required for protein synthesis is considered to be a major contributor to SDA, and it was hypothesised that a delay in digestion may have downstream effects, delaying the post-absorptive increase in protein synthesis rate. However, this did not appear to be the case here since the rate of protein synthesis was not affected by low salinity exposure. Preliminary observations suggested that changes in the activity of these crabs in low salinity might have explained the difference in MO_2 between these species. Nevertheless, when activity was examined in more detail, the data did not support this initial hypothesis. Given that digestive efficiency did not change over time or differ much between species or salinities, suggests that both *C. maenas* and *C. irroratus* can fully digest a meal in low salinity, however, the time needed to digest the meal in *C. irroratus* increases significantly with hyposaline exposure. The results of this study support the hypothesis that mechanical processing in decapod crustaceans is a large contributor to the energetic demand of SDA, and the short-term disruption of this was probably the cause of the differences in MO_2. This is different from what has previously been reported for fish, copepods and isopods (Tandler and Beamish 1979; Kiørboe et al. 1985; Carefoot 1990). Finally, many researchers fast animals prior to experimentation to eliminate the effect of digestion on the organisms' physiology. However, it is important to recognise that animals will have to deal with many competing physiological processes occurring simultaneously in a natural setting. This is

especially true for estuarine and intertidal animals that will have to deal with the interactive effects of digestion and salinity stress. This study provides valuable information that extends and diversifies our knowledge of crustacean physiology, and demonstrates the importance of understanding the interaction of multiple physiological processes on the condition of an organism.

Table 1: Characteristics of the specific dynamic action (SDA) and salinity response of *Carcinus maenas* (n = 9-10). Duration is not reported for any salinity treatment since the response lasted for the duration of the experiment. The SDA coefficient is only calculated for crabs fed in 32‰ since the end of the feeding response was too difficult to discern in the combined treatment experiments. A student's t-test was used to identify significant differences between the fasted groups, 1 h post-feeding groups, and the 5 h post-feeding groups; * indicates where a significant difference occurred between the two values. The SDA coefficient was calculated by dividing estimated energy expenditure (EEE) by the total energy content of the food consumed. Values are means ± 1 S. E.

	Fed 32‰	**Fasted 16‰**	**Fasted 8‰**	**Fed, 16‰ after 1 h**	**Fed, 8‰ after 1 h**	**Fed, 16‰ after 5 h**	**Fed, 8‰ after 5 h**
RMR (mg O_2/kg/h)	47.0 ± 3.8	47.5 ± 3.5	46.4 ± 3.5	41.9 ± 2.7	55.3 ± 6.7	50.2 ± 4.4	44.9 ± 4.7
Peak MO_2 (mg O_2/kg/h)	123.1 ± 5.4	129.6 ± 10.2	162.1 ± 10.8*	187.1 ± 14.0	215.8 ± 18.1	194.1 ± 14.7	205.0 ± 13.8
Time to peak (h)	2.7 ± 0.4	6.2 ± 1.7	14.8 ± 3.9	6.9 ± 1.2	6.2 ± 1.1	5.8 ± 1.8	7.6 ± 2.1
Scope (peak MO_2/RMR)	2.8 ± 0.3	2.9 ± 0.4	3.7 ± 0.5	4.5 ± 0.2	4.3 ± 0.5	4.3 ± 0.8	4.8 ± 0.4
Duration (h)	17.4 ± 1.2						
EEE (kJ)	0.8 ± 0.1	1.9 ± 0.4	2.5 ± 0.2	2.9 ± 0.3	3.2 ± 0.2	3.0 ± 0.5	3.9 ± 0.2
SDA coefficient (%)	32.3 ± 6.3						

Table 2: : Characteristics of the specific dynamic action (SDA) and salinity response of *Cancer irroratus* (n = 7-11). Duration is not reported for any salinity treatment since the response lasted for the duration of the experiment. The SDA coefficient is calculated only for crabs fed in 32‰ since the end of the feeding response was too difficult to discern in the combined treatment experiments. A student's t test was used to test for significant differences between the fasted groups, 1 h groups, and the 5 h post-feeding groups; * indicates where a significant difference occurred between the two values. The SDA coefficient was calculated by dividing estimated energy expenditure (EEE) by the total energy content of the food consumed. Values are means ± 1 S. E.

	Fed 32‰	**Fasted 24‰**	**Fasted 16‰**	**Fed, 24‰ after 1 h**	**Fed, 16‰ after 1 h**	**Fed, 24‰ after 5 h**	**Fed, 16‰ after 5 h**
RMR (mg O_2/kg/h)	48.9 ± 4.7	42.1 ± 3.4	44.5 ± 3.7	57.5 ± 5.6	55.7 ± 9.2	40.1 ± 3.5	45.7 ± 6.4
Peak MO_2 (mg O_2/kg/h)	119.0 ± 15.0	97.9 ± 9.2	139.3 ± 15.7*	152.0 ± 15.5	189.6 ± 21.8	118.0 ± 12.1	138.5 ± 12.0
Time to peak (h)	2.6 ± 0.5	12.8 ± 2.6	20.5 ± 3.6	9.0 ± 2.1	19.0 ± 4.7	13.9 ± 2.6	18.4 ± 4.1
Scope (peak MO_2/RMR)	2.5 ± 0.2	2.3 ± 0.1	3.2 ± 0.3*	2.7 ± 0.2	3.7 ± 0.3*	3.0 ± 0.2	3.2 ± 0.2
Duration (h)	20.3 ± 3.4						
EEE (kJ)	0.8 ± 0.1	1.1 ± 0.2	3.3 ± 0.5*	2.4 ± 0.2	4.7 ± 0.4*	2.3 ± 0.2	3.2 ± 0.2*
SDA coefficient (%)	25.2 ± 4.6						

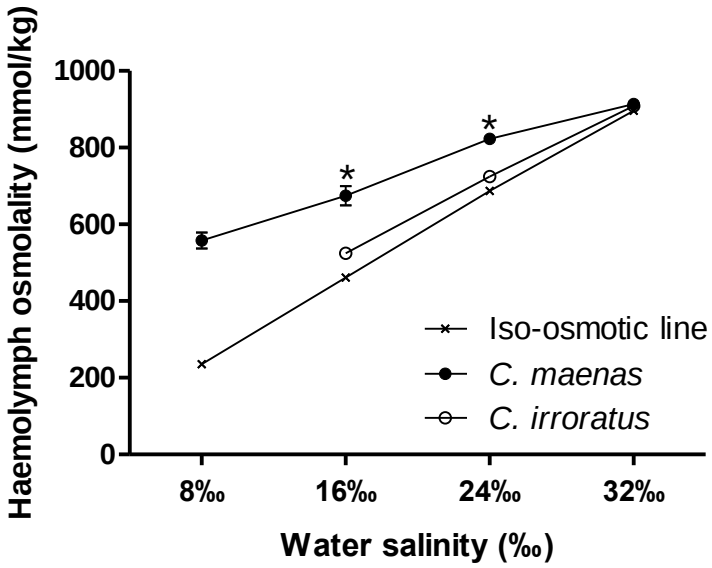

Figure 1: The haemolymph osmolality of 15°C acclimated, fasted *Carcinus maenas* (n = 8) and *Cancer irroratus* (n = 8) after 2 days of acclimation in 32, 24, 16 or 8‰ compared to the iso-osmotic line. * indicates where a significant difference occurred between the haemolymph osmolalities of *C. maenas* and *C. irroratus* ($p < 0.05$). Values are means ± 1 S. E. Note: most S. E. are too small to see on the graph.

