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Effects of hypersalinity on the behaviour, physiology and survival of commercially important North Sea crustaceans

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Glossary

List of abbreviations used in this thesis

NESFC	North Eastern Sea Fisheries Committee
MLS	minimum landing size
SSE	Scottish and Southern Energy
MIH	moult inhibiting hormone
EBP	early benthic phase
MARLIN	Marine Life Information Network
DEFRA	Department for Environment Food and Rural Affairs
IECS	Institute of Estuarine and Coastal Studies
CL	carapace length
CW	carapace width
SW	sea water
CHH	crustacean hyperglycaemic hormone
FIA	flow injection analysis
ICP-OES	inductively coupled plasma optical emission spectrometry
Aqb	artificial aquarium salt brine
Gcb	gas cavern brine
BTB	bromothymol blue
HCY	haemocyanin

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Abstract

Despite the increasing number of hypersaline discharges associated with desalination and, more recently, solute mining activities, there is little existing information relating to the effects such environmental disruptions may have on populations of commercially-important crustacean species. The present studies aim to redress this information-gap with novel investigations which have addressed some hypersalinity-induced behavioural and physiological responses of three crustacean species, the European lobster, *Homarus gammarus* (L), and two crab species, the brown crab, *Cancer pagurus* (L) and the velvet crab, *Necora puber* (L).

All three species feature prominently in the East Yorkshire fisheries, and are found typically in full salinity seawater environments that show little salinity variability. The development of extensive gas storage caverns underground in East Yorkshire, UK, has led to the commencement of the discharge offshore of large volumes of hypersaline brine effluent into the local, normally salinity-stable habitat of the three test species The combined volume and concentration of this discharge has the potential to raise the salinity in the local environment and these studies have focused on the possible ecological and commercial implications of such changes.

Each species was found to have a threshold value of hypersalinity above which halotaxic, preference behaviour was evoked (salinity 50 for *H. gammarus* and *N. puber* and salinity 45 for *C. pagurus*). The relationship between cardioventilatory activity and hypersalinity of *H. gammarus* and *N. puber* was examined under conditions when escape from the test salinity was prevented. Both showed a significantly decreased mean scaphognathite beat rate beyond a critical salinity value (salinity of 50 and 45 for *H. gammarus* and *N. puber* respectively). These salinities are consistent with the onset of the preference behaviour of these species. The heart rate of *H. gammarus* is also negatively related to increased salinity. These significant reductions in cardioventilation resulted in increased mortalities at salinities > 50–55. Significant changes to haemolymph pH and levels of haemolymph protein, haemocyanin, glucose and ammonia also occurred when test *H. gammarus* and *N. puber* were given sufficient time to acclimate to a test salinity. These changes made were typical of those under hypoxia in these and other decapod species and are consistent with the observed changes to the cardioventilatory behaviour.

These findings prompt the novel hypothesis that hypersaline exposure beyond limits, which vary inter-specifically, elicits a switch to anaerobic respiration, even when the animals are in a fully-oxygenated medium. Results showed that when exposed to hypersaline conditions, *H. gammarus* was a weak iono-regulator, with the haemolymph ionic concentration increasing directly with that of the external medium whilst remaining slightly hypo-ionic to it. Late-postmoult *H. gammarus* were found to be less tolerant of hypersalinity than intermoult ones and, even when the carapace was approaching full hardness, were intolerant of salinities > 40. Contrastingly, *C. pagurus* with 96h LC_{50} at a salinity of 55.5 was the most tolerant of the three species tested. The lack of significant haemolymph change in this species suggests a strong degree of osmo- and iono- regulation. Under hypersaline exposure *N. puber* regulated haemolymph variables within the range of salinity 35–50. Higher salinities were found to require a more protracted acclimation period.

The combined effects of haemolymph and cardioventilatory changes found for the test species demonstrate that unavoidable exposure to hypersaline conditions results in a lowered fitness and eventual death. Inevitably, this will impact negatively on commercial crustacean shellfisheries in and around the areas of brine discharge unless the discharge itself is managed and monitored appropriately.

Chapter 1
General Introduction

1.1 Salinity

1.1.1 Importance of salinity to aquatic organisms.

Salinity is one of the main factors affecting aquatic life. Environmental salinity is a master factor in the control of the reproduction, larval dispersal and recruitment, and geographical distribution of marine crustaceans (Anger 1991; Anger 1996; Spivak and Cuesta 2009) and hence salinity changes are likely to impact on community structure. Aquatic organisms that obtain their oxygen from the water (*e.g.* fish, crustaceans) rather than the air (*e.g.* seals, cetaceans) are adapted to the normal salinity of their environment and to taking saline water into their bodies in order to obtain the oxygen from it. Thus any change to ambient salinity has the potential to affect the ability of animals to carry out vital biological processes.

Salinity is determined by the amount of dissolved salts in water. The anion chloride is the main determinant of salinity in sea water. Traditionally salinity was measured in parts per thousand (ppt) or parts per million (ppm) – for example a salinity of 35 ppt is equal to 35 grams of salt dissolved in 1 litre of water. Now salinity is measured on the practical salinity scale, which has no units and hence salinity is just expressed as a number *e.g.* 35 (Lewis 1980; Lewis and Perkin 1980). These numbers equate to those of ppt. Thirty-five is considered as the salinity of normal sea water for the UK, however normal salinity can vary depending on factors such as location, currents, evaporation, precipitation and ice formation. At colder temperatures oxygen dissolves more readily in water and therefore colder waters have a greater oxygen saturation level. Also the higher the salinity of the water, the less oxygen it can carry (Table 1.1).

Table 1.1 The effect of temperature and salinity on oxygen saturation of sea water.

Salinity	Temperature °C	Oxygen saturation mg.L^{-1}
35	5	9.9
35	10	8.8
35	15	7.9
35	20	7.2
45	5	9.1
45	10	8.1
45	15	7.3
45	20	6.7
55	5	8.4
55	10	7.5
55	15	6.8
55	20	6.2

Created from data in Salinity Nomogram in Richards and Corwin (1956)

1.1.2 How crustaceans cope with salinity

Osmoregulation is the ability of an aquatic animal to maintain its internal fluid concentrations at an acceptable level for biological function, in response to changes in the concentration of the external media. This is very important in organisms that are faced with regular salinity challenges, such as those in estuarine areas.

Aquatic animals are either osmoconformers or osmoregulators with regard to salinity change (Kinne 1971; Schubart and Deisel 1999; Karleskint et al. 2009):

1. **Osmoconformer**: an animal that allows its internal fluid concentrations to change in line with those of the environment. They can only control the concentration of their body fluids by behavioural means, such as avoidance. Long term exposure to unfavourable salinity will often result in the death of an osmoconformer.
2. **Osmoregulator**: an animal that maintains its internal fluid concentrations at a level acceptable to the animal regardless of environmental changes such as a lowering or rising of salinity. In extremes of salinity they may need to supplement their physiological control methods by behavioural means. Osmoregulators can regulate hyper or hypo osmotically

depending on species, or both (Figure 1.1). Although the animal can regulate its body fluids, long term exposure to unfavourable salinity can still result in the death of the animal and may have impacts on its ability to grow or reproduce.

Another way of classifying salinity tolerance in aquatic organisms is according to their degree of salinity tolerance:

1. **Euryhaline**: an animal which can tolerate a range of environmental salinities and salinity fluctuations within a specified range.

2. **Stenohaline**: an animal which can tolerate only a small range of environmental salinities.

Often osmoconformers are euryhaline, but this is not always the case. Examples of different combinations include: the strong regulator crab *Eriocheir sinensis*, the weak regulator *Carcinus maenas*, both of them being euryhaline, and the stenohaline osmoconformer *Cancer pagurus* (Péqueux et al 1996).

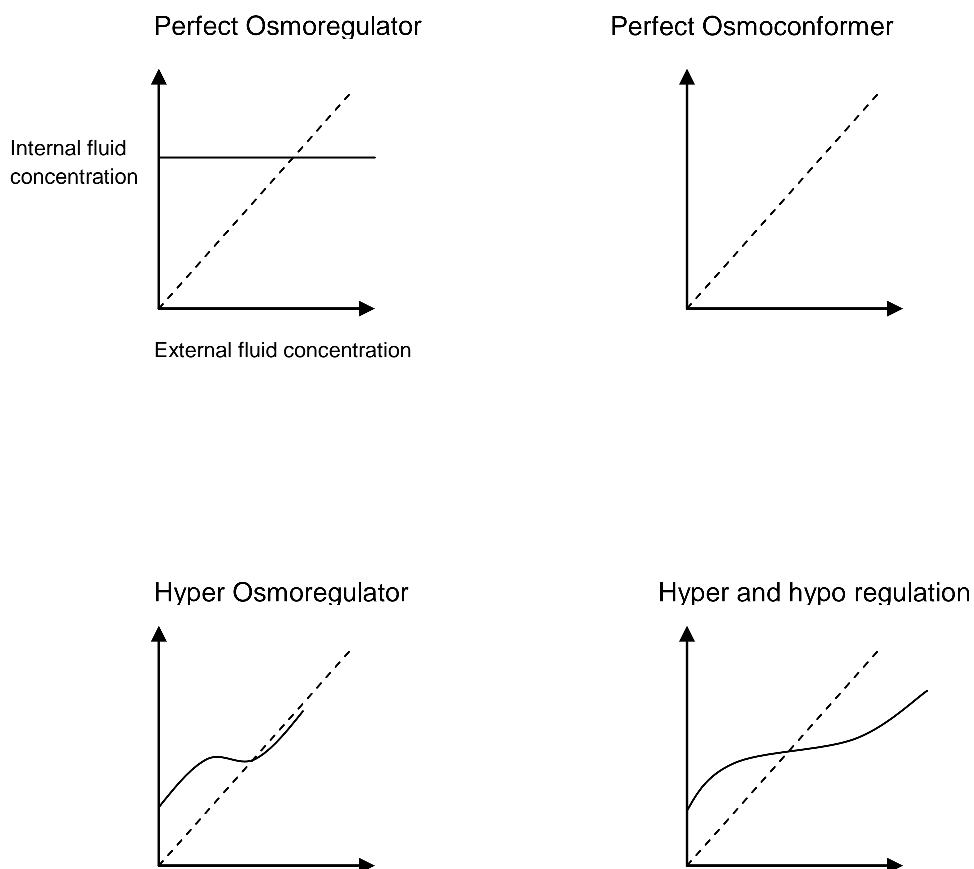

Figure 1.1 Examples of different osmoregulatory methods.

There are 5 main groups into which osmoregulation can be classified (Laverack 1985):

1. reduced permeability of body surface
2. active uptake or extrusion of ions
3. regulation of body water volume
4. conservation of salts or water by the excretory organs
5. regulation of cellular osmotic concentrations.

When dealing with salinity acclimation, the osmolality of the external medium is generally related to that of the internal environment (the haemolymph) (Péqueux 1995). Osmoregulatory capacity is the difference between the osmotic pressures of the haemolymph and of the external medium at a given salinity. In osmoregulatory crustaceans, exposure to water borne pollutants, environmental stressors and pathological agents often results in a decrease of Na^+ and Cl^- regulation and/or their osmoregulatory capacity (Lignot et al. 2000). Osmoregulation is very important in estuarine intertidal species but not so in fully marine species. Primary marine inhabitants (those that evolved in the sea) are mainly stenohaline, they live in the open sea and therefore encounter little osmotic stress resulting in a poor osmotic regulation ability. This is due to the inability of their cells to cope with or adapt to any change in body fluid composition, especially when coupled with their cells' high permeability to ions and water (Péqueux 1995). Their euryhaline relatives are found in the coastal zones where salinities can change regularly and hence rely heavily on osmotic control to regulate the concentrations of internal body fluids (Davenport 1985).

Extracellular osmoregulation is employed by fish and euryhaline crustacea and depends on salt pumps often located in the gut or gills (Davenport 1985). Many of the euryhaline crustacea and teleosts are capable of pumping salts in either direction across the gills so enabling them to expel salts when environmental salinities are high and to absorb salts when external salinities are low (Davenport 1985).

In aquatic crustaceans, especially the decapods, the primary sources of osmoregulation are the organs of the branchial chambers such as the gills, in other decapod crustaceans differentiated ion transporting epithelia are involved in osmoregulation. The tissues in these areas have a large

surface area to volume ratio as well as a large flow of water over the ionocytes (ion transporting cells) (Haond et al. 1998; Lignot et al. 2000). The gills of crustaceans are well known as having the main role in active osmoregulatory and excretory activities, being the main location for osmoregulation, acid-base regulation of the haemolymph and the excretion of the end products of nitrogen metabolism. In crustaceans that hyperosmoregulate (that is they keep the concentration of their blood higher than the external medium), the gills act as a site for active uptake of Cl^- and Na^+ ions (Péqueux 1995).

When exposed to hypersaline waters, many marine crabs in the families Ocypodidae, Grapsidae, and Varunidae regulate haemolymph NaCl at levels below the medium, apparently by excreting salts across the gill (Mantel and Farmer 1983). The brine shrimp *Artemia* is a powerful hypoosmoregulator in salinities above 30% seawater, tolerating hypersaline conditions by excreting NaCl through specialised salt glands in nauplii and through gills in adults (Croghan 1958).

There is no evidence of osmoregulatory structures in the gills of the lobster *Homarus gammarus*, but there are differentiated ion transporting epithelia in the branchial cavity, on the branchiostegite and on the epipodites which are probably used for osmoregulation (Haond et al. 1998). The European lobster *Homarus gammarus* is closely related to the American lobster *Homarus americanus* which has been shown to be a limited osmoregulator at salinities lower than 20 (Jury et al. 1994a; Haond et al. 1998). They allow their haemolymph osmolarity to drop with reducing environmental salinity but always maintain it slightly above the environmental osmolarity. At salinity 10, the metabolic rate of the animals is twice that of ambient conditions (Jury et al. 1994a).

1.1.3 Impacts of salinity change on crustacea.

Methods for surviving adverse salinity conditions include movement away from the unfavourable environment, osmoregulation or osmoconformation, and in the case of sessile and sedentary organisms, burrowing or closing shell valves. This behavioural control of internal osmotic concentrations is used by many euryhaline decapod crustaceans as a way of reducing their exposure to stressful conditions (Davenport 1985; Laverack 1985).

The effort needed by animals not moving away from unfavourable salinity change to maintain their bodies at a suitable osmolarity may have implications for their growth. Growth of an organism may

be affected by many factors, including changes to the chemical nature of its environment. Changes to the growth rate, or lack of growth completely can affect maturity, ageing and reproductive potential, as well as having implications for population structure through changes to birth and death rates (Moriarty 1993). The amount of energy left for growth of an organism after all other energy requirements are taken into account is known as “scope for growth” and is defined as the difference between the energy content of the food consumed and all energy losses apart from growth (Moriarty 1993). Environmental stressors such as salinity change may result in aquatic species needing to put more of their energy resources into regulating their internal osmolality so that their metabolic systems can function properly. It is possible that this would result in a lower growth rate and in the case of commercially important crustaceans a lower meat yield. It may also mean that it takes longer to reach a marketable size.

The tolerance of aquatic animals for salinity decreases at temperatures different from optimum, thus unfavourable salinities are best tolerated at the temperature optimum (Kinne 1964; 1971). Temperature changes may significantly affect the osmoregulatory capability of crustaceans. Hence what may be a tolerable salinity in winter months may be intolerable in the summer and vice versa. Changes in the ionic concentration of the ambient medium have also been shown to affect the tolerance of organisms to cold and heat.

The degree of physiological stress experienced by crustaceans during exposure to varying salinities may also be affected by changes in temperature. The majority of crustacean species studied show a greater tolerance to raised salinity levels at lower temperatures (see review in Kinne 1971). This effect is due in part to the impact of temperature on the osmoregulatory functions of the metabolism (Fincham and Rainbow 1988). It may be expected that at higher salinities there will be a higher oxygen demand from the animal as it requires more energy to be able to regulate its body fluids at an acceptable level, as it increases its ventilation rate and therefore metabolic demand. There is the possibility for the previously discussed (section 1.1.3) scope for growth implications over the long term due to energy going into regulation instead of growth and repair of the body tissues.