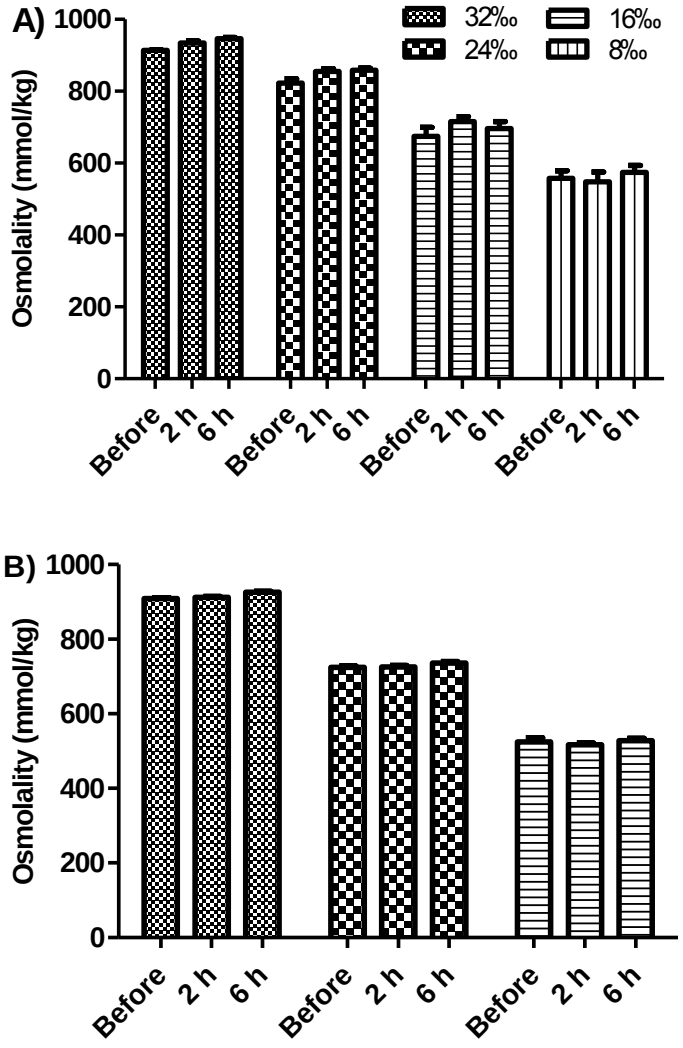

Figure 2: The effect of feeding on haemolymph osmolality (mmol/kg) in 15^{o}C acclimated A) *Carcinus maenas* (n = 8) and B) *Cancer irroratus* (n = 8). Crabs were acclimated to 32, 24, 16 or 8‰ for 2 days then haemolymph osmolality was measured before feeding, and 2 and 6 h after a meal of shrimp of 2% of their body mass. Values are means ± 1 S. E. There were no effects of feeding on the haemolymph osmolality of either species.

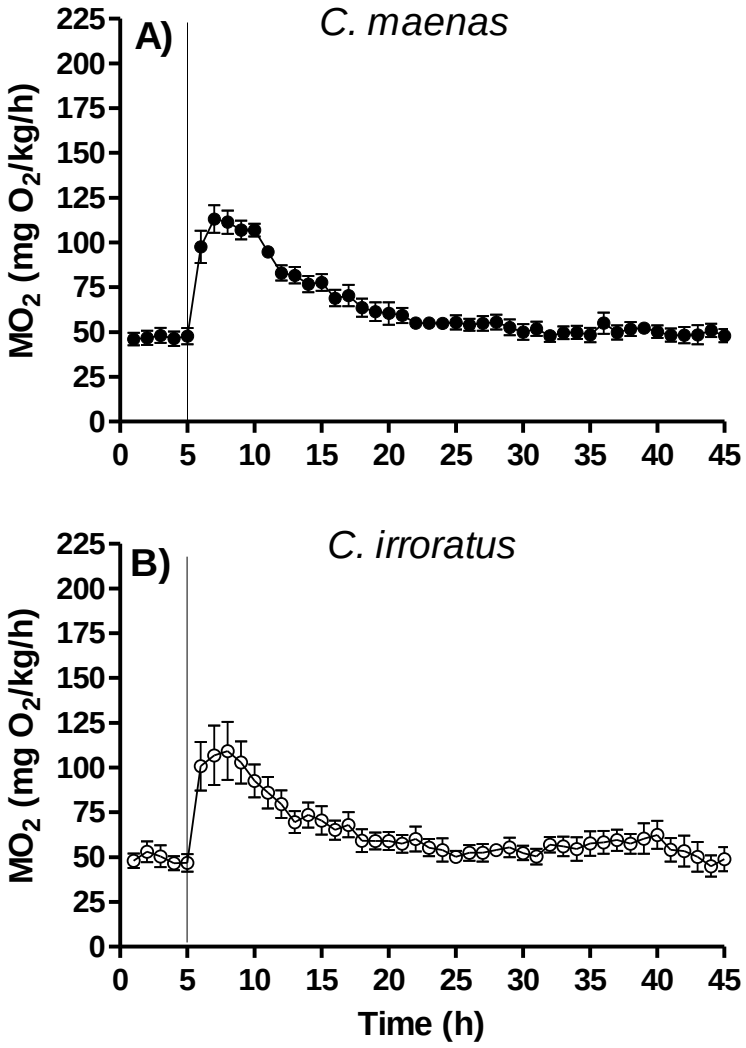

Figure 3: The change in oxygen consumption (mg O_2/kg/h) of 15°C acclimated A) *Carcinus maenas* (n = 10) and B) *Cancer irroratus* (n = 7). Oxygen consumption was monitored for a 5 h pre-feeding period, the animals were fed a piece of shrimp of 2% body mass in 32‰ at the time point indicated by the vertical line, and the oxygen consumption was then measured until it returned to pre-feeding values. Values are the means ± 1 S. E.

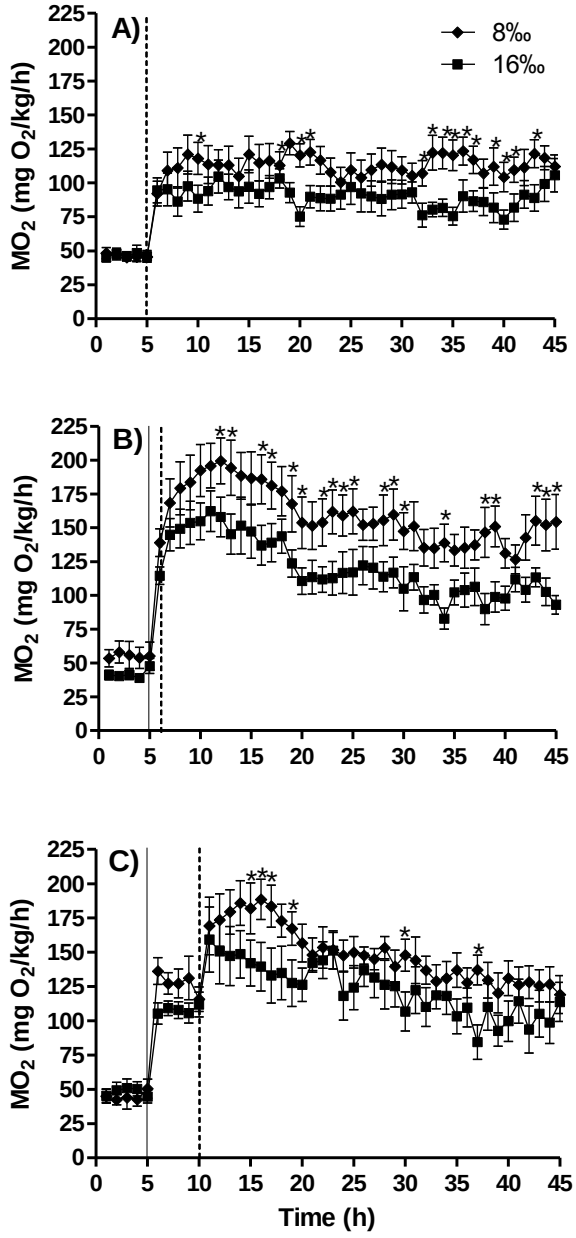

Figure 4: The oxygen consumption (mg O_2/kg/h) of 15°C acclimated *Carcinus maenas* when given a hyposaline treatment of A) 16‰ (n = 10) and 8‰ (n = 10) while in a fasted state; B) 16‰ (n = 9) and 8‰ (n = 10) 1 h after feeding; and C) 16‰ (n = 9) and 8‰ (n = 9) 5 h after feeding. Oxygen consumption was monitored for a 5 h pre-feeding period. The animals were then fed a piece of shrimp of 2% body mass at the time point indicated by the solid vertical line. After feeding, salinity was lowered from 32‰ to either 16‰ or 8‰ at the time point indicated by the dashed line. * indicates where significant differences occurred in oxygen consumption between salinities at that time point ($p < 0.05$). Values are means ± 1 S. E.