Salinity ranges encountered in the sea are not necessarily those that are tolerated for prolonged periods by animals in the laboratory. The degree of salinity tolerance is dependent upon several factors such as temperature, water movement, substratum composition, dissolved gas

concentrations, as well as food availability and competition. Deaths related to hypernormal or hyponormal salinities are caused by one or more of three main factors (Kinne 1971):

1. Functional or structural damage via osmotic phenomena manifesting at the protein, cell and tissue levels.
2. Functional or structural damage caused by changes in the relative proportions of body fluid solutes.
3. Damage caused by critical changes in the metabolic rate and/or performance.

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As water temperature increases the oxygen saturation decreases. Generally, in crustaceans, an increase in temperature causes an increase in respiration (Allan et al. 2006), so this coupled with an increase in salinity is likely to cause great physiological stress on animals that do not regularly experience the two together. Geddes (1975) found that in *Parartemia zietziana* (the Australian brine shrimp) at high temperature, an increase in the permeability of the body and the resultant stress imposed upon active regulatory mechanisms, rather than a shortage of dissolved oxygen, was responsible for a limitation in salinity tolerance at high temperatures. Salinity tolerance in the amphipod *Gmelinoides fasciatus* depends on water temperature, with highest tolerance at low temperatures (Berezina and Panov 2004). Rome et al (2005) found that in *Callinectes sapidus* (the blue crab), low temperature and salinity conditions yielded the highest mortality rates. They summarised that lower temperature tolerance at lower salinity is most likely the result of higher physiological demands and lower physiological abilities. McLusky (1969; 1970) studied the effects of temperature and salinity on the estuarine amphipod *Corophium volutator* and found the possibility of a shift in the energy requirements of different metabolic processes under osmotic and temperature stress from growth into osmoregulation.

The lowest (or highest) salinities that can be tolerated may vary within the same species, based on its geographical origin. *Carcinus maenas* (the green crab or green shore crab) from the Baltic sea can tolerate much lower salinities than those caught in the Atlantic Ocean (Péqueux 1995) due to adaptation/acclimation to the Baltic's lower salinity environment. After acclimation to hypersaline water (175% seawater) for 65 days the crab *Hemigrapsus oregonensis* (Pacific green shore crab) showed significant hypoosmotic regulation when compared to the little regulation shown after just 3 days (Gross 1963 in Laverack 1985). The effect of stressors on osmoregulation is usually detectable at sublethal levels and could be considered as a form of early warning system for sublethal stress in crustaceans (Lignot et al. 2000). Quintino et al (2008) studied the effects of brine discharges on marine fauna and showed an adverse effect in stenohaline organisms in terms of adult survival, larval abnormal development and sperm fertilisation success.

In the natural environment, osmotic stress caused by hypersaline conditions (those studied in this thesis) is normally associated with tropical and subtropical climates where evaporation occurs in shallow lagoons, mangrove swamps and salt marshes, causing increased salinity. However it can also occur in the polar regions where ice formation can cause the salinities of underlying water to increase due to the salts being expelled as water underlying the sea-ice freezes. Less well known sources of highly saline brines include deep ground waters, deep stable oceanic pools and near salt springs (Anati 1999). Hypersaline conditions can be caused by anthropogenic influences such as effluent from desalination plants and around salt pans. The main focus of previous hypersaline studies has been on the discharge from desalination plants in African and Middle Eastern areas where the scarcity of fresh water has resulted in the need for the desalination process (Meerganz von Medeazza 2005; Raventos et al. 2006).

1.1.4 Toxicological implications of salinity change

Stress is said to occur when changes to physiological (or other) processes occur which render an organism less fit for survival (Bayne 1980). When an organism is subjected to a gradient, for instance salinity, under experimental conditions, or in the field, there is usually an upper and a lower limit, beyond which the organism will not be able to survive. These are known as the upper and lower lethal limits (Figure 1.2). In toxicology, the median lethal dose, LD_{50}, or LC_{50} is the amount of substance that is required to kill half the members of a tested population in a given time

(usually 96 hours) and is an administrative standard used to describe the tolerances of species. LC_{50} figures are frequently used as a general indicator of a substance's acute toxicity. LC_{50} does not mean that all subjects will die at this concentration. Other standards used include the effective concentration used to produce a specific response (*e.g.* a behavioural movement) or effect in 50% of the population over a given time (EC_{50}), the lethal time in which 50% of a population will be killed at a specific concentration (LT_{50}) and the effective time at which 50% of a population will demonstrate a particular response or effect at a given concentration (ET_{50}) (Timbrell 1989; Moriarty 1993; Forbes and Forbes 1994). Some examples of different crustaceans' responses to salinity change are given in Table 1.2.

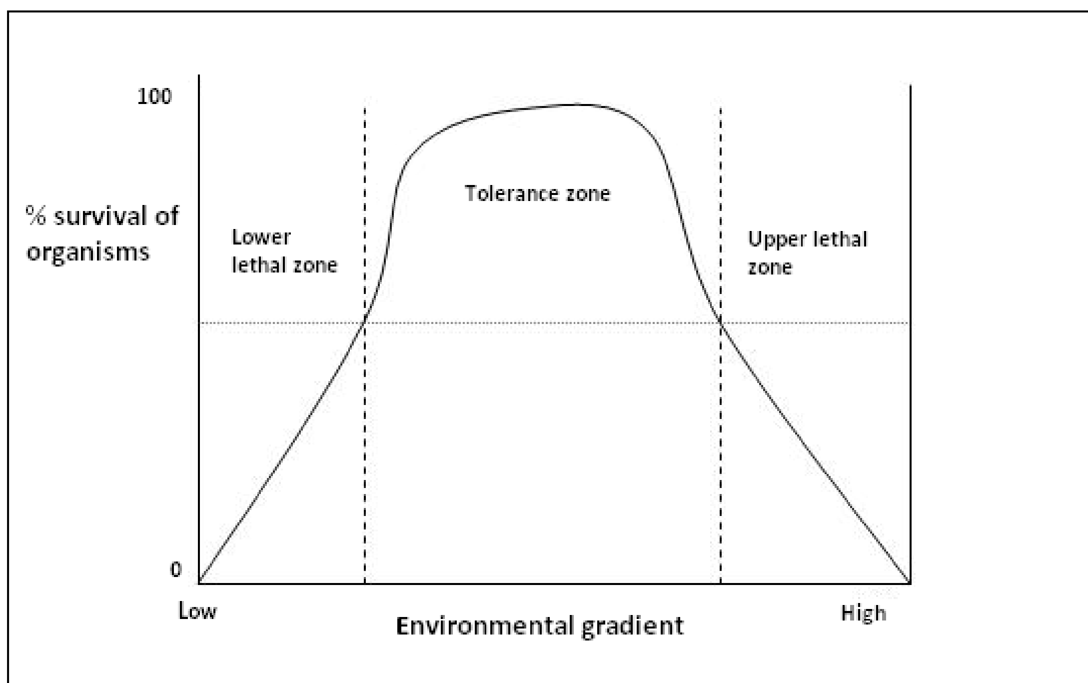


Figure 1.2 Generalised representation of tolerance, survival and lethal zones in response to an environmental gradient.

Table 1.2 Some examples of crustacean species demonstrating different salinity tolerances, responses and ranges.

Species	Tolerance / response
Armases miersii (a semi-terrestrial grapsid crab)	Salinity of 45-55 caused prolonged developmental stages as a juvenile (through a delayed metamorphosis) and increased mortality rates (Anger 1996).
Homarus americanus (American Lobster)	Has been show to be a limited osmoregulator at salinities lower than 20ppt. (Jury et al. 1994b; Haond et al. 1998) A 1-2 psu per minute reduction in salinity caused a rapid rise in heart rate once salinity decreased to 26.6 and a reduction once the salinity reached 22.1 (Dufort et al. 2001).
Pagurus bernhardus (common hermit crab)	The hermit crab *Pagurus bernhardus* has an asymmetrical response to salinity change. It will isolate itself inside its shell at a certain salinity but will not recover as soon as the salinity becomes favourable again such as in better adapted species. Instead it as to spend a long period of time in more favourable conditions before becoming active again, suggesting the body tissues are still suffering osmotic stress (Davenport 1985).
Palaemon peringueyi (caridean shrimp)	Although the caridean shrimp *Palaemon peringueyi* has been shown to be capable of growth and activity at salinities between 10 and 50, exposure to four different temperatures at salinities greater than 35 all caused an increase in the respiration rate, suggesting osmotic stress (Allan et al. 2006). At 15°C *Palaemon peringueyi* showed an increase in oxygen consumption from 0.2 to 0.9 µl/mg/wwt/h when salinity was increased from 5 to 45. This increased to nearly 1.1 µl/mg/wwt/h when temperature was increased to 30°C (Allan et al. 2006).
Scylla olivacea	Salinity had no effects on the catches of the estuarine mud crab *Scylla olivacea* in a Philippine mangrove forest (Walton et al. 2006).
Scylla paramamosain	*Scylla paramamosain* is a crab which shows a preference for estuarine habitats, and catch-per-unit-effort data indicates stable populations despite extended periods of low salinity or even freshwater conditions through a large part of the year (Le Vay et al. 2001).
Tigriopus fulvus, (a copepod)	Above a salinity of 90, the copepod *Tigriopus fulvus* falls into a state of locomotory inactivity and cannot recover after 60 hours at salinity 98 and only 3 hours in 225 salinity (Issel 1914 & Randall 1957 in Kinne 1971).

1.1.5 Salinity change as an environmental problem

Desalination of seawater is common in arid areas as a means of supplying fresh water for domestic, agricultural and industrial uses and results in a hypersaline brine discharge (Meerganz von Medeazza 2005; Raventos et al. 2006). Growing demand for fresh water and global population growth has led to increased demand for fresh water thus resulting in an increase in this process, not just in arid areas but in other areas of the globe. Desalination plants are principally located in southern areas of the northern hemisphere, where low rainfall means fresh water is less readily available (Raventos et al. 2006), for example the Middle East and the Americas. However, it is now increasing in more northern areas such as the European side of the Mediterranean Sea, and has been taking place on a small scale on the Island of Jersey since the 1970s (Jersey-Water undated). The first mainland UK desalination plant opened in London in June 2010 and the £270m centre is expected to deliver up to 140 million litres of water to 400,000 homes in times of drought (BBC-News 2010).

Solute mining, for reasons such as the storage of CO_2 and the creation of gas storage cavities in salt strata adjacent to coasts and is also a major source of brine (Dusseault et al. 2001; Shi and Durucan 2005; Quintino et al. 2008). Excavation is carried out by dissolution of the underlying salt deposits through the injection of heated seawater, resulting in the production of a hypersaline brine.

The brine generated by the desalination and solute mining processes is subsequently discharged into surrounding coastal or estuarine environments, potentially causing a rise in the ambient salinity with the concomitant impacts this may have for marine fauna.

1.2 Gas cavern construction in the Holderness area of Yorkshire

Scottish and Southern Energy Hornsea Ltd (SSE) in conjunction with Statoil Ltd is in the process of constructing an underground natural gas storage facility at Aldbrough on the Holderness coast (Fig 1.3). In Spring 2005, they started the creation of 9 caverns beneath the Holderness coastline at a depth of approximately 1800m (Proctor et al. 2006). These caverns are created by the dissolution of salt deposits in the Zechstein salt stratum underlying the coastline. Once the salt has been removed the space created will allow for the planned storage of natural gas of between 390 and 420 million m^3, supplying eight million homes a day (Reeves 2005; Statoil 2009).

The site of the caverns is located 2.5 km south-east of Aldbrough and 1.5km inland from the coast (Figure 1.3). The nine caverns are created by directional drilling from a central processing area down to the underlying salt stratum (approx 2 km deep). Heated seawater is then pumped into the boreholes to dissolve the salt and form the caverns in a process known as leaching. The initial phase of leaching the caverns will take approximately four years to complete with the first cavern expected to be ready to store gas by 2007 (Reeves 2005). Commercial operations began at the site on 1st June 2009 with capacity in another three caverns expected to become available by the end of 2010 (Statoil 2009). When fully operational (expected to be in 2012) (SSE 2011), the facility at Aldbrough will have the capacity to store up to 370 million m^3 in nine underground caverns and will be the largest onshore gas storage facility in the UK, being be able to deliver gas to the National Transmission System at a rate of 40 million m^3 per day and have up to 30 million m^3 of gas per day injected (Statoil 2009).

The success of this venture has prompted the possibility of further development of the area for gas storage and since 2007 the energy company E.ON has been applying to the East Riding of Yorkshire council to develop a 10 cavern gas storage site at Whitehall Farm near Aldbrough (EON undated).

Currently the resulting hypersaline effluent from the SSE site is being discharged under consent from the Environment Agency into the adjacent coastal waters of the North Sea (Proctor et al. 2006). The Environment Agency have set limits for the maximum temperature and salinity (dissolved solids) at which the brine can be discharged, these are 27 °C and 284 $g.L^{-1}$ respectively (Jacobs 2007) and discharges occur at or close to these limits. In 2006 SSE applied to the East Riding of Yorkshire Council to extend the project to include a further 9 caverns increasing the total

storage capacity for natural gas to 840 million m^3. Consent for this was granted in May 2007 (SSE 2006; SSE 2007). Salt caverns are typically much smaller than depleted gas field reservoirs and depleted aquifers, usually only $^{1}/_{100}$ of the volume of a depleted gas reservoir. For this reason they are especially suited for short-term storage of natural gas because of their high deliverability as well as the ability to quickly switch from injection to withdrawal (Shi and Durucan 2005). The brine contains a number of elements (Table 1.3), one of the elements of particular concern is copper which naturally occurs in the salt deposits to be leached at <5-10 µg l^{-1} and in the antifouling system used to stop faunal colonisation of the equipment at 12-25 µg l^{-1} (IECS 2004 *unpubl*). Copper is a naturally occurring part of crustacean physiology as the metallic element in their respiratory pigment haemocyanin (White and Rainbow 1985; Depledge 1989) but in high concentrations it is toxic to aquatic organisms (Flemming and Trevors 1989; Grosell et al. 2007).

Table 1.3 Composition of the Aldbrough brine discharge. Trace metals found in solution salt taken from Aldbrough No 1 borehole (IECS 2004 *Unpubl*).

Parameter	Concentration in brine discharge (µg l^{-1})
Aluminium	2 - 5 From antifouling system < 100 Naturally occurring within salt
Antimony	< 5.0
Arsenic	< 5
Boron	< 20
Cadmium	< 2.0
Chromium	< 2.0
Cobalt	< 4.0
Copper	12 - 25 From antifouling system < 5 - 10 Naturally occurring within salt
Iron	< 20
Lead	< 25
Mercury	< 0.25
Molybdenum	< 5.0
Nickel	< 5.0
Phosphorous, PO_4 as P	< 160
Selenium	< 5.0
Tin	< 10
Zinc	< 20

The E. Yorkshire diffuser is sited within an area important for commercial fishing of *Homarus gammarus, Cancer pagurus,* and *Necora puber* (Figure 1.3), the catches of which contribute significantly to the economy (Walmsley and Pawson 2007). Consequently although hypersalinity is not a common phenomenon for this region, its ecological effects may have a significant potential commercial relevance. This is true in terms of the success of fishing and post-harvest marketing operations as well as having potential impacts on larval recruitment and stock replenishment. The effluent is at a salinity of ≈284 and ≈26.3 °C which is considerably warmer than the normal range of temperatures for the area (6 °C to 14 °C winter/summer) as well as being over 8 times more saline (Cutts et al. 2004). The thermal and saline plume has been shown to extend for over 300m, suggesting that in addition to other effects of saline stress seen by animals in the area, they will have to contend with a lower level of oxygen availability (Table 1.1). The dimensions and area of the water column and seabed affected by the plume are dependent on tidal and wind action. In the year of operation 2006-2007, the maximum salinity recorded at 50m from the discharge point during routine monitoring was 47.9 psu, and at 250m of 37.1 psu (Jacobs 2007).

These coastal works have the potential to cause many environmental disturbances; this thesis focuses on the impacts of the high salinity brine discharge. This effluent is currently discharged through diffuser apparatus that is designed to help the brine disperse as quickly as possible into the surrounding waters with the aim of minimising the environmental impact. However as discussed in section 1.1, even small changes in ambient salinity can cause stenohaline organisms to alter their physiological or behavioural state. If organisms in areas around the brine discharge cannot cope with raised environmental salinity, it could result in a change in community structure through alterations in breeding patterns, larval dispersal and recruitment, food availability and both inter and intra species competition. Settlement, the stage in which a pelagic larvae metamorphose to a benthic stage (followed by the period as an early juvenile) is a critical period in the life cycle of many benthic organisms (Moksnes et al. 1998). Pressures at this stage include high predation and availability of both suitable habitat type and acceptable habitat condition. Due to this high predation pressure many crustacean larvae settle (and continue to permanently live) in areas where shelter is readily available (Cobb 1971; Smith and Herrkind 1992; Moksnes et al. 1998), for instance mussel beds, rocky areas of sea bed or seagrass beds. The area around the Aldbrough diffuser where the commercial lobster and crab fishery is located has an irregular bed topography with many small rocks and cobbles (underwater camera surveys IECS, University of Hull, *unpubl.*) which provide

shelter for adult specimens of these species. If the brine discharge was to affect this area so that the area became less favourable for settlement of both the crustaceans and their prey such as molluscs and polychaetes, the fishery could be adversely affected. Del Pilar Ruso et al (2007) found a shift in a community structure from a community characterised by polychaetes, crustaceans and molluscs to one up to 98% dominated by nematodes (an opportunistic species characteristic of high stress areas), in a soft bottomed area affected by a brine discharge.

1.3 The Holderness crustacean fishery area

1.3.1 Crustacean fishing

Since the Neolithic and Mesolithic eras, hunter gathering humans have probably eaten shellfish such as crabs and lobsters collected in the littoral or in shallow sublittoral tidal areas using primitive traps made with whatever materials were available such as reeds, wood and grasses (Mannino et al. 2007). *Cancer pagurus* was of domestic importance to the Romans and references to crab boats occur in the 12th century records of Whitby Abbey, Yorkshire (Edwards 1979). Today, the same species are still important food items, although the way they are collected has changed considerably. With the improvements of catching gears, catches have increased in volumes sufficient to allow them to be both a source of food and also an important source of income.

1.3.2 The East Yorkshire Shellfishery

All European marine fisheries are managed within the Common Fisheries Policy (CFP) which was agreed between Member States in 1983 and was reviewed and ratified by the Council of Ministers in 1992 (EC 3760/92). The Restrictive Shellfish Licensing Scheme came into effect in January 2006. Under the scheme all vessels of <10 m that have a shellfish entitlement are required to submit details of their daily landings of lobsters, crawfish, brown crabs, spider crabs, velvet swimming crabs and shore crabs together with the potting or netting effort used and the area fished every month, in much the same way as vessels >10m already did (Walmsley and Pawson 2007).

The current Yorkshire fishing fleet has both inshore and offshore fishing vessels. The inshore vessels are responsible for much of the crustacean catches. In the Humber estuary and along the coast of Lincolnshire there is a small-scale beam trawl fishery for brown shrimp (*Crangon crangon*) and pink shrimp (*Pandalus montagui*). From March onwards throughout the year potting for crab and lobster becomes very important to the static gear inshore fleet and is the main source of income for many of the Holderness region’s fishing vessels. Around the Bridlington and Whitby areas boats can put down up to 2000 pots each, going up to 40 miles offshore to lay them out. July to September is when the highest catches of *Homarus gammarus* can be obtained in this region and *Cancer pagurus* are fished for all year. Since June 1998, potting for lobsters, brown crabs,

velvet crabs and whelks within the North Eastern Sea Fisheries Committee (NESFC) district, has been by written permit only (Walmsley and Pawson 2007).

Table 1.4 gives details of the Yorkshire ports and the number of vessels that operate out of them. Bridlington is especially important regionally and nationally in terms of catches and vessel numbers (Walmsley and Pawson 2007). The fisheries for both *Homarus gammarus* and *Cancer pagurus* are the most important financially for both the Yorkshire ports (Table 1.5) and the whole of the UK shell fishing industry (Table 1.6).

Minimum landing size (MLS) is the most widely used measure in crustacean fisheries to manage stocks. It is normally set at the size at which the species matures, with the aim of allowing individuals to reproduce at least once before harvest (Tallack 2007). *Homarus gammarus* is an important commercially fished crustacean species in the UK (Lizárraga-Cubedo et al. 2003). The fishery for *H. gammarus* is the UK is regulated on the basis of a minimum landing size of 87mm set by the Department for Food Environment and Rural Affairs (DEFRA). This measurement is taken as the length of the carapace from the rear of the eye socket. Tully *et al.* (2001) found the size at which *H. gammarus* was 50% mature ranged from 92.5 mm to 96 mm, suggesting that the current minimum landing sizes are too small.

Minimum landing size of *Cancer pagurus* varies from 130 or 140 mm carapace width depending on the area the crab is caught from. These sizes were introduced on a European level in 2000. Soft shelled crabs and berried females must not be landed. In the NESFC district which governs the East Yorkshire shell fishery of this study, the minimum landing size for *Cancer pagurus* is 130mm carapace width.

In the NESFC district the minimum landing size for *Necora puber* is set at 65mm carapace width.

Figure 1.3 The fishing ports of the Holderness region, with Aldbrough included for reference. X marks the location of the gas caverns.

Table 1.4 Vessels and their principal catches for the Holderness ports.

Port	Number of vessels fishing for shellfish
Hornsea	Up to 8 beach boats of 5–7 m are active throughout the year potting for *Cancer pagurus* and *Homarus gammarus.*
Tunstall and Withernsea	Up to 15 beach boats regularly fish from this exposed coastline working up to 400 pots each for *C. pagurus* and *H. gammarus*. More vessels join during the summer.
Spurn Point, Kilnsea and Stone Creek	One beach-launched boat fishes full-time throughout the year, sets pots out a few miles offshore for *C. pagurus* and *H. gammarus*. Several part-time boats set pots during the summer months.
Bridlington	Thirty eight vessels target shellfish all year round up to 75 miles from port, each setting up to 2000 pots for *C. pagurus* and *H. gammarus*, whereas the smaller vessels in the fleet, including cobles, mini-keel-boats and fast-workers, set up to 800 pots each from spring onwards.
Filey	Five full-time cobles, launched from the beach use a variety of fishing gears including pots. From April, most work the pot fishery and some cobles each set up to 650 pots for *H. gammarus.*
Scarborough	Up to 20 small static gear boats, 16 of which set pots out to 6 miles all year round. Effort aimed at crabs has recently increased, and a few of the larger boats set pots further offshore for *C. pagurus* all year working as far south as the Wash. The *H. gammarus* fishery is busy during the summer.

Table 1.5 Volumes and values of the 2006 shellfish landings in NESFC district ports (from South Shields in the north to Tetney in the south)

Shellfish	Landed weight (t)	Landed value (£1000s)
Whelks	310	164
Gastropods		
Scallops	20	183
Cockles		
Other bivalves	0	=
Brown crab	**2,464**	**2,484**
Other crabs	**491**	**747**
Lobsters	**607**	**6,679**
Nephrops	458	1,359
Shrimps & prawns	23	40
Cephalopods	34	103
Total	**10,786**	**18,196**

From Walmsley and Pawson 2007

Table 1.6 Volumes and values of all UK shellfish landings (2005-2006)

Shellfish	Landed weight (t)	Landed value (£1000s)
Whelks	10,950	6,350
Gastropods	2	2
Scallops	5,482	10,745
Cockles	11,002	4,925
Other bivalves	10,280	1,821
Brown crab	**9,156**	**12,406**
Other crabs	**2,538**	**2,828**
Lobsters	**1,549**	**18,397**
Nephrops	2,636	9,957
Shrimps & prawns	498	1,257
Cephalopods	4,032	6,794
Total	**58,125**	**75,452**

From Walmsley and Pawson 2007

1.4 Site description

The coastline adjacent to the Aldbrough brine discharge site is made up of glacial till deposited at the end of the last ice-age (Cutts et al. 2004). The glacial till is mainly boulder clay, consisting of 72% mud, 27% sand and 1% pebbles and boulders. The soft nature and low cohesiveness of this material results in an average 2 $m.year^{-1}$ retreat of the cliff. These same materials make up the sea bed of the area, with a mainly boulder clay bottom, with patches of shingle, sand, pebbles and boulders. The erosion of this coastline is important for the accretion of sediment into the Humber Estuary where it contributes to mudflat creation, as well as for the formation of Spurn Head (Figure 1.3), the large spit that crosses the mouth of the Humber Estuary. Both the mudflats and Spurn Head play a role in flood prevention for the populous of the Humber region (Cutts et al. 2004).

The area's benthic community is characterised by species indicative of an environment subject to frequent physical disturbance such as the polychaete worm *Nephtys cirrosa*, where continual reworking of the sediments means that a climax community cannot develop. Sixty four benthic species were identified in a 2001 survey by the Institute of Estuarine and Coastal Studies (IECS) (*unpubl.* data), but this high diversity was not reflected by high abundance. Allowing for natural variability, this number is relatively consistent over time with 55 recorded species in 2004 and 73 in 2005 (Mazik and Allen 2006). An epifaunal survey (Proctor and Musk 2004) showed 39 species of epifaunal invertebrate, 33% were mobile crustacean species that accounted for over 90% of the total epifaunal abundance.

Homarus gammarus is the second most common crustacean species (the first being *Crangon crangon*) in the Aldbrough brine discharge area, making up 24% of the total crustacean assemblage (Cutts et al. 2004; Proctor and Musk 2004). Less than 3% were above the minimum landing size of 87 mm carapace length, demonstrating the importance of this region's near-shore habitats for sub-adult populations and recruitment for adult populations which support the local fishing industry (Cutts et al. 2004). The same survey found that in the study area, *Necora puber* composed 9% of the total crustacean assemblage. The proportion of the assemblage attributed to *Cancer pagurus* is unknown due to the absence of quantitative data, however it is clear from personal observations made at local ports such as Bridlington, that the species is a key one for commercial fishing operations in the area.

1.5 Biology of species studied

1.5.1 Species to be studied

This study focuses on the crustacean species *Homarus gammarus* (the European lobster), *Cancer pagurus* (the brown [or edible] crab) and *Necora puber* (the velvet crab), as these are the key species fished for by vessels in the area around the brine discharge, as well as being the most economically important. Chapter 3 also uses *Carcinus maenas* (the green shore crab) and *Pagurus bernhardus* (the common hermit crab) to provide an intertidal species comparison for salinity preference, however these are not important commercially.

1.5.2 Habitat and distribution – *Homarus gammarus*

Homarus gammarus (Figure 1.4) (syn: *Homarus vulgaris.* Common names: European Lobster or Common Lobster) is found in waters from the Arctic Circle to the Mediterranean, with the highest abundances around Norway, France, Ireland and the UK (for UK distribution see Figure 1.5) with the main fishing effort historically taking place along the east coast (Richards and Wickins 1979; Spence 1989).

Figure 1.4 *Homarus gammarus*

Figure 1.5 The distribution of *Homarus gammarus* around the British Isles (from Marlin.ac.uk)

The main habitat of *Homarus gammarus* is rocky sea beds down to a depth of around 100m. The distribution is related to sea bed topography, with distribution being limited to areas with rocky

outcrops, wrecks, piers etc. This is commonly attributed to the lobster's need for shelter during vulnerable periods of its life such as the moult but may actually be due to the need to avoid and shelter from tidal currents (Howard and Nunny 1983). Lack of shelter in sandy or muddy flat areas may be a limiting factor in the distribution of the species (Cobb 1971). Lobsters tend to be nocturnal and have been shown to be significantly more active during the hours of darkness (Mehrtens et al. 2006). During daylight hours, if no shelter is available, captively held lobsters will retreat to the most shaded or enclosed areas of tanks such as the corners (O'Farrell 1966), a habit which has also been noticed in this study. There are some reports of juvenile *H. gammarus* utilising the softer substrata such as mud and sand to construct tunnels similar to those of *Nephrops norvegicus* (Richards and Wickins 1979).

Large adult *Homarus gammarus* tend not to be found towards the edge of the continental shelf, but individuals can be found tens of kilometres offshore in areas of suitable habitat such as wrecks (Bannister & Addison 1995 in Smith et al 2001.) The reproduction of these may help sustain inshore populations. Natural mortality in lobsters > 80mm CL has been estimated at 10 – 22% year $^{-1}$ (Hepper 1978 and Bannister 1986) in Smith et al (2001).

1.5.3 Behaviour and physiology – *Homarus gammarus*

Lobsters belong to the phylum Arthropda and they have a hard exoskeleton made of calcified chitin. The head and thorax are fused to the carapace, and the abdomen (commonly known as lobster tail) posterior to the carapace contains 6 articulated segments (Figure 1.6). The abdomen is utilised as a behavioural adaptation for escaping from predators by using rapid tailflips to propel the lobster backwards. The antennae are used to sense vibrations in the environment and shorter antennules are used to detect chemical changes in the environment (Spence 1989).

H. gammarus are primarily nocturnal and emerge at night to hunt for food including fish, other crustaceans, molluscs, polychaete worms and possibly plankton (Forsyth 1960; Ingram 1985), returning to shelter with increased light levels (Richards and Wickins 1979). It has been suggested that *H. gammarus* is a central base forager (Smith et al. 2001), having a home shelter or group of shelters and returning to this after excursions to look for a mate, food etc. The limited movement of *H. gammarus* (Smith et al. 2001) may be tied with this need for shelter. This is in contrast to the closely related *Homarus americanus* which is well known to undertake seasonal migrations of

hundreds of kilometres. The type of benthic environment available to settling juvenile lobsters in the early benthic phase (EBP) of their life cycle appears to have a marked effect on their survival. In field experiments EBP *H. gammarus* had significantly greater chance of survival from predation when presented with shelter providing substrata such as cobbles (Ball et al. 2001).

The moulting period of a lobster is a critical stage in its life history as during this time the organism is soft shelled and therefore susceptible to both predation and physical damage. During this time the physiology of the animal changes, which may be extensive enough to alter its resistance to environmental stressors. The survival of soft lobsters to lowering of salinity and dissolved oxygen, and rising temperatures was found to be lower than that of hard shelled lobsters (McLeese 1956).

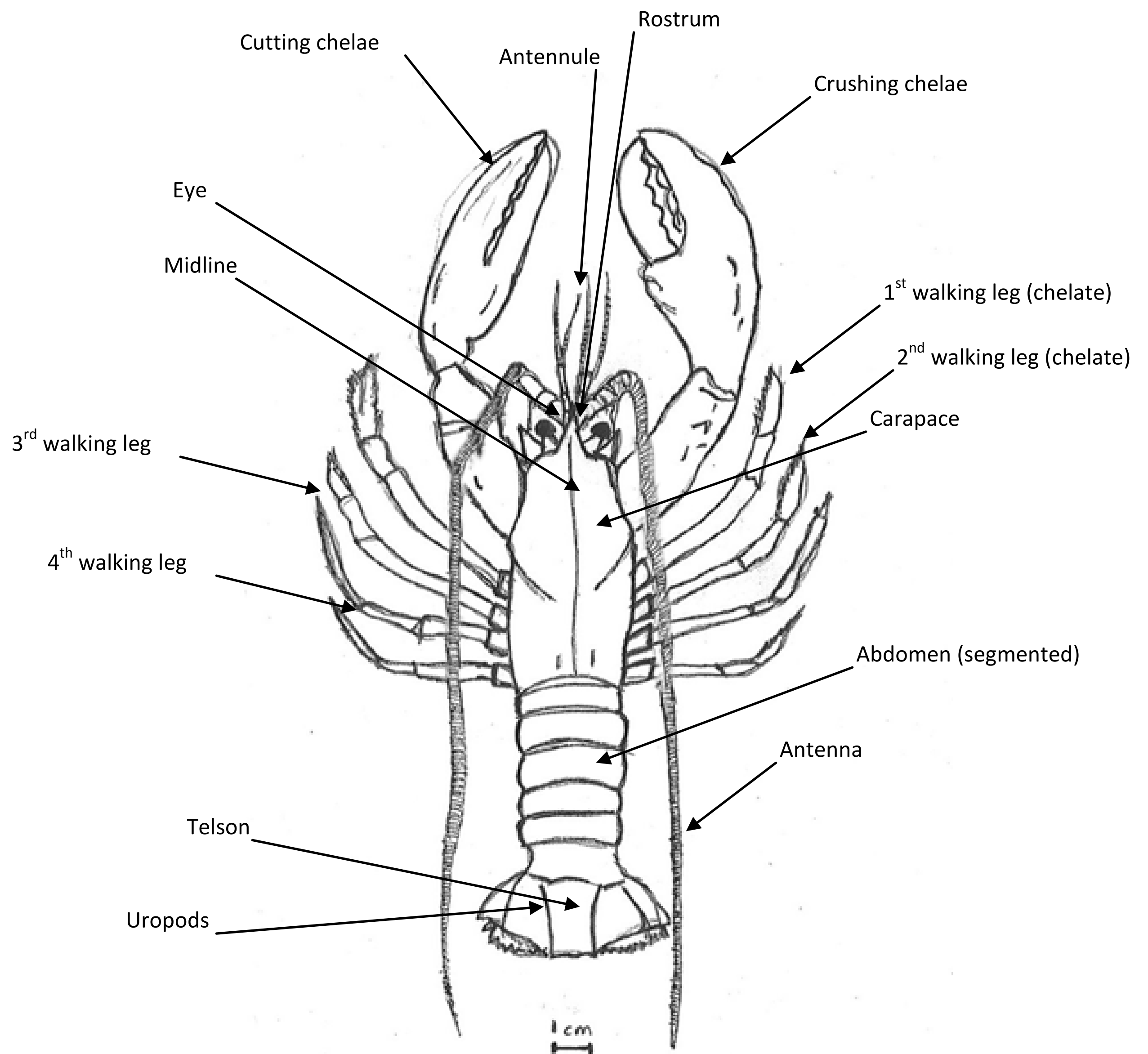


Figure 1.6 ***Homarus gammarus*** **showing the main body features. Dorsal view. (drawn by author)**

1.5.4 Habitat and distribution – *Cancer pagurus*.

Cancer pagurus (common names; brown crab, edible crab) (Figure 1.7) has a similar distribution to *H. gammarus* but is found mainly in North West Europe. It is found around the whole coastline of the British Isles (Figure 1.8), where it is a common member of the subtidal community (Hall et al. 1991). The main habitat is rocky or stony sea beds down to a depth of around 100m with higher abundance where more shelters are available, although the species can also be found on softer grounds such as sand and mud. *C. pagurus* is able to partially bury itself in unconsolidated sediments, offering low resistance to tidal currents (Howard and Nunny 1983).