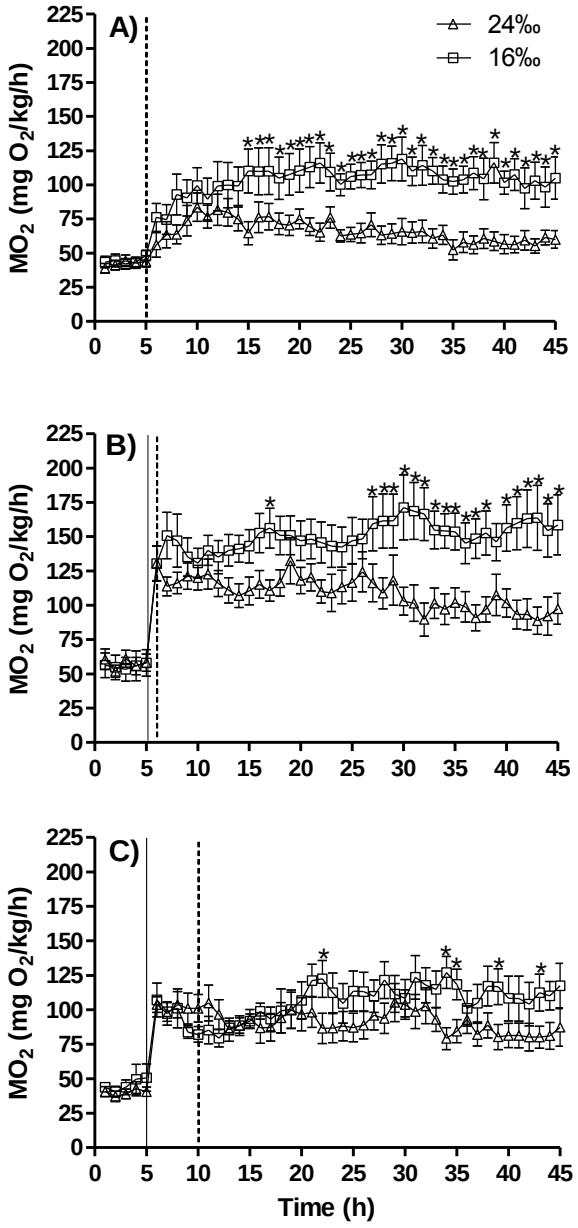

Figure 5: The oxygen consumption (mg O_2/kg/h) of 15°C acclimated *Cancer irroratus* when given a hyposaline treatment of A) 24‰ (n = 10) and 16‰ (n = 11) while in a fasted state; B) 24‰ (n = 9) and 16‰ (n = 7) 1 h after feeding; and C) 24‰ (n = 10) and 16‰ (n = 10) 5 h after feeding. Oxygen consumption was monitored for a 5 h pre-feeding period. The animals were then fed a piece of shrimp of 2% body mass at the time point indicated by the solid vertical line. After feeding, salinity was lowered from 32‰ to either 24‰ or 16‰ at the time point indicated by the dashed line. * indicates where significant differences in oxygen consumption occurred between salinities at that time point ($p < 0.05$). Values are means ± 1 S. E.

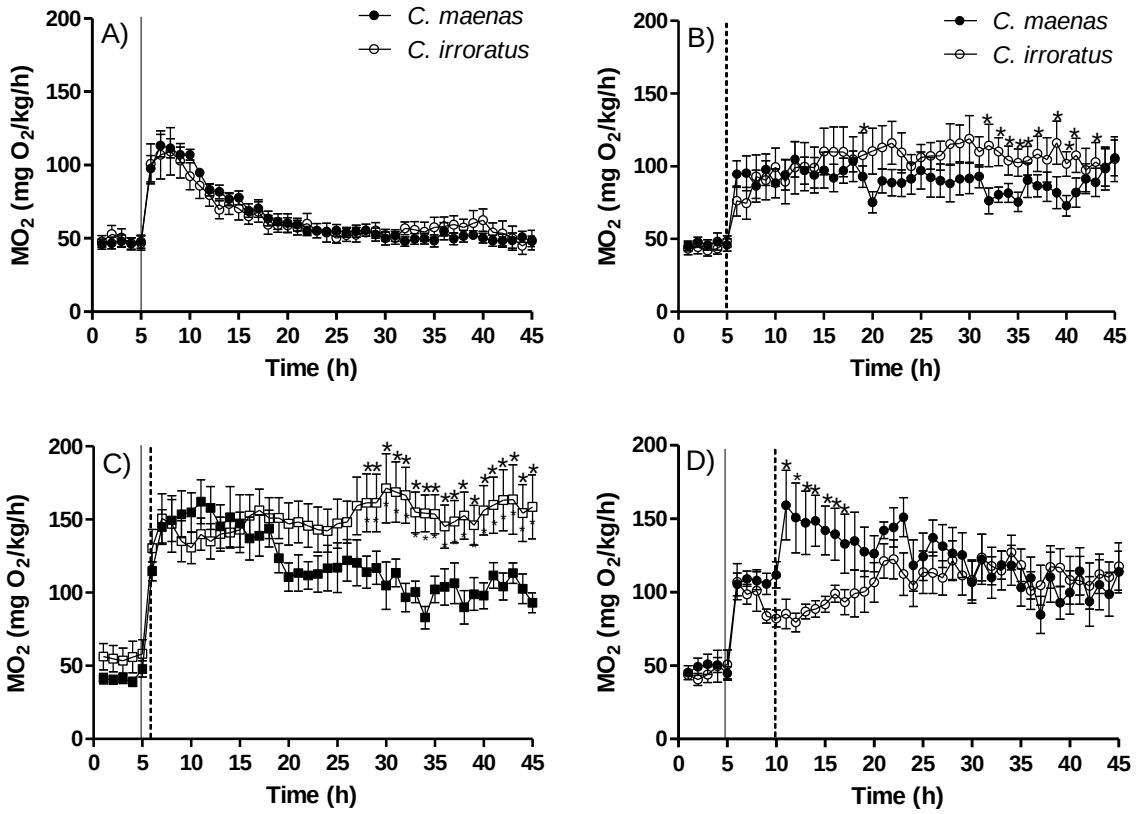

Figure 6: The oxygen consumption (mg O_2/kg/h) of 15°C acclimated *Carcinus maenas* and *Cancer irroratus* plotted on the same axis to allow for direct comparison of the A) SDA response in 32‰, B) the osmoregulatory response of fasted crabs to 16‰, C) the 16‰ treatment 1 h after feeding, and D) the 16‰ treatment 5 h after feeding. Oxygen consumption was monitored for a 5 h pre-feeding period, the animals were then fed a piece of shrimp of 2% body mass at the time point indicated by the solid vertical line. After feeding, salinity was lowered from 32‰ to 16‰ at the time point indicated by the dashed line. * indicates where significant differences in oxygen consumption occurred between species at that time point ($p < 0.05$). Values are means ± 1 S. E.

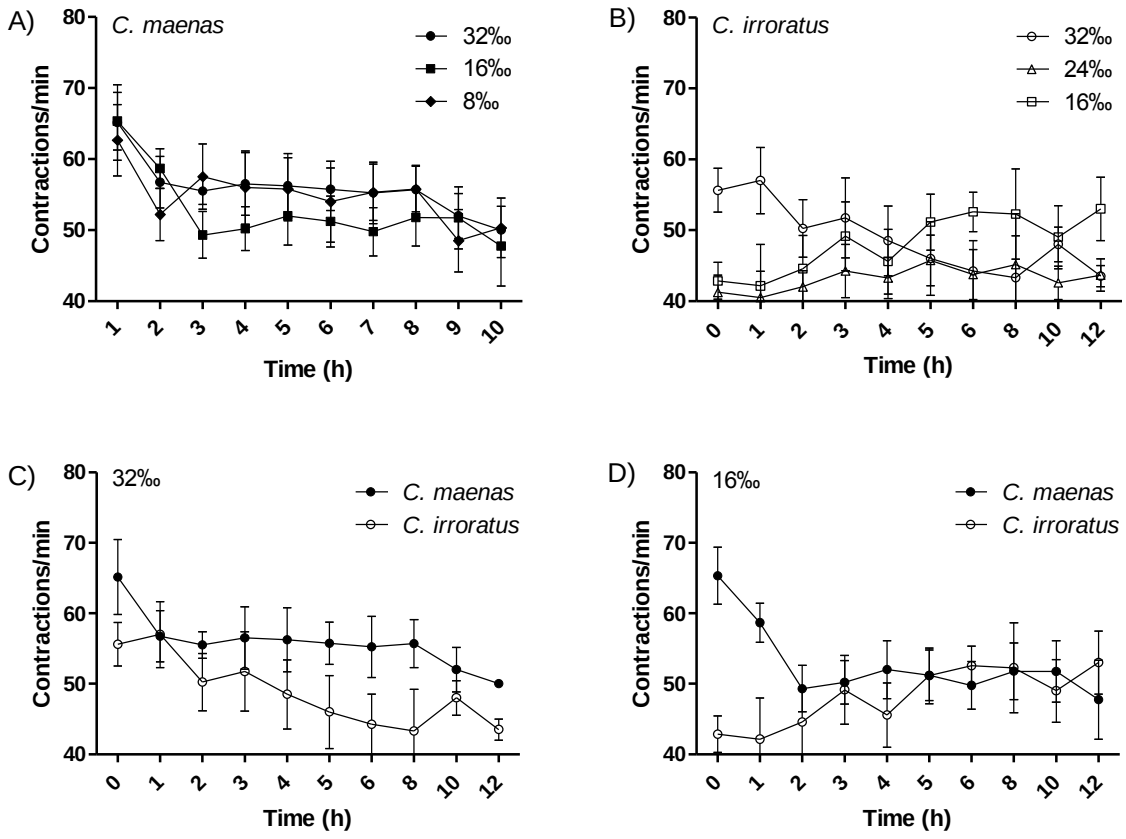

Figure 7: The rate of pyloric contraction (contractions/min) in 15°C acclimated A) *Carcinus maenas* (n = 8) and B) *Cancer irroratus* (n = 8) in 32, 24, 16 or 8‰. Crabs were given a radio-opaque meal of shrimp in 32‰ and allowed to feed for 1 h or until the meal was consumed. Then, they were transferred to 24, 16 or 8‰ and the contractions of the pyloric region were measured via x-ray every h until the foregut was clear of the meal. The contraction rates of both species in C) 32‰ and D) 16‰ were plotted on the same axis to provide a direct comparison. Values are means ± 1 S. E.