Figure 1.7 *Cancer pagurus*

Figure 1.8 Distribution of *Cancer pagurus* around the British Isles (from marlin.ac.uk).

1.5.5 Behaviour and physiology – *Cancer pagurus*

In general, the anatomy of *C. pagurus* is loosely similar to that of *H. gammarus* in that it has a head, thorax and articulated abdomen all covered in a calcified exoskeleton. In *C. pagurus* however, the abdomen is curled underneath and the whole body is therefore protected by the carapace, taking an oval, rather than elongated, form (Figure 1.9). Both of the chelae are used for crushing and as the crab possesses no other claw appendages, if it loses both claws it is very difficult to eat as food cannot be dissected and passed to the mouth.

When inhabiting softer benthic habitats, the main prey for this species appears to be large bivalves (Shelton et al. 1979), excavating pits in order to obtain them (Hall et al. 1991). *C. pagurus* is also known to feed on other decapod crustaceans including *Galathea squamifera, Pilumnus hirtellus, Pisidia longicornis* and *Porcellana platycheles* (Lawton 1989). The main moulting period on the east coast of England is July-August (Edwards 1966), however on the south coast the moult period is less well defined with soft crabs occurring throughout the year (Bennett and Brown 1970).

Cancer pagurus is a commercially important species in Europe (Tully et al. 2006; Stentiford 2008; Barrento et al. 2011). Adult crabs undertake extensive migrations, which may be associated with the reproductive cycle (Tully et al. 2006).

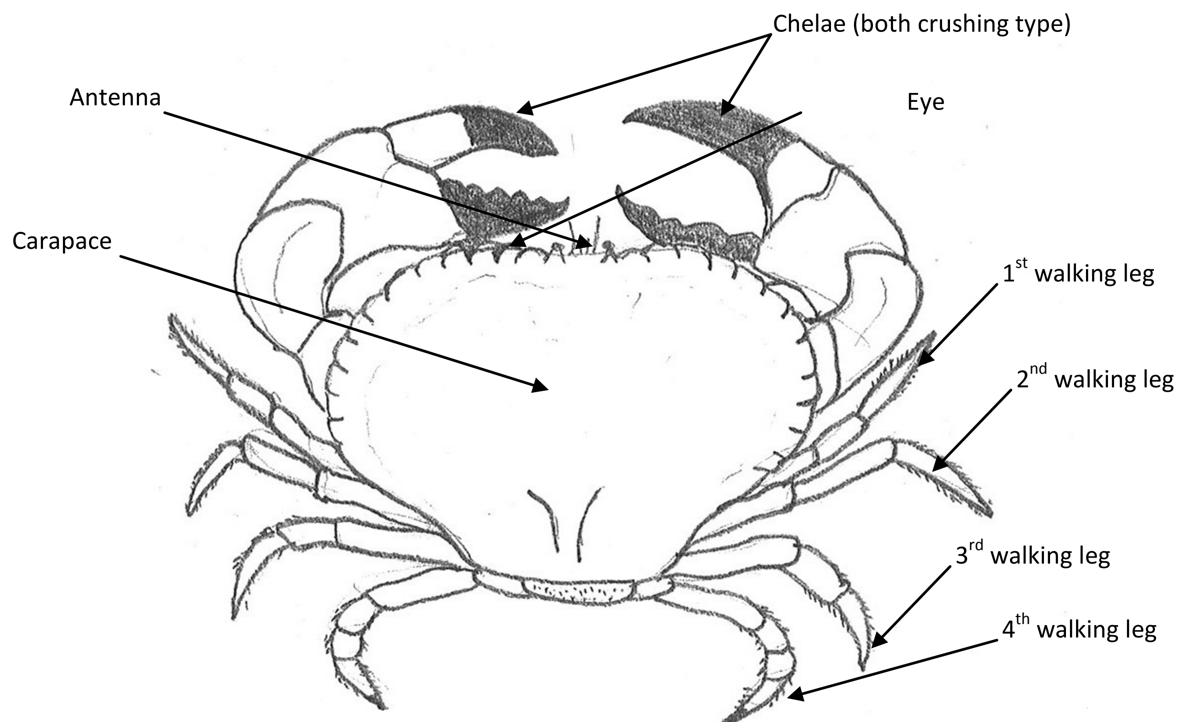


Figure 1.9 Diagram showing the main features of *Cancer pagurus,* Dorsal view. (drawn by author).

1.5.6 Habitat and distribution – *Necora puber*

Necora puber (Figure 1.10) (syn: *Liocarcinus puber*, *Portunus puber, Macropipus puber,* common names: the velvet crab, the velvet swimming crab or the red eyed crab), is a commercially fished species, found all over north-west Europe, most commonly found on stony and rocky substrata on the lower shore and in shallow sublittoral water. It occurs in its highest abundances on moderately sheltered shores all around the British Isles (Wilson 1999) (Figure 1.11) and can be found down to depths of about 80 m (Fish and Fish 1996). There is an absence of published literature stating this crab's presence in the Yorkshire area. According to information compiled by the Marine Life Information Network (MARLIN) *N. puber* is not found off the Holderness coastline (Figure 1.11), however many specimens have been found on both the sandy beach and rocky shore at Filey, (*Pers. obs.*) and the crab is fished for commercially from Bridlington southwards to the Aldbrough diffuser. This is evidence of increasing numbers locally which is important for the UK fishery and also perhaps indicative of warming of the North Sea. *N. puber* originates from the south-western corner of Europe, namely the coastline of Spain and Portugal (Wicks 2004; Pollard 2008). It has been considered as an invasive species in UK waters, although whether its presence is due to an extension of its range due to changing biogeographic factors or due to unintentional introduction by man is unclear (Wicks 2004; Pollard 2008). Changing consumer demand in the 1970s changed this species from a pest that stripped pots of their bait and damaged catches (Hearn 2002) to commercially fished species (Harwood 2000). Recent catches in the Holderness and Wash regions may therefore be evidence of climate change as the water warms up enough for *N. puber* to be establish itself in these previously unfavourable environments. It has already been suggested that the increase in swimming crab larvae from the decapod subfamily *polybiinae* in the North Sea is indicative of climate amplification (Lindley et al. 2010)

Although there is little market for *N. puber* in the United Kingdom, it is a commonly eaten food in Spain, where most of the UK catch is transported to. In the Shetland fishery a much higher meat yield is obtained from male *N. puber* rather than females (Tallack 2007). *N. puber* is much less robust to handling and environmental change than *C. pagurus* and *Carcinus maenas* (the shore or green crab) (Wyman et al. 1985).

Figure 1.10 ***Necora puber***

Figure 1.11 Map showing the distribution of *Necora puber* around the British Isles (from marlin.ac.uk)

1.5.7 Behaviour and physiology – *Necora puber*

N. puber has a generally high metabolic rate compared with brown or spider crabs, which is increased further by the stresses of handling and packing. An average sized velvet crab can pump around 1 litre of water over its gills per minute (Cumberlidge and Uglow 1977a), indicating the quantity of clean aerated sea water needed to keep an animal in an optimum condition. *N. puber* quickly becomes stressed under conditions which *H. gammarus* and *C. pagurus* would readily tolerate, such as during shipping and transportation and it is a very aggressive species so holding it in large numbers can be additionally difficult due to it attacking others in the tank. Even under favourable conditions it is the largest (and therefore the most commercially valuable) specimens of *N. puber* that succumb first to stress (Wyman et al. 1985). The high metabolic rate and susceptibility of this species to stress during the commercial harvesting process is indicative that it may also respond poorly to natural, environmental stresses such as changes in salinity, pollution, disturbance *etc*.

N. puber is not considered as an intertidal species, although they may be found low down on rocky shores and some sandy shores at low tide they do not tolerate exposure. The anatomy of *N. puber* is very similar to that of *C. pagurus*. The whole body is protected by the carapace and takes the oval shape (Figure 1.12). Both of the chelae are the crushing type and as the crab possesses no

other claw appendages, if it loses both claws it is difficult to eat as food cannot be dissected and passed to the mouth. The fourth pair of periopods has the dactyls flattened out into paddles which the crab uses to swim. The carapace is covered in short hairs which give it a soft texture hence the common name “velvet crab”.

The main time for feeding in *N. puber* is at night, but to a lesser extent during the day when they are covered by the tide. Adult, wild caught *N. puber* mostly eat brown algae, especially *Laminaria* and *Fucus*. This is followed by other crustaceans, especially other crabs and barnacles, followed by the mussel *Mytilus edulis*. However when in laboratory conditions the food preference was found to be as such crustaceans>mussels>algae with only severely starved (> 5 days) crabs eating the algae, however an adult crab of carapace width 70mm could eat a 30x50mm piece of *Laminaria* in <3 minutes (Choy 1986). Both adult and juvenile *N. puber* were found by Choy (1986) to have empty stomachs during the winter especially berried females from the littoral zone. Also recently moulted animals had empty stomachs.

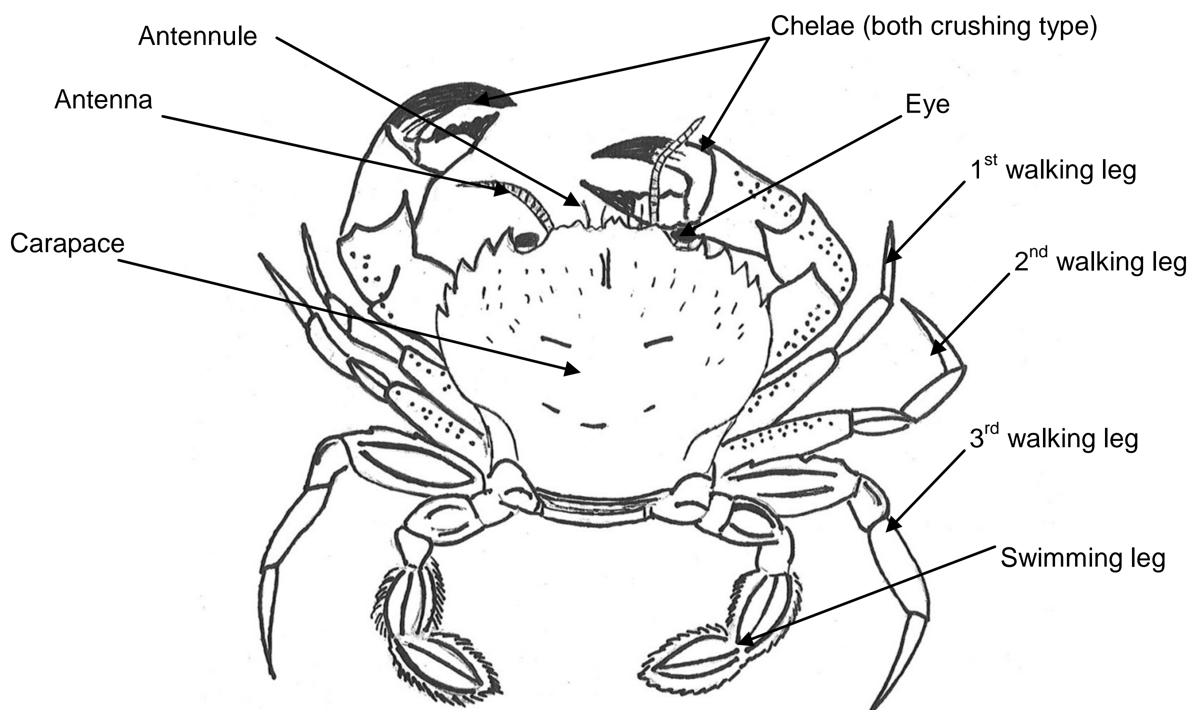


Figure 1.12 Diagram of the main features of *Necora puber*. Dorsal view (drawn by author).

1.5.8 Salinity tolerances in the test species

The adults of the three species tested, *H. gammarus, C. pagurus* and *N. puber* are all widely considered as being principally subtidal species, with adults only occasionally occurring out of water in the lower littoral zone (*pers obs.*). *C. pagurus* (Péqueux 1995) and the portunid crab *N. puber* (Dorgelo 1979), are considered to be osmoconformers and there is some evidence to suggest that despite being a subtidal species *H. gammarus* is a limited osmoregulator (Charmantier et al. 1984).

There is a lack of existing data on the salinity preferences and tolerances of these three species in the literature, especially for *C. pagurus* and *N. puber*. Whilst for *H. gammarus* there is a some information known about its responses to lowered environmental salinity (hyposalinity) (Charmantier et al. 1984; Lucu and Devesconi 1999; Torres et al. 2002; Pavicic-Hamer et al. 2003) there is almost nothing that describes how these species react in hypersaline waters (Charmantier et al. 1984). It is known that *H. gammarus* has been successfully acclimated down to salinity 20 in the lab (Lucu and Devesconi 1999) and in juveniles reared at 15°C, mortality occurs only in salinities below 17 and above 46 with regulation being isosmotic in high salinities, and slightly hyperosmotic in low salinities (Charmantier et al. 1984). In the related *H. americanus* there was an almost linear increase in oxygen consumption, heart and scaphognathite beat rates in animals exposed to dilute seawater, with almost a twofold increase in metabolic rate when animals were moved from salinity 20 to 15 to 10. It has been shown that after acclimation to a low salinity of 25 from salinity 33 *N. puber* can reduce its apparent water permeability (Rainbow and Black 2001) possibly as an adaptive measure for coping with a more dilute environment.

The combination of salinity change and other environmental factors is also known to have an effect on these species. In *H. americanus,* lead exposure was shown to override some of the normal adaptive adjustments to hyposalinity when compared with control animals (Gould and Greig 1983). Dall (1970) used *H. americanus* for osmoregulatory studies because considerable hyperosmoregulation had been observed at the lower end of its salinity tolerance range. In the same species haemolymph glucose, crustacean hyperglycaemic hormone (CHH), lactate, total protein, cholesterol, triglycerides, chloride and calcium concentration, pH and density were all influenced negatively by high temperature both in average of alteration from the physiological value and in recovering time (Lorenzon et al. 2007).

Further adaptations and tolerances for salinity for each species are discussed in detail in subsequent chapters.

1.6 Crustacean stress, health and metabolism

Crustaceans have aquatic, terrestrial and semi terrestrial representatives and so have had to adapt to the huge physical and chemical diversity of these environments. Brackish, estuarine and intertidal environments are probably among the most stressful aquatic habitats, and the establishment of crustaceans in these environments implies highly adapted physiological features (Péqueux 1995). The stronger the adaptive response, the more likely the detection of stress-induced change in that response (Gould and Greig 1983). The species used in this study are found in a fully marine, sublittoral and therefore normally stable environment, however crustaceans are very sensitive to changes in water quality, chemistry, light and temperature. It is important that these balances are maintained in order for them to survive in the aquarium or outside their natural environment. Each species has its own tolerances to levels of applied stressors such as temperature, oxygen saturation, pH and toxins. *Cancer pagurus* and *Carcinus maenas* have a much higher tolerance to these than *Necora puber* (Bernasconi 2006). Environmental stressors of this nature may rapidly become lethal to some species and can negatively affect their quality and hence the market price. Intertidal species generally have a greater range of environmental tolerances than subtidal species. When an environmental stressor such as salinity moves outside the limits of homeostasis the organism adapts its metabolism to cope and this increase or decrease in metabolic processes is what can eventually lead to death, or sublethal impacts decreasing the marketability. Sublethal effects may impact the long term sustainability of populations (and therefore the commercial market) by affecting growth and reproduction.

Increases in oxygen consumption as salinity is reduced (or increased maybe) may be due to increased energy needed to actively pump ions needed to maintain homeostasis. It is uncertain whether animals with higher respiration rates would be able to survive in these unfavourable conditions for any length of time and may need to migrate into areas of more ambient salinity to survive. This will be one of the factors addressed in this thesis.

1.6.1 Cardioventilatory activity

Blood circulation in decapod crustaceans is achieved by rhythmic contractions of a single-chambered, neurogenic heart. Heart rates between 15-150 beats per minute (bpm) are not unusual in individuals however rates are normally at levels between these extremes (Cumberlidge and

Uglow 1977a). Heart rate is typically elevated in response to physical and environmental stress and this ability to increase respiration rate is probably a factor in the ability of crustaceans to escape from predators or unfavourable conditions. Variability in cardiac activity has been shown to be an indicator of the Darwinian fitness of decapods (Depledge and Lundebye 1996), and therefore their physical ability to cope with changing environmental gradients and high variability in any measured behaviour or parameter may itself be an indicator of stress as animals do not necessarily respond in a uniform way to environmental changes.

Branchial irrigation in decapod crustaceans is maintained by the pumping action of the scaphognathites. The scaphognathites are enlarged blade shaped exopodites of the second maxillae, one in each of the left and right branchial chambers. They are capable of both synchronous and independent activity (Cumberlidge and Uglow 1977a). In crustaceans the scaphognathites pump water over the gills in a forward direction in most species including the lobsters, but this is interrupted at irregular intervals by reversed beats which propel water backward over the gills (Wilkens and McMahon 1972). The scaphognathite in *Homarus americanus* moves as a rigid blade which does not flex but instead effects water propulsion by changing its angle of attack during each half beat (Wilkens and McMahon 1972). Change in the cardioventilatory activity of crustaceans is used as a sublethal indicator of the impact of environmental change (Ansell 1973; Cumberlidge and Uglow 1977a; Walters and Uglow 1981).

1.6.2 Autotomy

This is the ability to regrow lost limbs and is an adaptation that is shared by all the crustaceans included in this study. Under stressful conditions, a limb can be shed and a new one is grown in replacement, *e.g*. due to injury, or to aid escape from a predator. The limb is lost at a fracture plane at its base which is covered in a thin membrane which has a hole in the centre through which blood passes into the rest of the limb, but on fracture this hole is quickly sealed by a blood clot. In a premoult animal, the new limb grows out of the old stump and is soft and encased in a membrane, only becoming hard when the animal moults. The new limb will eventually attain a size that is slightly smaller than the previous one (Spence 1989). If an animal is already experiencing environmental stress in the form of changes to salinity, pollution etc, it might have an impact on the ability of the animal to complete this process successfully, if the fracture plane is not closed quickly

pathogens could enter the body. In addition if energy is going into maintaining homeostasis under environmental stress, there will be less available for growth of a new limb at the next moult, if the onset of the moult is not delayed by the stressor. As the main feeding appendages of the lobster are the subchelate walking legs, loss of the crushing and/or cutting claw (which are used mainly for defence and opening hard food) does not mean starvation (Ingram 1985). However in crabs the chelae are used for feeding and loss of one or both can lead to malnutrition and eventually death.

1.6.3 Haemolymph O_2 affinity and anaerobic metabolism in crustaceans

Hypersaline conditions have already been shown to affect mobility in both *H. gammarus* and *C. pagurus* (Macdonald and Elliott 2005). If this reduction of mobility also involves the scaphognathites (gill bailers), it could mean that even in a fully oxygenated media, the crustacean could face an internal hypoxia as it cannot draw enough water across the gills for respiration, thus affecting the acid base balance of the haemolymph. This hypothesis is investigated in Chapter 6.

Crustaceans are ectothermic, gaining most of their heat from their environment. For most ectothermic animals, the colder the environment the less active they are, both in terms of behavioural activity and internal processes such as metabolism and respiration. In crustaceans metabolic rates are determined largely by external temperatures. Within lethal limits the higher the temperature the higher the metabolic rate and the more rapidly animals will become distressed by external stressors such as a low oxygen supply (Wyman et al. 1985), water borne pollutants, or environmental salinity change (*i.e.* hypersalinity). Stress of this kind often induces anaerobic respiration which results in the production of lactic acid and the build up of lactate in the blood and tissues as a shortage of oxygen means the tissues respire inefficiently. Lactate is a toxic substance which, if high concentrations persist, will ultimately kill the animal, but before that will reduce its survival and its condition so as to make it a less attractive commercial product (Wyman et al. 1985). Therefore the raised temperature of hypersaline discharges is a concern with regards to the metabolic processes of affected species. Lactate can also accumulate under conditions other than aerial exposure, such as during exercise. $_{L}$-Lactate is the primary metabolic acid product produced by decapod crustaceans and exercise induced acidosis is caused almost totally by $_{L}$-Lactate (McDonald et al. 1979; Wood and Randall 1981).

1.6.4 Nitrogen metabolism in crustaceans

Ammonia is the primary nitrogenous waste excreted by aquatic crustaceans and is excreted constantly via the gills by a passive diffusion into the water in the branchial chamber. Here the water in is continuously renewed, so protecting the animal from the toxic effects of high ammonia levels (Durand et al. 1999). Other important waste products are urea and uric acid. Some minor excretory products of nitrogen metabolism are guanine, trimethylamine oxide (TMAO), creatine, creatinine and amino acids. Increased ammonia production may indicate a higher rate of protein metabolism (Emerson 1969) and elevated ammonia levels in *H. americanus* in low salinity media have been shown to disrupt ionoregulatory functions (Young-Lai et al. 1991). So it is important that crustaceans have an effective ammonia excretion or detoxification system in order to iono-osmoregulate efficiently and respond to changing environmental conditions (*i.e.* salinity).

Quantifying the changes in ammonia in crustaceans in response to hyper/hypo salinity, shows the initiation of changes in the physiology in response to environmental change and is considered as indicative of a sublethal response to stress in aquatic organisms. Therefore assessing ammonia production in crustaceans in response to changing environmental variables (*i.e.* salinity) may be a way of assessing not only their tolerance levels, but also the degree of stress imposed.

For a comprehensive review of the effects of ammonia on the body (though not just applicable to crustacea) see Wright (1995), but briefly summarised they are:

- Modification of the properties of the blood brain barrier and disruption of cerebral blood flow.
- NH_4^+ can directly substitute itself for K^+ in nerve conduction.
- Interfere with transport of amino acids and impede the process of amino acid excretion
- Cause mutations in astrocytes and neurons (nervous system/brain tissues).
- Alter carbohydrate and fat metabolism
- Alter ATP levels in the brain and other tissues

Many aquatic species also can be uricotelic (they can produce uric acid as well as ammonia). They can switch to an alternative biochemical pathway so that they produce alternate forms of waste to ammonia in higher amounts. This is an energetically expensive option but other forms of waste

such as urea and uric acid are less toxic to the organism if they build up in the body (Uglow and Williams 2001).

Hence it is important to establish how nitrogen metabolism, especially ammonia production and excretion is affected by changing environmental salinity in order to determine the potential sublethal impacts that may occur in salinity stressed species.

1.7 Aims, objectives, hypotheses and structure of the thesis

1.7.1 Aims and objectives

The discharge of any effluent into the marine environment is of concern as any change to what is normally a relatively (within the boundaries of normal fluctuation) stable environment has the potential to impact on many of the biotic and abiotic components of that ecosystem. As the area around the Aldbrough brine discharge site is an important commercial fishery, any impacts the brine may have on the commercially fished species need to be assessed. This information is required, not only for the local discharge but for helping to predict the impacts of any brine discharge in temperate regions.

The main aim of this investigation is to determine whether, and if so how, commercially important crustaceans will respond, both behaviourally and physiologically, to a hypersaline discharge that causes an increase in the osmotic concentration of their environment. Both sublethal and lethal toxicity testing will also be carried out in which elevated salinity is the toxicant.

The objectives of this study are as follows:

1. to assess the behavioural salinity preferences of the animals;
2. to assess both the behavioural and physiological salinity tolerances of the animals;
3. to determine the salinity concentrations that cause 50% (LC_{50}) of the animals to die when acutely exposed (as may happen in field situations), the time it takes in a given hypersalinity to cause a 50% mortality (LT_{50});
4. to assess the responses of the study species to hypersaline media, as shown by body movements and changes in heart and scaphognathite beat patterns;
5. to predict the impact on commercially important crustacean species in areas affected by a brine discharge.

Individual hypotheses are given in each individual chapter. The brief outline of each chapter is as follows:

1.7.2 Structure of the thesis

- **Chapter 2** gives an overview of the general methodology used, with more detail given in the relevant chapters as needed.

- **Chapter 3** examines at the behavioural responses of the study species when given a choice of different environmental salinities. The data have been used to determine behavioural preferences and salinity ranges.

- **Chapter 4** examines the mortality rates and times for *Homarus gammarus, Cancer pagurus* and *Necora puber* in different hypersaline concentrations.

- **Chapter 5** takes the information obtained in chapters 3 and 4 and examines the cause of reactions observed by analysing haemolymph samples from animals subjected to both hypo and hypersaline environments (focusing mainly on the hypersaline). The crustaceans have been both acclimated to hypersalinity and acutely exposed to hypersaline media with no prior acclimation – to gauge their physiological reactions.

- **Chapter 6** further investigates the responses to hypersalinity by looking at the concealed responses that cannot be seen by eye. The heart and scaphognathite beats of *Homarus. gammarus* and *Necora puber* were analysed during acclimation to hypersaline conditions.

- **Chapter 7** the data from chapter 6 is related to the results obtained in chapters 3, 4 and 5 to give an overall view of what exactly happens to the study species when exposed to hypersaline conditions. This is then related to the results of chapter 6 to make predictions of what might happen to commercially important crustacean species subjected to raised salinity environments (such as the Holderness brine discharge) in temperate regions of the world.

Chapter 2

General Methodology

Methods common to all chapters

2.1 Supply and husbandry of experimental animals.

Although most crustaceans have a hard exoskeleton that protects them from most surface wounds and that helps to prevent the entrance of microorganisms, normal fishing and handling operations produce a high risk of damage occurring, including fracturing and puncturing of the exoskeleton and more commonly, limb loss. Stress-inducing post capture conditions are experienced by crustaceans. The most stressful of these is disturbance, both physical and visual, which can cause a large amount of stress (Paterson et al. 1993).

Hosie (1993) constructed a damage index (Table 2.1) and an activity index (Table 2.2) based on a study of *Cancer pagurus* and the physical damage it sustains during live transport as a way of assessing the viability of specimens for experimental use. These indices have been slightly modified and used here for the same purpose as well as to assess the health of those undergoing treatment.

Specimens were obtained from the local fishery at Bridlington harbour, or collected from the local rocky shore at Filey. Animals were transported dry with damp paper and ice packs to minimise temperature fluctuations and to lower their metabolic rates during transport. Intermoult adults, both male and non-ovigerous females were used. *Homarus gammarus* in the late-postmoult stage was used additionally in one of the trials (Chapter 5) to assess the impacts of hypersalinity on part of the moult cycle.

On arrival at the laboratory the animals were checked for mortalities and their viability assessed using the damage index and activity index described above. Creel or hand-collected rather than trawl caught animals were used for this study due to the lower likelihood of damage. Only animals scoring a 1 or 2 on the activity index were chosen for this study, with most of animals scoring a 1. Damaged animals were used if they scored sufficiently high on the index (1–2) and also scored well on the activity index (1).

Table 2.1 Damage index used for grading experimental crustaceans (after Hosie 1993)

Damage Index
1. Broken dactyls - (the final segment on the walking legs)
2. Damaged or torn joints (including damage to scar tissue where legs have been lost)
3. Broken or snapped legs and/or claws
4. Cracked/punctured or otherwise damaged carapace
5. Damage to other body parts, e.g. abdomen, mouthparts.

Table 2.2 Activity index used for grading experimental crustaceans (modified from Hosie 1993)

Activity Index	Description
1. Strong	Strong limb movement, no drooping of limbs
2. Medium	Limb and claw movement, relatively strong but with slight drooping of limbs
3. Weak	Minimal limb movement, drooping of limbs but slight strength in claws. Mouthparts slightly open
4. Moribund (approaching death)	No limb or claw movement. Complete hanging of all limbs, eyes sunken, mouthparts open. Some movement of antennae or mouthparts. Pale carapace colour. Blood can still be drawn from walking legs with a syringe.
5. Dead	As above (4) but no movement at all. Blood impossible to draw from legs with a syringe.

Surviving and viable animals were put into tanks to acclimatise and recover from their capture. The animals were stored in the aquarium in sea water obtained via tanker lorry from Bridlington which has a salinity of 35, in large opaque plastic tanks with between two and eight animals kept in each depending on the size of the tank and the animals. The opaque tanks minimised potential stress from visual disturbance. Lobsters had their chelae secured with elastic bands to prevent injury to both other lobsters and to handlers. The aquarium was maintained in a temperature controlled room at 8°C (± 1°C) with a 12:12 hour light:dark photoperiod. Animals were held for a minimum of 48 hours to a maximum of 5 days before experimentation. This time was allowed to ensure full temperature acclimation, recovery from capture and transport and to allow the loss of any tidal

rhythms that may have affected metabolism. This temperature and photoperiod were maintained throughout the experiments.

A dispensation was obtained from the North Eastern Sea Fisheries Committee (NESFC), to allow the capture of undersized animals to be used in the trials. This was a budgetary requirement that allowed travel on a regular fishing vessel without the need to personally charter it as the dispensation allowed the collection of specimens that would not detract from the boat crew's livelihoods. Specimens were not selected if more than 5 mm below the minimum landing size. Specific size ranges were used for each test species (Table 2.3).

Table 2.3 Sizes of the crustacean specimens used

Species	**Sizes used**	**Minimum landing size (in 2008)**
Homarus gammarus	82 – 90 mm carapace length	87mm
Cancer pagurus	125 – 140 mm carapace width	130 mm
Necora puber	60 – 75 mm carapace width	65mm
Carcinus maenas	45 – 77 mm carapace width	N/A
Pagurus bernhardus	Inside *Nucella lapillus* and *Littorina littorea* shells of 25-35mm length	N/A

Animals were not fed during the holding period before experimentation, in order not to influence the metabolism and to ensure all animals were in a similar nutritional state. Animals that survived experiments and blood sampling procedures were returned to 35 sea water and fed on *Mytilus edulis* (common blue mussel) once a week, or killed following RSPCA guidelines for mechanical killing (RSPCA 2007). Between ½ to 1 mussel was allowed per animal depending on size. Any uneaten food was removed the next day and the water changed after two days as feeding prompted the production of a large amount of faecal matter, adversely affecting the water quality. *Mytilus edulis* is the preferred food of many decapod crustaceans (Richards and Wickins 1979; Mascaró and Seed 2001).