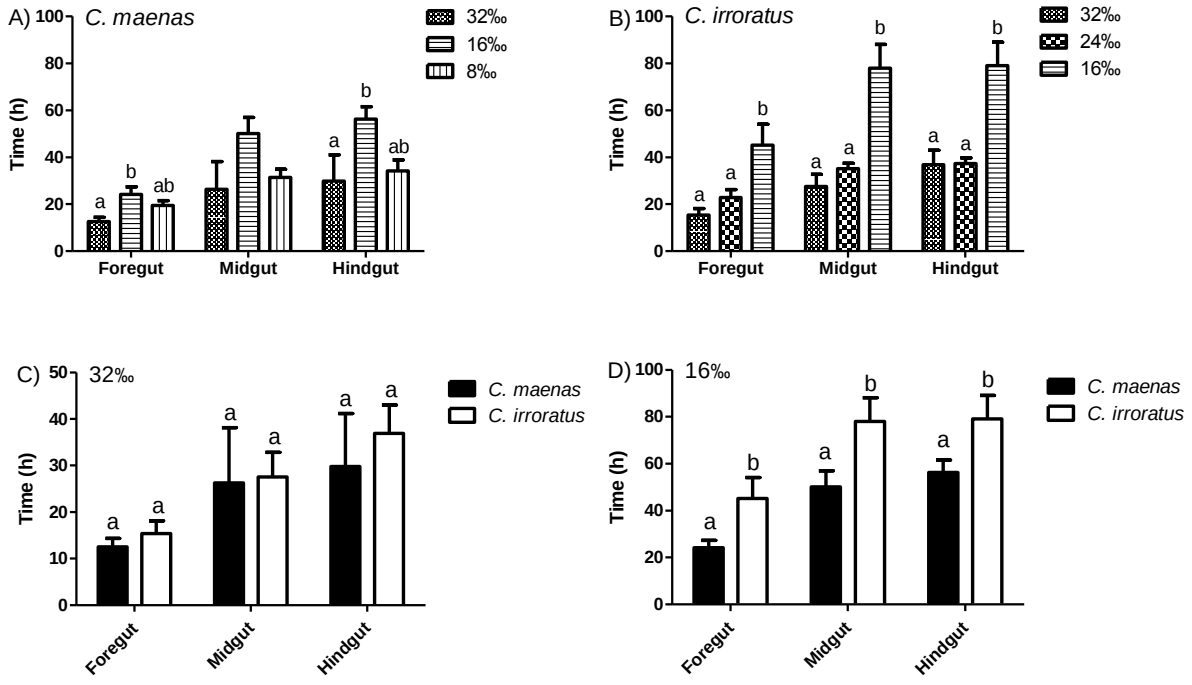

Figure 8: The time (h) taken to clear the meal from the foregut, midgut and hindgut in 32, 24, 16 or 8‰ of 15°C acclimated A) *Carcinus maenas* (n = 8) and B) *Cancer irroratus* (n = 8). Crabs were given a radio-opaque meal of shrimp and allowed to feed in 32‰ for 1 h or until the meal was consumed. Then they were transferred to 24, 16 or 8‰ and the meal was followed through each section of the gut until it was cleared. The clearance times of the foregut, midgut and hindgut of both species in C) 32‰ and D) 16‰ were plotted on the same axis to provide a direct comparison. The bars represent the total time taken for meal clearance (i.e. from the time the meal was ingested to the time it was completely cleared from each gut region). Different letters indicate where a significant difference occurred between salinity (or species) in that region of the gut ($p < 0.05$). Values are means ± 1 S. E.

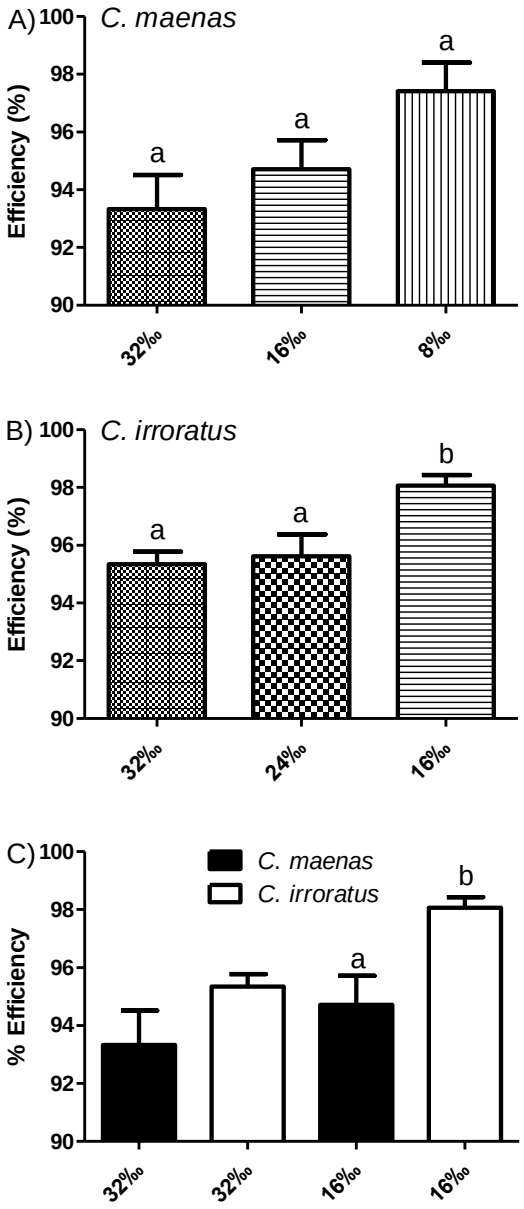

Figure 9: The digestive efficiency (%) of 15°C acclimated A) *Carcinus maenas* (n = 6-8) and B) *Cancer irroratus* (n = 8-10) in 32, 24, 16 and 8‰. Crabs were given a piece of shrimp in 32‰ and allowed to feed *ad libitum*. Then they were transferred to 32, 24, 16 and 8‰ and their feces was collected over the following 3 days. The digestive efficiency of both species in C) 32‰ and 16‰ were plotted on the same axis to provide a direct comparison between the two species. Different letters indicate where a significant difference occurred between salinities (or species) ($p < 0.05$) Values are means ± 1 S. E.

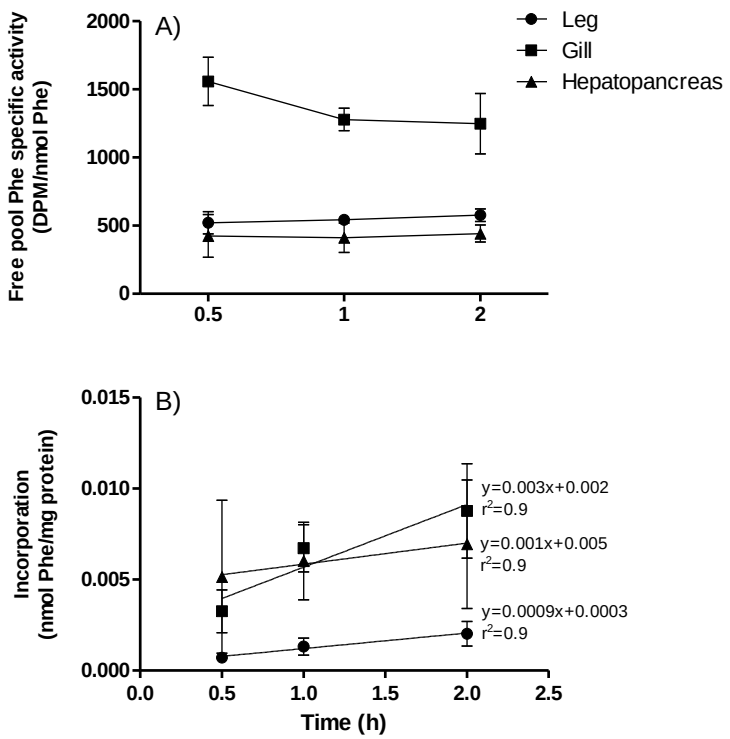

Figure 10: The time course of changes in A) the specific activity of the free pool tritiated phenylalanine (DPM/nmol Phe) and B) the incorporation (nmol Phe/mg protein) of tritiated phenylalanine into the leg, gill and hepatopancreas tissue of 15°C acclimated *Cancer irroratus* (n = 8 per time period). Values are means ± 1 S.E.