Determination of death of the animals followed the method of McLeese (1956) in which death was indicated by no movement of any body part when observed upon a close examination or even in

response to mechanical stimulus, and no "recovery" was recorded when returned to ambient conditions. A thorough examination of the animals was required as animals that appeared to be dead from salinity could, according to McLeese, recover completely within a few hours of returning to ambient conditions. In an addition to this method, death could be determined in more difficult cases by attempting to take a small blood sample from the rear walking legs of the animals. From preliminary trials it was discovered that in a truly dead animal a blood sample was impossible to take, but in animals that were still alive the blood would still flow freely into the syringe despite appearing dead. Upon inspection under the microscope, dead blood was either extremely clotted or all the cells had lysed.

2.2 Experimentation procedure

Due to the varying techniques used for assessment of salinity impact on the study species, detailed explanations of the experimental methods used for each procedure are given in the relevant chapters.

2.3 Making hypersaline brine

Hypersaline brine was produced using natural sea water at salinity 35 as a base then either adding Instant Ocean™ aquarium salts, or brine collected from the gas caverns at Aldbrough to raise the salinity to that required for the particular trial. Instant Ocean™ is a clean and sterile product that was used for most of the trials so that a salinity only response could be studied, rather than one prompted by any other chemicals that may or may not be present in the gas cavern brine. Thus enabling predictions for areas other than the local one to be made based on the salinity-dependent responses of the study species. Hyposaline water was made by adding Milli-Q™ ultrapure water to a natural sea water base.

2.4 Monitoring salinity and water quality

Salinity was monitored on a daily basis using a refractometer (Bellingham & Stanley 45-119) calibrated with Milli-Q™ water at aquarium temperature. Aquarium water quality was monitored

before each trial and otherwise on a weekly basis using a saltwater master test kit by Aquarium Pharmaceuticals. The bacterial filter was maintained from a starter culture obtained from J. Garland at Clearwater Seafoods, Canada. Mechanical filter media were changed as needed.

2.5 Statistical analyses

Statistical analyses were carried out using SPSS versions 15 to 18, or by hand following the methodology in Fowler et al (1998). Graphs were drawn in SPSS, Microsoft Excel or SigmaPlot version 11. Normality checking prior to each statistical test was carried out via a Kolmogorov Smirnov test. Homogeneity of variance was tested for with a Levene’s test. Details of the specific statistical tests are given in the appropriate chapters.

Chapter 3

Behavioural assays

Behavioural responses of crustaceans to hypersaline exposure

3.1 Introduction

There are four main survival strategies employed by aquatic crustaceans when challenged with high stressor intensities in their environments:

1. locomotory escape or avoidance responses, such as movement of the whole organism, or part of it, away from the affected area (e.g. by fleeing, retreating into a burrow, or, as with barnacles, withdrawing behind protective opercular plates);

2. reduction of contact with external media (*e.g.* by reducing or ceasing ventilatory behaviour, or burrowing); normally a temporary measure;

3. using a physiological response to regulate the immediate effects of the stressor;

4. adapting to the altered stressor intensity level, either by acclimating to a new steady-state response to the stressor (a non-genetic response) or, over several generations, adapting genetically to the altered conditions. Often, a combination of both types of response occurs (Kinne 1964).

The species studied here are all active decapod crustaceans and this chapter focuses on their locomotor responses to changes of environmental salinity. Locomotor movements (*e.g.* walking, swimming) are important adaptations of crustaceans which link their behaviour and ecology. Thus, short term ecological changes can alter population and community structure through motile species avoiding any adverse conditions, or congregating because of an abundance of food or mating opportunities.

Altered behaviour is usually the first response to changed salinity and can help organisms to avoid adverse conditions (Curtis et al. 2007) and failure of a behavioural response system can lead to reduced individual fitness and associated adverse consequences for the population (Miller 1980).

Decapod crustaceans are motile species and a rapid avoidance response to adverse conditions would therefore have a presumed survival value. A prerequisite of such behavioural modifications, however, is the ability to detect the change in salinity. Due to the presence of the exoskeleton, crustaceans need specialised sensors to be able to detect environmental change such as salinity. These are in the form of hairlike extensions of the cuticle (setae/sensilla) and contain the dendrites of sensory neurons (Schmidt 1989; Garm et al. 2004). Sites of salinity detection in decapod crustacea have been shown to be in the mouthparts (Garm et al. 2004), legs (Davenport and Wankowski 1973; Schmidt 1989), antennae (Tazaki 1975) and branchial chambers (Hume and Berlind 1976; Dufort et al. 2001).

Short term locomotory avoidance responses effectively protect the organism from the higher energetic costs associated with increased ion- and osmoregulation. The magnitude of the organism's behavioural responses to a salinity change may be a species-related one that reflects its osmoregulatory ability. Thus the porcelain crab, *Porcellana platycheles* shows salinity preference behaviour only at salinities below its tolerance limit of < 40 % seawater (Davenport 1972a; Davenport and Wankowski 1973) whereas the mud crab, *Scylla serrata*, shows no preference within its normal salinity range of 2 – 42 (Davenport and Wong 1987). When exposed to a hyposalinity gradient, the osmoconforming crab *Cancer gracilis* briefly explores, then moves to the highest salinity in the gradient (Curtis et al. 2007).

Salinity is a limiting factor in the distribution of many aquatic organisms and hypersalinity, whilst not as common marine phenomenon as hyposalinity, does have a limiting effect on marine biotic distributions (Gunter 1961). Seasonal salinity changes can influence the distribution of coastal and estuarine species such as the various penaeid shrimp species (Gunter et al. 1964) and salinity gradients result in different species occupying different parts of the gradient (*e.g.* the portunid crab species of the Caribbean (Norse 1978) and the North Sea crab species such as *Liocarcinus spp* and *Carcinus maenas* (Mathieson and Berry 1997).

Habitat structure can directly influence the physiological and behavioural mechanisms of organisms and must be considered when interpreting the responses of animals in relation to physicochemical variables (McGaw 2001). The use of shelters has been widely noted for a number of both lobster and crab species with most lobster and many crab species shown to live in rocky areas which provide good opportunities for shelter from light, predation and/or currents. Such shelters are rarely

found in sandy or muddy substrata. For some species, the presence of shelter may induce a crustacean to stay in an area when conditions become sufficiently unfavourable as to cause it to otherwise it vacate the area (Table 3.1). Physical factors and seabed topography have been shown to affect the size composition of *Homarus gammarus* and *H. americanus* populations, with the substratum type and current strength also having major influences (Howard and Nunny 1983; Robichaud and Campbell 1991).

Table 3.1 Shelter preferences of some lobster and crab species.

Lobster species	**Shelter preferences**	**Reference**
Panulirus interruptus	Rocky areas with shaded/dark shelters	(Spanier and Zimmer-Faust 1988)
Panulirus argus	Caves, holes in coral or rock. Juveniles can be found in sponges	(Smith and Herrkind 1992; Butler et al. 1995)
Homarus gammarus	Prefers areas where rocks reduce current speeds	(Howard and Nunny 1983)
Homarus americanus	Rocky bottom, no lobsters found inhabiting sandy or muddy bottoms	(Cobb 1971)
Scyllarides latus	Prefers shaded or opaque shelter to identical transparent one in laboratory. In the field specimens found in rocky areas or man made reefs	(Spanier and Almog-Shtayer 1992)
Crab species	**Shelter preferences**	**Reference**
Carcinus maenas (juv)	Mussel beds, eelgrass, filamentous green algae	(Moksnes et al. 1998)
Hemigrapsus nudus	Display a weak negative phototaxis and prefer environments with available shelter. Uncommon where areas have high sediment load. Presence of shelter means it will tolerate unfavourable salinities for longer	(McGaw 2001)
Pagurus bernhardus	Uses its shell as protection from fluctuating environmental salinities	(Shumway 1978)

3.2 Aims, objectives and hypotheses

The aim of this study is to determine whether hypersalinity causes halokinesis / halotaxis in decapod crustaceans (a movement in response to salinity) and if so to what extent? This resulted in the following null hypotheses;

1. Hypersalinity does not cause an obvious behavioural response in the crustacean species tested (visible physical movements rather than concealed behavioural response *i.e.* altered scaphognathite beat behaviours).

2. The crustaceans tested cannot distinguish between normal and hypo/hypersalinity.

3. When hypersalinity challenged, the crustaceans tested prefer to be in a shelter rather than in ambient open water.

For the purposes of this investigation, hypersalinity is defined as any salinity above, and hyposalinity any salinity below, what the species normally experiences in the wild in the Yorkshire area (in the case of those tested here, that salinity is 35). In addition to the three key species of this study (*Homarus gammarus, Cancer pagurus* and *Necora puber*) which are mainly sublittoral in nature, two additional species, *Carcinus maenas* and *Pagurus bernhardus* have been included to provide a comparison with intertidal crustaceans.

3.3 Materials and Methods

3.3.1 Animal husbandry

Creel-caught, intermoult specimens of *Necora puber* (carapace width 65-75 mm), *Cancer pagurus* (minimum landing size -5 mm +10 mm) and *Homarus gammarus* (minimum landing size ± 3 mm) were obtained from commercial landings at Bridlington, East Yorkshire, U.K. in the autumn of 2007. *Carcinus maenas* (45 mm - 77 mm) and small *Pagurus bernhardus* (inside *Nucella lapillus* and *Littorina littorea* shells of 25-35 mm length) were collected from the shore at Filey Brigg, Yorkshire, U.K. *N. puber* and *C. maenas* were maintained in opaque plastic tanks at a stocking density of 4 – 8 animals per tank and an average of 3 litres of water per crab. *H. gammarus* and *C. pagurus* were held 2 per tank with 6L water per animal. *P. bernhardus* were held 6 per tank with 1 L of sea water per crab. An unobtrusive drain/refill method was used daily to change 50% of the holding water.

3.3.2 Salinity induced behavioural preference test – Two salinity choice trial

A two way choice chamber was constructed (Figure 3.1) which consisted of two slopes (30°) with a flat central apex inside a large opaque plastic tank, (100 cm * 35 cm * 25 cm). This construction allowed separate pools of water on each side of the tank. Different choices of salinity were given on each side with one side always being at the normal salinity for the study area at 35, then the other side being a higher salinity (within the range 35 to 65). The construction of the experimental chamber was such that there was no mixing of the two pools, thus maintaining a stable salinity on each side, however test specimens could still crawl easily over the central apex. The slopes were constructed from rough-textured polystyrene which allowed the animals to maintain purchase so they could not simply ‘slip’ into a pool by mistake. Pools were aerated gently, maintained at a constant temperature (8 °C ± 1 °C) to ensure that there were a minimum number of variables that could affect the choice response of test specimens. The aquarium was maintained at a 12h light/dark cycle and all tests were carried out during the light periods. Lights were directly above the tank to ensure any behaviour was induced by salinity rather than light conditions.

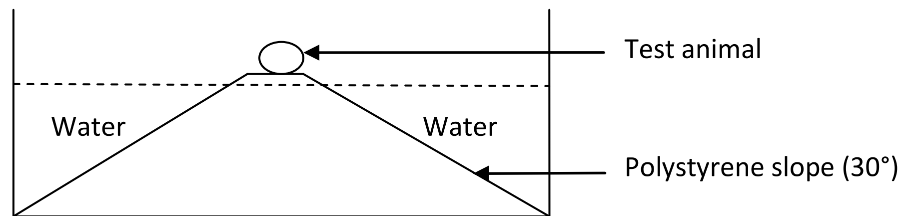


Figure 3.1 Two choice testing chamber (not to scale). Separate pools (one hypo/hypersaline and one normal salinity) were used to observe salinity preferences.

Different species were tested in varying numbers and combinations of gender, dependent on availability at time of capture (Table 3.2). Each test involved the placing of a single specimen in the chamber at the apex of the slopes thus presenting it with the choice of remaining emersed or descending into either pool of water. Each test involved observing the specimen from behind a screen and recording the time taken to move into a pool and the pool choice made. Assuming that a 6 h emersion period represented the maximum time for which a mid to low shore animal would be tidally exposed, those animals that took longer than 6 h to make a choice were deemed as being unresponsive. A positive choice was counted when the animal had its legs and mouthparts submerged (principal sites of salinity detection in decapods as discussed in section 3.1 and on the advice of Dr. R. Uglow, University of Hull). If, after choosing, a subsequent choice was made to change pools, only the initial excursion into a pool was recorded. This cut off was chosen as, for the purposes of this experiment, only the initial response to salinity was wanted, rather than one which may have occurred after a period of acclimation in the chosen pool. A parallel (control) series of tests were run alongside where a choice of normal (35 psu) salinity was given on both sides to ensure there were no other factors controlling any salinity preferences and indicated there was no preference for a particular side of the tank (see the 35-35 choice in tables 3.13 to 3.17). Not all species were tested in the hyposaline range as this thesis is about hypersaline mediated responses, hyposaline options were tested for, merely as an aside when time allowed, however the results are included here for interest.

On placement on the central apex, crabs tended to orient themselves so that the legs on one side of the body could be dipped into one pool, and the legs on the other side of the body could be dipped into the other pool. There then proceeded a period in which legs were alternately dipped into the normal and hypo/hypersaline pool until a choice was made and the crab submerged itself.

Occasionally in between the dipping behaviour and submergence, crabs would shuffle their bodies between the shallowest parts of the pools. Lobsters did not exhibit such marked 'choosing' behaviour. On submergence, both crabs and lobsters settled at the deepest part of the tank, often backing into a corner. Only one single animal (*Carcinus maenas*) out of all tested switched sides after the initial choice. Where numbers tested were sufficient to allow a gender-dependant analysis it was shown that there were no gender-dependent differences in behaviour in response to salinity (χ^2 test, $p > 0.05$ in all cases) hence gender responses have not been investigated further (Table 3.3, Table 3.4).

Table 3.2 Details of the animals used in the two way choice tests

Species	Number/Gender details
Necora puber	16 male, 4 female (minimum)
Cancer pagurus	15 male, 15 female
Homarus gammarus	10 male, 10female
Carcinus maenas	6 male, 6 female
Pagurus bernhardus	6 male, 6 female

Table 3.3 Results of χ^2 tests to ascertain if there was a difference in the preference behaviour of *Cancer pagurus* to hypo/hypersalinities based on sex. 1 df.
Using Fisher's correction when n<5. (5% significance threshold = 3.84, 1% = 6.63).

Salinity choice	χ^2 value	p-value	p with Fisher's correction
35-35 control	0.133	0.715	1.000
35-40	0.715	0.705	1.000
35-45	0.186	0.666	1.000
35-50	1.154	0.283	0.598
35-55	0.000	1.000	1.000
35-60	0.240	0.624	0.068
35-65	3.333	0.068	0.224

Table 3.4 Results of χ^2 tests to ascertain if there was a difference in the preference behaviour of *Homarus gammarus* to hypo/hypersalinities based on sex.
1df. Using Fisher's correction when n<5. (5% significance threshold = 3.84, 1% = 6.63)

Salinity choice	χ^2 value	p-value	p with Fisher's correction
25-35	Unable to calculate as all chose salinity 35	n/a	n/a
30-35	0.833	0.361	0.650
35-35 control	5.051	0.025	0.070
35-40	1.818	0.178	0.370
35-45	0.220	0.639	1.000
35-50	0.392	0.531	1.000
35-55	0.392	0.531	1.000
35-60	Unable to calculate as all chose salinity 35	n/a	n/a

3.3.3 Salinity induced behavioural preference test – Multiple salinity choice trial

The experimental set up for these tests comprised a circular tank (depth 60 cm, diameter 80 cm), filled with sand to a depth of 15 cm into which 4 shelters were buried at a 30° angle (Figure 3.2). Each shelter was constructed from a 2L soft plastic bottle with the rounded neck removed to create a large opening. Each was strengthened by sections of round guttering and each had a sea water delivery pipe fixed at the rear. Shelters were connected via the delivery pipe to a peristaltic pump, with 3 hypersaline reservoirs (100, 85 and 65 psu) and one fresh water reservoir (both *cf.* normal seawater salinity of 35) (Figure 3.2). The pumps were calibrated to each deliver water at 2 $ml.min^{-1}$ to the shelters. The salinity of the reservoirs was calibrated so that on mixing with the 35 psu sea water in the experimental tank, the salinity of the shelters equated to 85 ± 5, 65 ± 5, 45 ± 5 and 37.5 ± 2.5. As hypersaline water is denser than ambient sea water, the low flow rate into the shelters ensured that, to a large extent, the hypersaline conditions remained in the shelter only. The shelter with a hyposaline flow (which when mixing with the tank water equated to 37.5 ± 2.5 psu) was included to counteract any major increase in the overall salinity of the tank.

When each shelter had reached the desired salinity a test animal was introduced to the centre of the tank and the set-up left for 24 hours. The tank was inspected every 15 minutes during the first 4 h and then hourly for 4 h and a final check after 24 h. The position of the test animal was noted each time. After 24 h the experiment was complete and the final position of the specimen was

recorded and taken as the choice made. Salinity inside the shelters and in the open water was monitored using 0.5 ml water samples obtained during the checks via a syringe that was able to reach the bottom of the tank and determined using a calibrated refractometer.

Two species were used in the multi choice test (*Homarus gammarus:* 10 males / 10 females and *Cancer pagurus:* 8 males / 8 females). The aim of this test was to determine whether the test animals would prefer a sheltered location but a challenging salinity or an exposed location of normal (35) salinity. A control test was run where the shelters all received a flow of salinity 35 seawater to ensure there was no preference for a particular shelter or that the flow was not affecting the behaviour.

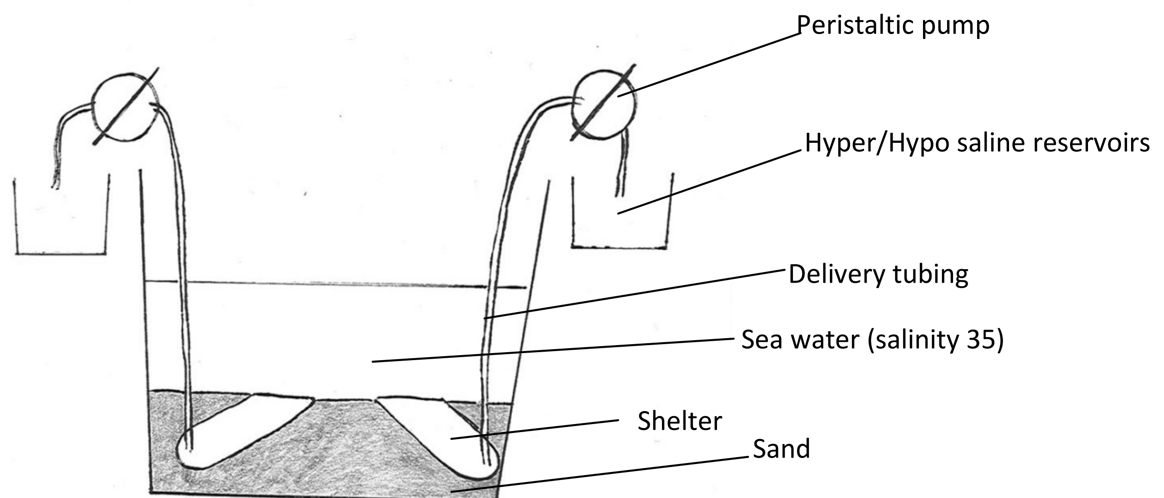


Figure 3.2 Multi choice testing chamber

3.3.4 Salinity dependent gender testing for *Homarus gammarus*

The results of a χ^2 test on the lobster preference data for the test group (Table 3.5, Table 3.6) did not show any significant gender-dependent the salinity preferences (χ^2 = 1.511, p = 0.470, df = 2). Consequently the gender data have been pooled for analyses. No gender-dependent difference were found for the control trial either (χ^2 = 2.533, p = 0.639, df = 4, Table 3.7, Table 3.8).

Table 3.5 χ^2 test on the *Homarus gammarus* preference data. Crosstabulation.
As no lobsters of either sex chose shelter a or b, these were excluded these from the calculation (due to calculating an expected value of 0).

			choice			Total
			shelter c 40-50	shelter d 35-40	open water	
sex	male lobster	Count	0	4	6	10
		Expected Count	0.5	4.5	5.0	10.0
	female lobster	Count	1	5	4	10
		Expected Count	0.5	4.5	5.0	10.0
Total		Count	1	9	10	20
		Expected Count	1.0	9.0	10.0	20.0

Table 3.6 χ^2 test on the *Homarus gammarus* preference data. χ^2results.
(df 2, 5% critical significance threshold =5.99, 1% = 9.21)

	χ^2 Value	df	P value
Pearson Chi-Square	1.511[a]	2	0.470
Likelihood Ratio	1.900	2	0.387
Linear-by-Linear Association	1.230	1	0.267
No. of Valid Cases	20		

[a] 4 cells (66.7%) have expected count less than 5. The minimum expected count is 0.50.

Table 3.7 χ^2 test on the *Homarus gammarus* multi choice control preference data. Crosstabulation.
Expected values calculated by SPSS v17.

			choice control					Total
			shelter a 35	shelter b 35	shelter c 35	shelter d 35	Open water 35	
sex control	male lobster	Count	3	1	2	2	2	10
		Expected Count	3.0	1.5	2.5	1.0	2.0	10.0
	female lobster	Count	3	2	3	0	2	10
		Expected Count	3.0	1.5	2.5	1.0	2.0	10.0
Total		Count	6	3	5	2	4	20
		Expected Count	6.0	3.0	5.0	2.0	4.0	20.0

Table 3.8 χ^2 test on the *Homarus gammarus* multi choice preference data. Results. Critical significance thresholds, 5% = 9.49, 1% = 13.28)

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	2.533[a]	4	.639
Likelihood Ratio	3.314	4	.507
Linear-by-Linear Association	.195	1	.658
N of Valid Cases	20		

3.3.5 Salinity dependent gender testing for *Cancer pagurus*

There was no significant gender-dependent salinity preference in the test group of *C. pagurus* (χ^2 = 1.333, p = 0.721, df = 3, Table 3.9, Table 3.10) or in the control group (χ^2 = 8.333, p = 0.080, df = 4,

Table 3.11 and Table 3.12).

Table 3.9 χ^2 test on the *Cancer pagurus* preference data. Crosstabulation. Expected values calculated by SPSS v17. As no lobsters of either sex chose shelter a, SPSS excluded it from the calculation (due to calculating an expected value of 0).

			choice				Total
			shelter b 60-70	shelter c 40-50	shelter d 35-40	open water 35	
sex	male crab	Count	1	1	2	4	8
		Expected Count	0.5	1.5	2.0	4.0	8.0
	female crab	Count	0	2	2	4	8
		Expected Count	0.5	1.5	2.0	4.0	8.0
Total		Count	1	3	4	8	16
		Expected Count	1.0	3.0	4.0	8.0	16.0

Table 3.10 χ^2 test on the *Cancer pagurus* preference data. Results.

	Value	df	P value
Pearson Chi-Square	1.333[a]	3	0.721
Likelihood Ratio	1.726	3	0.631
Linear-by-Linear Association	.065	1	0.799
No. of Valid Cases	16		

[a.] 8 cells (100.0%) have expected count less than 5. The minimum expected count is 0.50

Table 3.11 χ^2 test on the *Cancer pagurus* preference data – control results. Crosstabulation. Expected values calculated by SPSS v17. As no lobsters of either sex chose shelter a or b, SPSS excluded these from the calculation (due to calculating an expected value of 0).

			choice control crabs					Total
			a 35	b 35	c 35	d 35	open water	
sex_ctrl	male crab	Count	3	3	0	1	1	8
		Expected Count	1.5	2.0	2.0	1.5	1.0	8.0
	female crab	Count	0	1	4	2	1	8
		Expected Count	1.5	2.0	2.0	1.5	1.0	8.0
Total		Count	3	4	4	3	2	16
		Expected Count	3.0	4.0	4.0	3.0	2.0	16.0

Table 3.12 χ^2 test on the *Cancer pagurus* preference data control test. Results.

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	8.333[a]	4	.080
Likelihood Ratio	11.090	4	.026
Linear-by-Linear Association	2.872	1	.090
N of Valid Cases	16		

3.3.6 Statistical analyses

Behavioural choices were analysed using a G-test (Fowler et al. 1998), with a Ln(x+1) transformation for tests in which zero observed values occurred for some choice options (*Pers. comm* Dr Jim Fowler[1]) or a Chi^2 (χ^2) test (SPSS v17). The G-test was used due to being unable to construct the 2 by 2 table and hence the minimum 1 degree of freedom required for the χ^2 test when combining the data for the both males and females. When the crustaceans were assessed for gender-dependent salinity responses a χ^2 was appropriate. Expected values were calculated by

[1] Dr Jim Fowler. Retired Principal Lecturer, Dept of Biological Sciences, DeMontfort University, Leicester, UK. Author of Fowler et al 1998.

taking the total number of observations and dividing equally between the number of salinity choices available.

3.4 Results

3.4.1 Two choice testing; *Necora puber*

All the species of crustaceans tested clearly showed a strengthened preference for ambient salinity in direct relationship with the size of the difference with either the hypo- or hypersalinity options available.

Necora puber showed an increasing preference for the normal salinity of 35 increasing from a 65% preference when the choice was 35-40 to a 95% preference when the choice was 35-65. This preference for the ambient salinity (35 psu) over hypo/hypersalinities was statistically significant once the choice became 35psu/50psu in the hypersaline range and 25psu/35psu in the hyposaline range (Table 3.13).

Table 3.13 Preferences of *Necora puber* when exposed to a choice of normal (35psu), hyposalinity or hypersalinity (25-65 psu).
(N=22 to 26, df = 1, 5% significance threshold = 3.84, 1% = 6.63)

Salinity Choice	25-35	30-35	35-35 control	35-40	35-45	35-50	35-55	35-60	35-65
Number choosing 35/hypersalinity	6/16	11/11	13/11	17/9	16/10	17/5	18/4	20/2	21/1
G value	4.61 *	0.00	0.16	2.46	1.37	4.85 *	9.42 **	14.80 **	21.90 **

* = significant at the 5% level, ** = significant at the 1% level

3.4.2 Two choice testing; *Cancer pagurus*

Cancer pagurus showed a preference for the normal salinity of 35 increasing from a 63.3% preference when the choice is 35-40 to a 90% preference when the choice is 35-65. Strong preferences for salinity 35 occur from option 35-45 upwards with over 75% choosing salinity 35 over the hypersaline alternative (Table 3.14). *C. pagurus* showed a significant ($p < 0.01$) preference for the normal salinity (35) over hypersalinities once the choice became 35/45. There was no significant preference between salinities 35 and 40.

Table 3.14 Salinity preferences of *Cancer pagurus* when exposed to a choice of normal (35psu) or hypersalinity (40-65 psu).
(N=30, Degrees of freedom = 1, 5% significance threshold = 3.84, 1% = 6.63)

Salinity Choice	35-35 control	35-40	35-45	35-50	35-55	35-60	35-65
Number choosing 35/hypersalinity	15/15	19/11	23/7	27/3	26/4	26/4	27/3
G value	0	2.12	8.85 **	21.72 **	17.73 **	17.73 **	21.72 **

* = significant at the 5% level, ** = significant at the 1% level

3.4.3 Two choice testing; *Homarus gammarus*

Homarus gammarus showed an increasing preference for the normal salinity of 35. This occurred both in the hypo and hypersaline range. At a choice of 25-35, 100% chose salinity 35. In the hypersaline range the strongest preference occurred at the choice 35-60 with 90% choosing salinity 35. Preference for the normal salinity (35) became significant once the salinity choice offered with normal reached salinity 50 in the hypersaline range or 25 in the hyposaline range, (p = 0.01) preference for the ambient salinity (Table 3.15). At salinities between these there was no significant behavioural preference.

Table 3.15 Salinity preferences of *Homarus gammarus* when exposed to a choice of normal (35) or hypo/hypersalinity (25-65 psu).
(N=20, df = 1, 5% significance threshold = 3.84, 1% = 6.63)

Salinity Choice	25-35	30-35	35-35 control	35-40	35-45	35-50	35-55	35-60
Number choosing lower/higher salinity	0/20	8/12	9/11	11/9	13/7	17/3	17/3	18/2
G value	26.91 **	2.72	0.20	0.20	1.78	10.55 **	10.55 **	14.36 **

* = significant at the 5% level, ** = significant at the 1% level

3.4.4 Two choice testing; *Carcinus maenas*

Carcinus maenas showed a significant preference for normal salinity (35) over hypersalinities once the choice became 35/40 (Table 3.16). Due to the limited number of adult specimens available of this species at the time of field collection no attempt was made to ascertain if there was a gender-dependent response. *C. maenas* showed the most definitive preference for normal salinity with all animals choosing salinity 35 when the other options were 45, 55, 60 and 65.

Table 3.16 Salinity preferences of *Carcinus maenas* when exposed to a choice of normal (35psu) or hypersalinity (40-65).
(N=12, Degrees of freedom = 1, 5% significance threshold = 3.84, 1% = 6.63)*

Salinity Choice	35-35 control	35-40	35-45	35-50	35-55	35-60	35-65
Number choosing 35/hypersalinity	7/7	11/3	13/1	11/3	13/1	13/1	13/1
G value	0	4.69 *	11.78 **	4.69 *	11.78 **	11.78 **	11.78 **

* = significant at the 5% level, ** = significant at the 1% level

3.4.5 Two choice testing; *Pagurus bernhardus*

Pagurus bernhardus showed a significant preference for normal salinity (35) over hypersalinities once the choice became 35/55 (Table 3.17). No attempt was made to ascertain if there was a difference in the responses of the two sexes due to limited availability of specimens. *P. bernhardus* showed a less defined response, however a greater than 75% preference occurred once the hypersaline option reached salinity 50 or above.

Table 3.17 Salinity preferences of *Pagurus bernhardus* when exposed to a choice of normal (35psu) or hypersalinity (40-65 psu).
(n = 8 to 12, df = 1, 5% significance threshold = 3.84, 1% = 6.63)*

Salinity Choice	35-35 control	35-40	35-45	35-50	35-55	35-60	35-65
Number choosing 35/hypersalinity	6/6	7/5	4/4	8/2	12/0	9/2	9/2
G value	0	0.30	0	3.40	14.86 **	4.30 *	4.30 *

* = significant at the 5% level, ** = significant at the 1% level

3.4.6 Multi choice testing; *Homarus gammarus*

As many decapods have been shown to prefer to inhabit shelters (see section 3.1), with some even staying in unfavourable environmental conditions in order to remain in a shelter, this test aimed to investigate the trade-off between safety (a shelter) but unfavourable conditions (hypersaline water within the shelter) and exposure (no shelter) but more favourable conditions (ambient water salinity), as a way of investigating what might potentially happen in the wild if an area inhabited by these species underwent hypersaline flooding.

The results of the control multi choice testing on *H. gammarus* have shown that the experiment was unbiased as there was no significant preference for any shelter, or an exposed location (G = 1.22, df = 4, Table 3.18).

Table 3.18 G test for salinity preference of *Homarus gammarus* multi choice control group. df = 4. Critical significance threshold: 5% = 9.49, 1%=13.28. Shows no significant preferences (G adj < 9.49).

Choice and salinity	shelter a 35	shelter b 35	shelter c 35	shelter d 35	Open water 35
observed	6	3	5	2	4
expected	4	4	4	4	4
G step1	2.43	-0.86	1.12	-1.39	0.00

G	1.30
correction factor	1.07
G adjusted	1.22

In the multi choice test on *Homarus gammarus,* all but one animal (19 out of 20) avoided the hypersaline areas of the tank. Ten lobsters chose open water (6 males, 4 females), nine lobsters chose the lowest salinity shelter (shelter d) at 35-40 psu (4 males, 5 females). The exception was a female that chose shelter c (40-50 psu) (Table 3.19). Thus lobsters, even when given the choice of a shelter within a hypersaline area, will avoid hypersalinity above 40 psu.

Table 3.19 Results of the multi choice test for *Homarus gammarus*.
Position in tank recorded after 24 hours. Positions: a=shelter at 80-90 psu, b=60-70, c=40-50, d=35-40, open water =35 psu.