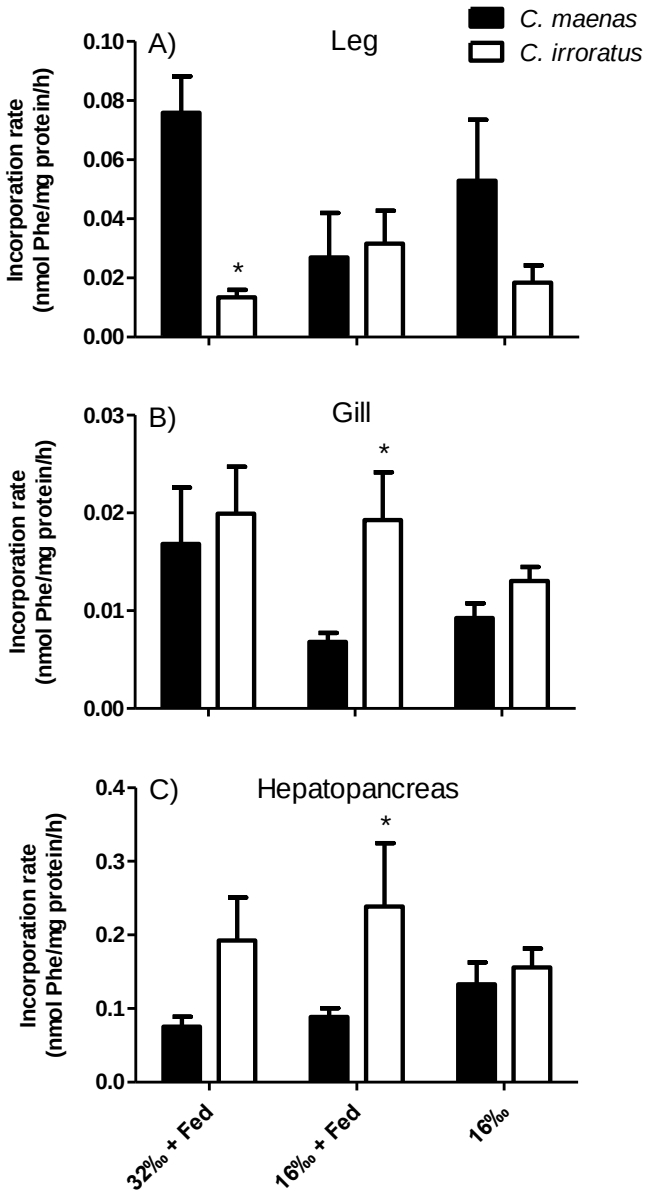

Figure 11: The incorporation rate (nmol Phe/mg protein/h) of tritiated phenylalanine into the A) leg, B) gill and C) hepatopancreas tissue of 15°C acclimated *Carcinus maenas* (n = 8) and *Cancer irroratus* (n = 8) when fed in 32‰, fed then transferred to 16‰, and fasted in 16‰. Fed crabs were given a piece of shrimp of 2% of their body mass. Tissue samples were collected 1 h post-injection. No significant differences occurred between treatments, * indicates where significant differences occurred between species ($p < 0.05$). Values are means ± 1 S. E.

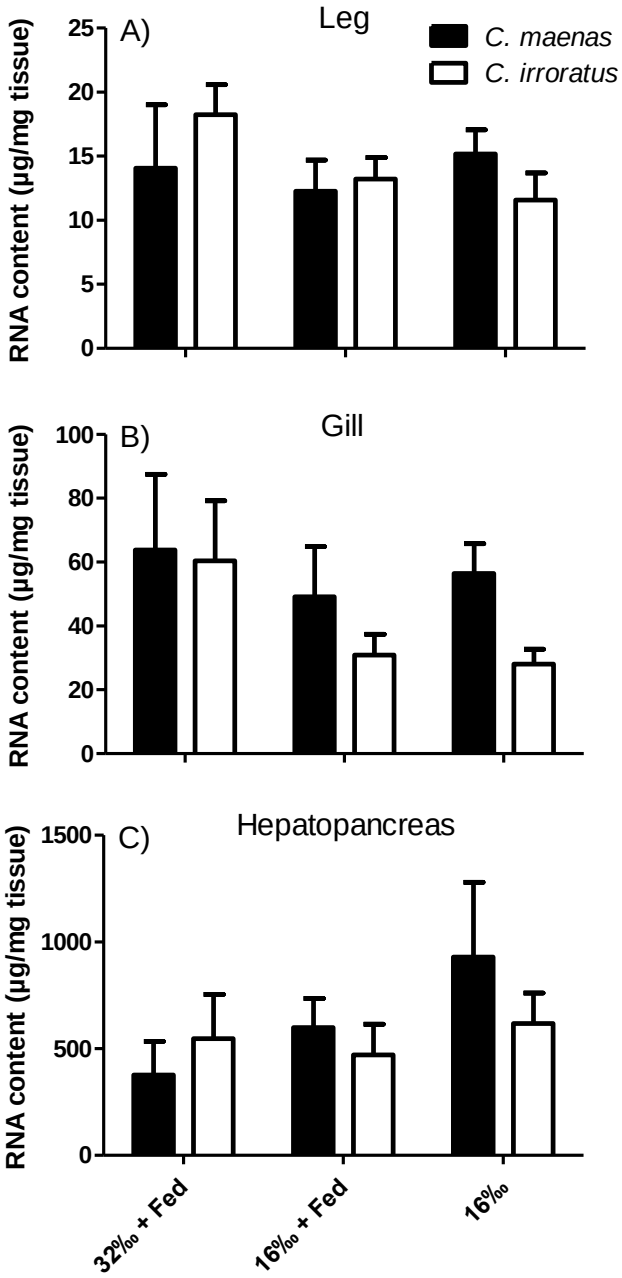

Figure 12: The RNA content (µg/mg tissue) of the A) leg, B) gill and C) hepatopancreas tissue of 15°C acclimated *Carcinus maenas* (n = 8) and *Cancer irroratus* (n = 8) when fed in 32‰, fed then transferred to 16‰, and fasted in 16‰. Fed crabs were given a piece of shrimp at 2% of their body mass. Tissue samples were collected 1 h post-injection. Values are means ± 1 S. E. No significant differences occurred between species in any of the treatments.

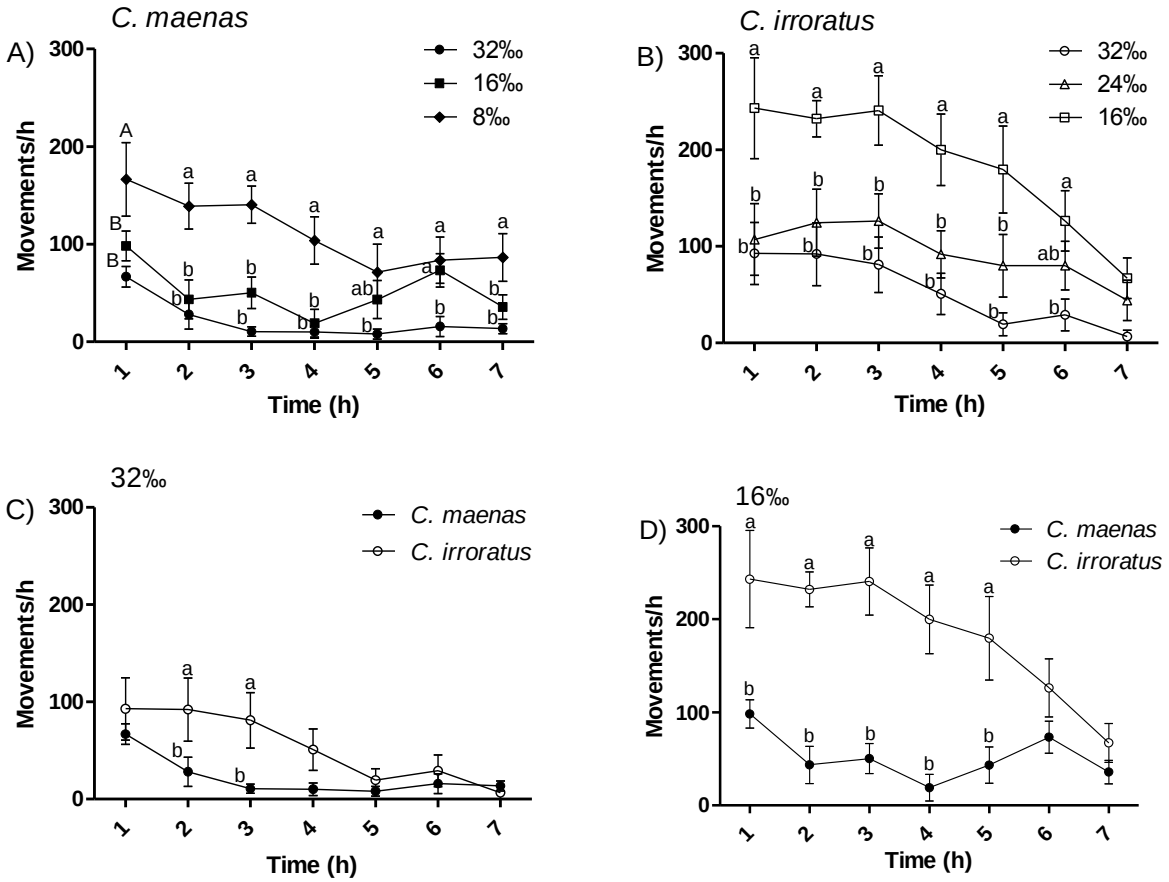

Figure 13: The activity level (movements/h) of 15°C acclimated A) *Carcinus maenas* (n = 8) and B) *Cancer irroratus* (n = 8) when fed in 32‰ then transferred to 32, 24, 16 or 8‰. The activity level of both species in C) 32‰ and D) 16‰ were plotted on the same axis to provide a direct comparison between the species. Values without a letter in common indicate where a significant difference occurred between salinities (or species), and letters differing in case indicate where a significant difference in activity occurred with time ($p < 0.05$). Letters are placed next to the symbols in cases where they do not fit above the symbol. Values are means ± 1 S. E.

REFERENCES

Andrade, D.V., Cruz-Neto, A.P., Abe, A.S., 1997. Meal size and specific dynamic action in the rattlesnake *Crotalus durissus* (Serpentes: Viperidae). *Herpetologica* **53** (4): 485-493.

Bamber, S.D., Depledge, M.H. 1997. Evaluation in the changes in the adaptive physiology of shore crabs (*Carcinus maenas*) as an indicator of pollution in estuarine environments. *Mar. Biol*. 129: 667-672.

Binns, R., 1969. The physiology of the anntenal gland of *Carcinus maenas* (L.) II: Urine production rates. *J. Exp. Biol*. **51**: 11-16.

Broekhuysen, G.J., 1936. On development, growth and distribution of *Carcinides maenas* (L.). Arch. Néerl. Zool. 2: 257-399.

Brown, A.C, Terwilliger, N.B., 1999. Developmental changes in oxygen uptake in *Cancer magister* (Dana) in response to changes in salinity and temperature. *J. Exp. Mar. Biol. Ecol.* **241**: 179-192.

Brown, C.R., Cameron, J.N., 1991a. The induction of specific dynamic action in the channel catfish by infusion of essential amino acids. *Physiol. Zool.* **64** (1): 276-297.

Brown, C.R., Cameron, J.N., 1991b. The relationship between specific dynamic action (SDA) and protein synthesis rates in the channel catfish. *Physiol. Zool.* **64** (1): 298-309.

Campbell, K.L., McIntyre, I.W., MacArthur, R.A., 2000. Postprandial heat increment does not substitute for active thermogenesis in cold-challenged star-nosed moles (*Condylura cristata*). *J. Exp. Biol.* **203**: 301-310.

Canjani, C., Andrade, D.V., Cruz-Neto, A.P., Abe, A.S., 2003. Aerobic metabolism during predation by a boid snake. *Comp. Biochem. Physiol. A* **133**: 487-498.

Cantelmo, A.C., Cantelmo, F.R., Langsam, D.M., 1975. Osmoregulatory ability of the rock crab *Cancer irroratus* under osmotic stress. *Comp. Biochem. Physiol. A* **51**: 537-542.

Carefoot, T.H., 1990. Specific dynamic action (SDA) in the supralittoral isopod *Ligia pallasii*: Identification of components of apparent SDA and effects of dietary amino acid quality and content on SDA. *Comp. Biochem. Physiol. A* **95** (3): 309-316.

Chakraborty, S.C., Ross, L.G., Ross, B., 1992. Specific dynamic action and feeding metabolism in common carp, *Cyprinus carpio* L. *Comp. Biochem. Physiol. A* **101**: 77-82.

Charmantier, G., Charmantier-Daures, M., 1991. Ontogeny of osmoregulation and salinity tolerance in *Cancer irroratus*: Elements of comparison with *C. borealis*. *Biol. Bull.* **180**: 125-134.

Chomczynski, P., Sacchi, N., 1986. Single step method of RNA isolation by acid guanidinium thiocyanate phenol chloroform extraction. *Anal. Biochem.* **162**: 156-159.

Cruz-Neto, A.P., Andrade, D.V., Abe, A.S., 1999. Aerobic metabolism during prey ingestion by juvenile rattlesnakes *Crotalua durissus*. *J. Herpetol.* **33**: 229-234.

Cruz-Neto, A.P., Andrade, D.V., Abe, A.S., 2001. Energetic and physiological correlates of prey handling and ingestion in lizards and snakes. *Comp. Biochem. Physiol. A* **128**: 515-533.

Curtis, D.L., Jensen, E.K., McGaw, I.J., 2007. Behavioral influences on the physiological responses of *Cancer gracilis*, the graceful crab, during hyposaline exposure. *Biol. Bull.* **212**: 222-231.

Curtis, D. L., McGaw, I. J., 2010. Respiratory and digestive responses of postprandial Dungeness crabs, *Cancer magister*, and blue crabs, *Callinectes sapidus*, during hyposaline exposure. *J. Comp. Physiol. B* **180**: 189-198.

Curtis, D.L., van Breukelen, F. McGaw, I.J., 2013. Extracellular digestion during hyposaline exposure in the Dungeness crab, *Cancer magister*, and the blue crab, *Callinectes sapidus*. *Comp. Biochem. Physiol. A* **166**: 564-570.

El Haj, A. J., Houlihan, D. F., 1987. In vitro and in vivo protein synthesis rates in a crustacean muscle during the moult cycle. *J. Exp. Biol.* **127**: 413-426.

Ellington, W.R., Lawrence, J.M., 1974. Coelomic fluid volume regulation and isosmotic intracellular regulation by *Luidia clathrata* (Echinodermata: Asteroidea) in response to hyposmotic stress. *Biol. Bull.* **146**: 20-31.

Forsum, E., Hillman, P.E., Nesheim, M.C., 1981. Effect of energy restriction on heat production, basal metabolic rate and specific dynamic action of food in rats. *J. N.* **111**: 1691-1697.

Fraser, K.P.P., Rogers, A.D., 2007. Protein metabolism in marine animals: the underlying mechanism of growth. *Adv. Mar. Biol.* **52**: 267-362.

Freire, C.A., Amado, E.M., Souza, L.R., Veiga, M.P.T., Vitule, J.R.S., Souza, M.M., Prodocimo, V., 2008. Muscle water control in crustaceans and fishes as a function of habitat, osmoregulatory capacity, and degree of euryhalinity. *Comp. Biochem. Physiol. A* **149**: 435-446.

Gallivan, G.J., Best, R.C., 1986. The influence of feeding and fasting on the metabolic rate and ventilation of the Amazonian manatee (*Trichechus inunguis*). *Physiol. Zool.* **59** (5): 552-557.

Garlick, P. J., McNurlan, M. A., Preedy, V. R., 1980. A rapid and convenient technique for measuring the rate of protein synthesis in tissues by injection of 3H phenylalanine. *Biochem. J.* **192**: 719-723.

Gilles, R., 1973. Oxygen consumption as related to the amino acid metabolism during osmoregulation in the blue crab *Callinectes sapidus*. *Neth. J. Sea Res.* **7**: 280-289.
Gnanamuthu, C.P., 1966. Changes in volume of a brackish water prawn in different media. *P. Indian Acad. Sci. B* **64** (2): 96-109.

Guerin, J. L. and Stickle, W. B., 1992. Effects of salinity gradients on the tolerance and bioenergetics of juvenile blue crabs (*Callinectes sapidus*) from waters of different environmental salinities. *Mar. Biol.* **114**, 391-396.

Guinea, J., Fernandez, F., 1997. Effect of feeding frequency, feeding level and temperature on energy metabolism in *Sparus aurata*. *Aquaculture* **148**: 125-142.

Harris, R.R., Andrews, M.B., 1982. Extracellular fluid volume changes in *Carcinus maenas* during acclimation to low and high salinities. *J. Exp. Biol*. **99**: 161-173.

Haschermeyer, A.E.V., Smith, M.A.K., 1979. Protein synthesis in liver, muscle and gill of mullet (*Mugil cephalus* L.) in vivo. *Biol. Bull.* **156**: 93-102.

Henry, R.P., Garrelts, E.E., McCarty, M.M., Towle, D.W., 2002. Differential induction of branchial carbonic anhydrase and Na^+/K^+ ATPase activity in the euryhaline crab, *Carcinus maenas*, in response to low salinity exposure. *J. Exp. Biol.* **292**: 595-603.

Herera, M., Aragão, C., Hachero, I., Ruiz-Jarabo, I., Vargas-Chacoff, L., Mancera, J.M., Conceição, L.E.C., 2012. Physiological short-term response to sudden salinity change in the Senegalese sole (*Solea senegalensis*). *Fish Physiol. Biochem.* **38**: 1741-1751.

Hopkin, S.P., Nott, J.A., 1980. Studies on the digestive cycle of the shore crab *Carcinus maenas* (L.) with special reference to the B cells in the hepatopancreas. *J. Mar. Biol. Ass. U.K.* **60**: 891-907.

Houlihan, D.F., Waring, C.P., Mathers, E., Gray, C., 1990. Protein synthesis and oxygen consumption of the shore crab *Carcinus maenas* after a meal. *Physiol. Zool.* **63** (4): 735-756.

Hwang, P.P., Lee, T.H., 2007. New insights into fish ion regulation and mitochondrion rich cells. *Comp. Biochem. Physiol. A* **148**: 479-497.

Intanai, I., Taylor, E.W., Whiteley, N.M., 2009. Effects of salinity on rates of protein synthesis and oxygen uptake in the post-larvae and juveniles of the tropical prawn *Macrobrachium rosenbergii* (de Man). *Comp. Biochem. Physiol. A* **152**: 372-378.

Janes, D.N., Chapelle, M.A., 1995. The effect of ration size and body size on specific dynamic action Adélie penguin chicks, *Pygoscelis adeliae*. *Physiol. Zool.* **68**: 1029-1044.

Johnson, R.N., Lillywhite, H.B., 1979. Digestive efficiency of the omnivorous lizard *Klauberina riversiana*. *Copeia* **1979**: 431-437.

Jury, S.H., Kinnison, M.T., Hunting Howell, W., Watson III, W.H., 1994. The effects of reduced salinity on lobster (*Homarus americanus* Milne-Edwards) metabolism: implications for estuarine populations. *J. Exp. Mar. Biol. Ecol.* **176**: 167-185.

Katersky, R.S., Carter, C.G., 2010. The effect of temperature on postprandial protein synthesis in juvenile barramundi, *Lates calcarifer*. *Comp. Biochem. Physiol. A* **156**: 529-536.

Kiørboe, T., Møhlenberg, F., Hamburger, K., 1985. Bioenergetics of the planktonic copepod *Acartia tonsa*: relation between feeding, egg production and respiration, and composition of specific dynamic action. *Mar. Ecol. Prog. Ser*. **26**: 85-97.

Klassen, G., Locke, A., 2007. A biological synopsis of the European green crab, *Carcinus maenas*. Can. *Manuscr. Rep. Fish. Aquat. Sci*. no. 2818: vii-75.

Kleiber, M., 1961. *The fire of life, an introduction to animal energetics*. New York, N.Y., U.S.A.: Wiley.

Le Vay, L., Jones, D.A., Puello-Cruz, A.C., Sangha, R.S., Ngamphongsai, C., 2001. Digestion in relation to feeding strategies exhibited by crustacean larvae. *Comp. Biochem. Physiol. A* **128**: 623-630.

Lewis, J. M., Driedzic, W. R. (2007). Tissues specific changes in protein synthesis associated with seasonal metabolic depression and recovery in the north temperate labrid, *Tautogolabrus adspersus*. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **293**: R474-R481.

Lovett, D.L., Verzi, M.P., Burgents, J.E., Tanner, C.A., Glomski, K., Lee, J.J., Towle, D.W., 2006. Expression profiles of Na^+/K^+-ATPase during acute and chronic hypo-osmotic stress in the blue crab *Callinectes sapidus*. *Biol. Bull*. **211**: 58-65.

Lucu, C., Flik, G., 1999. Na^+-K^+-ATPase and Na^+/Ca^{2+} exchange activities in gills of hyperregulating *Carcinus maenas. Am. J. Physiol. Regul. Integr. Comp. Physiol.* **276** (2): R490-R499.

MacMillan, D.N., Houlihan, D.F., 1989. Short-term responses of protein synthesis to re-feeding in rainbow trout. *Aquaculture* **79**: 37-46.

Mathers, E.M., Houlihan, D.F., McCarthy, I.D., Burren, L.J., 1993. Rates of growth and protein synthesis correlated with nucleic acid content in fry of rainbow trout, *Oncorhynchus mykiss*: Effects of age and temperature. *J. Fish Biol.* **43**: 245-263.

McCarthy, I.D., Moksness, E., Pavlov, D.A., Houlihan D.F., 1999. Effects of water temperature on protein synthesis and protein growth in juvenile Atlantic wolffish (*Anarhichas lupus*). *Can. J. Fish. Aquat. Sci.* **56**: 231–241.

McCue, M.D., Bennett, A.F., Hicks, J.W., 2005. The effect of meal composition on specific dynamic action in Burmese pythons (*Python molurus*). *Physiol. Biochem. Zool.* **78** (2): 182-192.

McCue, M.D., 2006. Specific dynamic action: A century of investigation. *Comp. Biochem. Physiol. A* **144**: 381-391.

McGaw, I.J., Reiber, C.L., Guadagnoli, J.A., 1999. Behavioural physiology of four crab species in low salinity. *Biol. Bull*. **196**: 163-176.

McGaw, I.J., 2001. Impacts of habitat complexity on physiology: Purple shore crabs tolerate osmotic stress for shelter. *Estuar. Coast. Shelf S.* **53**: 865-876.

McGaw, I.J., 2006a. Feeding and digestion in low salinity in an osmoconforming crab, *Cancer gracilis* I. Cardiovascular and respiratory responses. *J. Exp. Biol*. **209**: 3766-3776.

McGaw, I.J., 2006b. Prioritization or summation of events? Cardiovascular physiology of postprandial dungeness crabs in low salinity. *Physiol. Biochemical Zool.* **79** (1): 169-177.

McGaw, I.J., 2007. The interactive effects of exercise and feeding on oxygen uptake, activity levels, and gastric processing in the graceful crab *Cancer gracilis*. *Physiol. Biochem. Zool.* **80** (3): 335-343.

McGaw. I.J., Curtis, D.L., 2013. The effect of meal size and body size on specific dynamic action and gastric processing in decapod crustaceans. *Comp. Biochem. Physiol. A* **166**: 414-425.

McGaw, I.J., Penney, C.M., 2014. Effect of meal type on specific dynamic action in the green shore crab, *Carcinus maenas*. *J. Comp. Physiol. B* **184**: 425-436.

McNamara, J.C., Faria, S.C., 2012. Evolution of osmoregulatory patterns and gill ion transport mechanisms in the decapod Crustacea: A review. *J. Comp. Physiol. B* **182**: 997-1014.

Mente, E., 2003. *Nutrition, physiology and metabolism of crustaceans*. New Hampshire, U.S.A.: Science Publishers Inc.

Mente, E., Legeay, A., Houlihan, D.F., Massabuau, J., 2003. Influence of oxygen partial pressures on protein synthesis in feeding crabs. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **284**: R500-R510.

Mente, E., Carter, C.G., Barnes, K.S., Karapanagiotidis, I.T., 2011. Protein synthesis in wild-caught Norway lobster (*Nephrops norvegicus* L.). *J. Exp. Mar. Biol. Ecol.* **409**: 208-214.

Mitra, A., Flynn, K.J., 2007. Importance of interactions between food quality, quantity and gut transit time on consumer feeding, growth, and trophic dynamics. *Am. Nat.* **169** (5): 632-646.

Moran, W.M., Pierce, S.K., 1984. The mechanism of crustacean salinity tolerance: cell volume regulation by K^+ and glycine effluxes. *Mar. Biol.* **81**: 41-46.

Neufield, G.J., Pritchard, J.B., 1979. Osmoregulation and gill Na, K-ATPase in the rock crab, *Cancer irroratus*: response to DDT. *Comp. Biochem. Physiol. C* **62**: 165-172.

Norfolk, J.R.W., 1978. Intertidal volume and pressure regulation in *Carcinus maenas*. *J. Exp. Biol*. **74**: 123-132.

Overgaard, J., Andersen, J.B., Wang, T., 2002. The effects of fasting and duration on the metabolic response to feeding in *Python molurus*: an evaluation of the energetic costs associated with gastrointestinal growth an upregulation. *Physiol. Biochem. Zool.* **75**: 360-368.

Pan, Z.C., Ji, X., Lu, H.L., Ma, X.M., 2005. Influence of food type on specific dynamic action of the Chinese skink *Eumeces chinensis*. *J. Biochem. Physiol. A* **140**: 151-155.

Pannevis, M.C., Houlihan, D.F., 1992. The energetic cost of protein synthesis in isolated hepatocytes of rainbow trout *Oncohynchus mykiss*. *J. Comp. Physiol. B* **162**: 393-400.

Pequeux, A., 1995. Osmotic regulation in Crustaceans. *J. Crustacean Biol.* **15** (1): 1-60

Pierce, S.K., 1982. Invertebrate cell volume control mechanisms: a coordinated use of intracellular amino acids and inorganic ions as osmotic solute. *Biol. Bull*. **163**: 405-419.

Piersma, T., Dekinga, A., van Gils, J.A., Achtercamp, B., Visser, G.H., 2003. Cost-benefit analysis of mollusc eating in a shorebird: I. Foraging and processing costs estimated by the doubly labelled water method. *J. Exp. Biol*. **206**: 3361-3368.

Piller, C.S., Henry, R.P., Doeller, J.E., Kraus, D.W., 1995. A comparison of the gill physiology of two euryhaline crab species, *Callinectes sapidus* and *Callinectes similis*: energy production, transport-related enzymes and osmoregulation as a function of acclimation salinity. *J. Exp. Biol.* **198**: 349-358.

Robertson, R.F., El Haj, A.J., Clarke, A., Taylor, E.W., 2001. The effect of specific dynamic action and protein synthesis on the Baltic isopod crustacean, *Saduria entomon*. *J. Exp. Mar. Biol. Ecol*. **262**: 113-129.

Romero, M.C., Vanella, F., Tapella, F., Lovrich, G.A., 2006. Assimilation and oxygen uptake associated with two different feeding habits of *Munida gregaria* (=*M. subrugosa*) (Crustacea, Decapoda). *J. Exp. Mar. Biol. Ecol.* **333**: 40-48.

Rosas, C., Bolongaro-Crevenna, A., Sánchez, A., Gaxiola, G., Soto, L., Escobar, E., 1995. Role of digestive gland in the energetic metabolism of *Panaeus setiferus*. *Biol. Bull*. **189**: 168-174.

Secor, S.M., Diamond, J., 1995. Adaptive responses to feeding in Burmese pythons: Pay before pumping. *J. Exp. Biol*. **198**: 1313-1325.

Secor, S.M., Diamond, J., 1997. Effects of meal size on postprandial responses in juvenile Burmese pythons (*Python molurus*). *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **272** (3): R902-R912.

Secor, S.M., Faulkner, A.C., 2002. Effects of meal size, meal type, body temperature, and body size on the specific dynamic action of the marine toad, *Bufo marinus*. *Physiol. Biochem. Zool.* **75** (6): 557-571.

Secor, S.M., 2003. Gastric function and its contribution to the postprandial metabolic response of the Burmese python *Python molurus*. *J. Exp. Biol*. **206**: 1621-1630.

Secor, S.M. 2009. Specific dynamic action: a review of the postprandial metabolic response. *J. Comp. Physiol. B* **179**: 1-56.

Siebers, D., Leweck, K., Markus, H., Winkler, A., 1982. Sodium regulation in the shore crab *Carcinus maenas* as related to ambient salinity. *Mar. Biol.* **69**: 37-43.

Shumway, S.E., Koehn, R.K., 1982. Oxygen consumption in the American oyster *Crassostrea virginica*. *Mar. Ecol. Prog. Ser*. **9**: 59-68.

Smith, R.I., 1970. The apparent water-permeability of *Carcinus maenas* (Crustacea, Brachyura, Portunidae) as a function of salinity. *Biol. Bull*. **139**: 351-362.

Smith, R.W., Houlihan, D.F., Nilsson, G.E., Brechin, J.G., 1996. Tissue specific changes in protein synthesis rates in vivo during anoxia in crucian carp. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 271 (4): R897-R904.

Spaargaren, D.H., 1975. Changes in permeability in the shore crab, *Carcinus maenas* (L.), as a response to salinity. *Comp. Biochem. Physiol. A* **51**: 549-552.

Storch, D., Pörtner, H.O., 2003. The protein synthesis machinery operates at the same expense in eurythermal and cold stenothermal Pectinids. *Physiol. Biochem. Zool.* **76** (1): 28-40.

Sugarman, P.C., Pearson, W.H., Woodruff, D.L., 1983. Salinity detection and associated behavior in the dungeness crab *Cancer magister*. *Estuaries* **6** (4): 380-386.

Tan, E.C., van Engel, W.A., 1966. Osmoregulation in the adult blue crab, *Callinectes sapidus* rathbun. *Chesapeake Sci.* **7**: 30–35.

Tandler, A., Beamish, F.W.H., 1979. Mechanical and biochemical components of apparent specific dynamic action in largemouth bass *Micropterus salmoides* Lacépède. *J. Fish Biol*. **14**: 343-350.

Taylor, A.C., Butler, P.J., Al-Wassia, A., 1977. The effect of a decrease in salinity on respiration, osmoregulation and activity in the shore crab *Carcinus maenas* (L.) at different acclimation temperatures. *J. Comp. Physiol*. **119**: 155-170.

Taylor, A.C., Naylor, E., 1977. Entrainment of the locomotor rhythm of *Carcinus* by cycles of salinity change. *J. Mar. Biol. Ass. U.K.* **57**: 273-277.

Tirard, C.T., Grossfeld, R.M., Levine, J.F., Kennedy-Stoskopf, S., 1997. Effect of osmotic shock on protein synthesis of oyster hemocytes in vitro. *Comp. Biochem. Physiol. A* **116**: 43-49.

Thurberg, F.P., Dawson, M.A., Collier, R.S., 1973. Effects of copper and cadmium on osmoregulation and oxygen consumption in two species of estuarine crabs. *Mar. Biol.* **23**: 171-175.

Thomas, N.J., Lasiak, T.A., Naylor, E., 1981. Salinity preference behaviour in *Carcinus*. *Marine Behaviour and Physiology* **7**: 277-283.

Towle, D.W., Weihrauch, D., 2001. Osmoregulation by gills of euryhaline crabs: molecular analysis of transporters. *Am. Zool.* **41**: 770-780.

Treberg, J.R., Hall, J.R., Driedzic, W.R., 2005. Enhanced protein synthetic capacity in Atlantic cod (*Gadus morhua*) is associated with temperature induced compensatory growth. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **288**: R205–R211.

Urbina, M., Paschke, K., Gebauer, P., Chaparro, O.R., 2010. Physiological energetics of the estuarine crab *Hemigrapsus crenulatus* (Crustacea Decapoda Varunidae): responses to different salinity. *J. Mar. Biol. Ass. U.K.* **90** (2): 267-273.

Wallace, J.C., 1973. Feeding, starvation and metabolic rate in the shore crab *Carcinus maenas*. *Mar. Biol.* **20**: 277-281.

Wang, T., Burggren, W., Nobrega, E., 1995. Metabolic, ventilatory and acid-base responses associated with specific dynamic action in the toad *Bufo marinus*. *Physiol. Zool.* **66**: 192-205.

Whiteley, N.M., Taylor, E.W., El Haj, A.J., 1996. A comparison of the metabolic cost of protein synthesis in stenothermal and eurythermal isopod crustaceans. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **271**: 1295-1303.

Whiteley, N.M., Robertson, R.F., Meagor, J., El Haj, A.J., Taylor, E.W., 2001. Protein synthesis and specific dynamic action in crustaceans: effects of temperature. *Comp. Biochem. Physiol. A* **128**: 595-606.

Whiteley, N.M., Faulkner, L.S., 2005. Temperature influences whole-animal rates of metabolism but not protein synthesis in a temperate intertidal isopod. *Physiol. Biochem. Zool.* **78** (2): 227-238.

Williams, A.B., 1984. Shrimps, lobsters and crabs of the Atlantic coast of the Eastern Unites States, Maine to Florida. Washington D.C., U.S.A.: Smithsonian Institution Press.

Willmer, P., Stone, G., Johnston, I., 2004. *Environmental Physiology of Animals (2nd ed.)*. Hoboken, New Jersey, U.S.A.: Wiley-Blackwell.

Zaidan III, F., Beaupre, S.J., 2003. Effects of body mass, meal size, fast length and temperature on specific dynamic action in the timber rattlesnake (*Crotalus horridus*). *Physiol. Biochem. Zool.* **76** (4): 447-458.