Lobster	Sex	Position after 1 h	Position after 24h	Choice
1	male	d	open water	open water
2	male	open water	open water	open water
3	male	open water	open water	open water
4	male	open water	open water	open water
5	male	b	d	d
6	male	c	d	d
7	male	open water	d	d
8	male	open water	open water	open water
9	male	d	open water	open water
10	male	d	d	d
11	female	d	d	d
12	female	c	d	d
13	female	b	c	c
14	female	c	d	d
15	female	open water	open water	open water
16	female	open water	open water	open
17	female	b	d	d
18	female	open water	open water	open water
19	female	open water	open water	open water
20	female	a	d	d

The results of the multi-choice lobster experiment have shown that lobsters preferred ($p < 0.01$ in each case) both shelters or open water of normal salinity ($G = 10.15$, $df = 4$, Table 3.20)**.** Since the control trial found no preferences, these findings therefore suggest that it is the effects of salinity that are causing the preference behaviour exhibited in this species.

Table 3.20 G test for salinity preference of *Homarus gammarus*.
Ln x+1 transformation used on all observed values due to 0 values. df = 4. Critical significance threshold: 5% = 9.49, 1%=13.28.

Choice and salinity	shelter a 80-90	shelter b 60-70	shelter c 40-50	shelter d 35-40	Open water 35
observed	1	1	2	10	11
expected	5	5	5	5	5
G step1	-1.61	-1.61	-1.83	6.93	8.67

G value for whole test	10.55
correction factor	1.04
G adjusted	**10.15** **

** = significant at the 1% level

3.4.7 Multi choice testing; *Cancer pagurus*

The control multi choice testing on *C. pagurus* below showed that the experiment was unbiased as there was no significant preference for any shelter, or an exposed location (G = 0.43, df = 4, Table 3.21), with all areas of the tank being used evenly.

Table 3.21 G-test on the *Cancer pagurus* – control results preference data.
df = 4. Critical significance threshold: 5% = 9.49, 1%=13.28

Control Choice and salinity	shelter a 35	shelter b 35	shelter c 35	shelter d 35	Open water 35
Obs	3	4	4	3	2
Exp	3.2	3.2	3.2	3.2	3.2
G step1	-0.194	0.893	0.893	-0.194	-0.940

G vale for whole test	0.458
correction factor	1.063
G adjusted	**0.43**

In the trial using hypersaline water, all but 4 of the *C. pagurus* chose the 35-40 salinity shelter or open water at salinity 35. Eight crabs chose the open water (4 males, 4 females), four crabs chose shelter ‘d’ at 35-40 psu (2 males, 2 females), three crabs chose shelter ‘c’ at 40-50 psu (1 male, 2 females) and one crab chose shelter ‘b’ at 60-70 psu (male) (Table 3.22). Therefore, in total, 75% of crabs avoided the hypersaline areas of the tank, which suggests that they will show avoidance behaviour when exposed to a hypersaline environment. The results highlighted in ***bold and italics*** also potentially indicate that crabs chose a location then stayed there regardless of prevailing salinity. Seven crabs chose a shelter/open water then stayed there for 24 hours regardless of whether it was hypersaline.

When analysed statistically however, this 75% preference for the lowest salinity areas of the tank was proved not to be statistically significant ($G = 4.407$, $df = 4$, $p > 0.05$, Table 3.23).

Table 3.22 Results of the multi choice test for *Cancer pagurus*.
Position in tank recorded after 24 hours. Positions: a=shelter at salinity 80-90, b=60-70, c=40-50, d=35-40, open water =35. Bold and italics highlight where a choice was made and then no further movements occurred in 24 hours.

Lobster	Sex	Carapace width	Position after 1 h	Position after 24h	Choice
1	1	13	***c***	***c***	***c***
2	1	13.5	***b***	***b***	***b***
3	1	12.7	***open water***	***open water***	***open water***
4	1	13.4	a	open water	open
5	1	14.2	a	open water	open
6	1	13.5	a	d	d
7	1	13	***open water***	***open water***	***open water***
8	1	14.2	b	d	d
9	2	15.8	a	d	d
10	2	15.5	***open water***	***open water***	***open water***
11	2	13.6	***d***	***d***	***d***
12	2	14.1	b	c	c
13	2	13.4	a	open water	open water
14	2	13.9	***c***	***c***	***c***
15	2	13.4	c	d	open water
16	2	15.1	open water	d	open water

Table 3.23 G-test on the *Cancer pagurus* preference data.
df = 4. Critical significance threshold: 5% = 9.49, 1%=13.28.

Choice and salinity	shelter a 80-90	shelter b 60-70	shelter c 40-50	shelter d 35-40	Open water 35
Observed	1.000	2.000	4.000	5.000	9.000
Expected	4.200	4.200	4.200	4.200	4.200
G step1	-1.435	-1.484	-0.195	0.872	6.859

G	4.617
correction factor	1.048
G adjusted	**4.407**

3.5 Discussion

In the two choice tests, all species tested were able to distinguish between salinities and all made a choice of the normal seawater once their particular threshold was reached (Table 3.24). For both *Cancer pagurus* and *Homarus gammarus* there was no significant gender-related salinity preference. In the multi-choice tests *H. gammarus* showed a significant preference for either a shelter of ambient to low hypersalinity (35-40) or to be in open water, (potentially exposed to predators), at salinity 35. *C. pagurus* also showed a strong (75%) (though not statistically significant) orientation towards the lowest salinity areas of the tank. There was no difference in the salinity preferences of males and females. In both the two choice and multi choice control groups there was no significant preference for any location in the experimental tanks, suggesting the preferences shown by the test groups were prompted by salinity alone and not any other variables.

Table 3.24 Summary of salinities which prompt a movement into ambient (35 psu) water. Two choice experiment. Statistically significant at the 5% * or 1% ** level using a G-test.

Species	Hypersaline threshold [a]	Hyposaline threshold [a]
Necora puber	50 psu *	25 psu *
Cancer pagurus	45 psu **	Not tested
Homarus gammarus	50 psu **	25 psu **
Carcinus maenas	40 psu *	Not tested
Pagurus bernhardus	55 psu **	Not tested

[a] measured in increments of 5 salinity units (psu)

Null hypothesis*: Hypersalinity does not cause an obvious behavioural response in the crustacean species tested.* **REJECTED** as all species show a preference for avoiding high/low salinities at certain choice thresholds (Table 3.24).

Null hypothesis*: Crustaceans tested cannot distinguish between normal and hypo/hypersalinity.* **REJECTED** as all species have shown avoidance of high salinities at certain choice thresholds (Table 3.24)**.** A multi-choice test on *Cancer pagurus* and *Homarus gammarus* has shown that even when presented with a range of hypersalinities, the normal 35 psu is still preferential.

<u>Null hypothesis</u>: *Crustaceans will prefer to be in a shelter of hypersalinity rather than in normal salinity open water.* **REJECTED** *Homarus gammarus* has been shown in the multi-choice test to significantly prefer either a shelter at 35-40 psu or open water at 35 psu. Hypersaline shelters are not preferred. *Cancer pagurus* also showed a preference (though not statistically significant) for salinities closer to ambient.

The most intertidal species *Carcinus maenas* was found to have an upper salinity behavioural threshold of salinity 40, the lowest of the 5 species tested. *Pagurus bernhardus,* the common hermit crab and another intertidal species, has a hypersalinity threshold of salinity 55. No behavioural preference was shown in the control tests. It could be expected that due to the littoral environment experiencing a range of both hypo and hypersalinities brought about through precipitation, runoff and evaporation, that these species would tolerate the largest range of hypersalinity and therefore be less selective in their choice due to this adaptation. This was not the case for *C. maenas*. The upper threshold of salinity 40 is consistent with the results found by other authors who found that *C. maenas* has a preference for salinities 27-41 (Thomas et al. 1981) and 27-40 with a lower threshold of 17 (Ameyaw-Akumfi and Naylor 1987). *C. maenas* is sensitive to salinity change and known to distinguish between salinities with as little difference between them as 0.5 psu (McGaw and Naylor 1992). In the UK, in the mid to low littoral environment where *C. maenas* is found, hypersalinity (brought about by evaporation from tidal pools during hot weather) is infrequent compared with diluting phenomena such as rainfall and runoff (Morris and Taylor 1983), therefore these results do not seem unusual. However, in high-shore rock pools, salinities can vary between 4 and 150 psu (McAllen et al. 1998) so it is plausible that under neap tides *C. maenas* on the mid shore could experience some increase in the salinity of its environment due to natural evaporation and followed by the water in tidal pools not being completely refreshed on the high tides.

There is a current lack of information on the effects of hypersalinity on *P. bernhardus*, although studies on the effects of hyposaline media have shown it to be an osmoconforming species which shows increasing locomotor activity in dilute media (Shumway 1978; Davenport et al. 1980) and in the field it is restricted to tidal pools of 25 psu minimum, although under laboratory conditions it can be acclimated to 15.5 psu over a two week period (Davenport et al. 1980). This laboratory

response is a physiological tolerance rather than the behavioural one observed for the field specimens, and may not be the sort of physiological tolerance that would be naturally present due to the non-natural nature of laboratory studies.

The adults of the other three species tested, *Homarus gammarus, Cancer pagurus* and *Necora puber* are all widely considered as being principally sublittoral species, with adults only occasionally occurring in the lower littoral zone (*pers obs.*), probably stranded in pools by the receding tide. Subtidal/sublittoral species generally are considered as ion- and osmoconformers as, due to the unlikelihood of being challenged by salinities other than 100% sea water, they do not have as much need for regulatory mechanisms. Consequently, the upper threshold values found for these three species are unexpected. Here, both *N. puber* and *C. pagurus* chose equally to be in normal seawater and hypersalinities up to but not including 50 psu - 15 psu above ambient. *H. gammarus* also showed no preference between normal and hypersalinities up to its behavioural threshold of 45 psu. As no preference was shown in the control tests, these findings suggest that all three species may be able to ion- and osmoregulate efficiently up to their threshold point even though naturally occurring hypersaline challenges are rare in their natural environments. Choice of a certain salinity hints that physiologically that salinity may be tolerable, at least for a certain amount of time. This tolerance can then lead to acclimation which is the point at which an organism is considered to have adapted physiologically to survive in the new environment. A median lethal time of above 500 hours in a challenging environment was taken as indicating survival (Davenport 1972b), see Chapter 4.

As with *Pagurus bernhardus* the salinity-based responses of *Homarus gammarus, Cancer pagurus* and *Necora puber* are poorly investigated in the literature, with the both the lower and upper salinity preferences and tolerances of these three species lacking. However *H. gammarus* has been successfully acclimated down to salinity 20 in the lab (Lucu and Devesconi 1999) and in juveniles reared at 15°C, mortality occurs only in salinities below 17 psu and above 46 psu with regulation being isosmotic in high salinities, and slightly hyperosmotic in low salinities (Charmantier et al. 1984).

The avoidance of both hypo and hypersalinities shown by *H. gammarus* in the two-choice salinity tests described here are not experimental abnormalities. *Homarus americanus* (the American lobster), shows similar avoidance behaviour, with seasonal salinity-dependent migrations to deeper

water in response to lowered salinity in its estuarine habitat (Robichaud and Campbell 1991; Jury et al. 1994a; Jury et al. 1994b). This species has also been shown to differentiate between ambient and hyposalinities with a lower limit of 12.6 psu that prompts avoidance behaviour (Jury et al. 1994b). *H. gammarus* and *H. americanus* are very closely related species that separated in the Pleistocene era. Slight morphological differences have been substantiated by biochemical studies but there is a low level of interspecific genetic variation and little intraspecific genetic differentiation between populations, with the species being able to hybridise with each other (Hedgecock et al. 1977).

Cancer pagurus is considered to be an osmoconformer (Péqueux 1995) and the related species *C. borealis* and *C. irroratus* are also sublittoral osmoconforming crabs (Charmantier and Charmantier-Daures 1991). The latter two species prefer rocky or gravelly bottoms, but can be found also on muddy/silty substrata (Robichaud and Frail 2006). Adult *C. irroratus* have a 48 h Lethal Salinity (LS_{50}) salinity tolerance range of 8.5 - 65 psu. In *C. borealis*, the corresponding LS_{50} values are 12 psu - 65 psu. The adults of both species were isosmotic in high salinities and weak hyper-regulators in low salinities (Charmantier and Charmantier-Daures 1991). The Dungeness crab, *Cancer magister,* can detect changes in salinity at 29.9 and 32.7 psu, values which correspond with 96% and 105% of its mean ambient of 31 psu (Sugarman et al. 1983). It is unknown whether these crabs also show preference behaviour associated with these LS_{50} values, however it is known that the osmoconforming *Cancer gracilis* when exposed to a hyposaline gradient, moves towards the higher salinities (Curtis et al. 2007), moving itself away from the unfavourable salinity.

The only portunid studied here*, N. puber,* is also considered to be an osmoconforming crab (Dorgelo 1979). Other portunid crabs that have been studied by other authors for their salinity responses include the swimming crabs *Liocarcinus (Macropipus) holastus* which enters the water column and allows water movements to carry it away from areas of adverse salinities (Venema and Creutzberg 1973) and *Callinectes ornartus* which shows limited tolerances for salinities below 25% sea water. Other portunids, *Arenaeus cribrarius* and *Portunus sebae* also show limited survival in salinities below 50% sea water (Norse 1978).

All species tested here in the two choice tests were able to distinguish between salinities and all made a choice of the 35 psu salinity once their particular threshold was reached. There was no significantly different salinity-dependent gender preferences of *Cancer pagurus* and *Homarus*

gammarus. Smith et al (2001) found no gender-dependent behaviour difference in lobsters relating to their general movements in their natural environment.

In the multi-choice tests *H. gammarus* showed a significant likelihood of choosing either a shelter of normal to low hypersalinity (35-40) or open water, (potentially exposed to predators), at salinity 35. *C. pagurus* appeared to indicate some preference behaviour with 75% choosing the lowest salinity areas of the tank, however this was not statistically significant. No preference behaviour was exhibited or indicated in the control tests for either species. This indicates a general preference for normal salinity over hypersaline areas. As with the two-choice tests, the multi-choice test results showed there was no gender-related difference in the salinity preference. The present finding, that lobsters choose equally to be either in a shelter at 35-40 psu or in open water at 35 psu, suggests they would actively avoid hypersalinity in their environment, therefore indicating that such behaviour may have a consequent influence on distributions of lobsters within areas affected by a brine plume. This preference for ambient salinity may have implications for fisheries located in brine discharge areas (*e.g.* the E. Yorkshire fishery), if adult (*i.e.* commercially valuable) lobsters relocate to more favourable habitats. It is known that the distribution of lobsters around the British Isles is related to sea bed topography, with distribution being limited to areas with rocky outcrops, wrecks, piers etc (Howard and Nunny 1983). During spring and summer *H. gammarus* in Poole Bay, England, only undertake excursions from their shelters during the night (Smith et al. 1999). These findings suggest a strong preference for shelter in this species as well as a possible negative phototaxis.

The species studied in this chapter are all also known for their shelter use and, in the multi-choice test, this behavioural preference for shelter was overridden by hypersalinity with both *H. gammarus* and *C. pagurus* choosing either a shelter of 35-40 psu or open water of 35 psu over shelters of hypersalinities. This suggests that in the field crab populations may avoid areas of the sea bed affected by a brine plume from any solute mining, regardless of whether there are opportunities for shelter in that area. The converse is true for *Hemigrapsus nudus;* when shelter is available it will endure longer exposure to salinities below its normal than when shelter is not available (McGaw 2001). In the case of *H. nudus* the benefits of shelter appear to outweigh the energetic costs of increased iono-osmoregulation when exposed to hyposaline conditions. The results of the multi-choice test suggest that avoidance of hypersaline environments overrides any negative phototaxis. Energetically, it is probably less costly to be in open ambient water than in a shelter which will

require a greater effort in iono-osmoregulation, especially as salinities over 48.9 are known to cause a 50% mortality in *H. gammarus* and 55.5 in *C. pagurus* (see Chapter 4). Jury et al (1994b) suggest that *Homarus americanus* uses behavioural adaptation to avoid potentially lethal low salinities. Females in particular seem more sensitive to changes and act first. The species displays a negative phototaxis with the lobsters consistently choosing an opaque shelter over a transparent one (Cobb 1971). *Cancer borealis* is also known to inhabit rocky crevices or burrows (Richards and Cobb 1986).

Laboratory behavioural responses occur in a small space and over a short period of time, often, as is the case here, with abrupt changes in tested parameters such as salinity, and caution is required in extrapolating such laboratory-based responses to the field. Salinity changes in the natural environment may be gradual and will most likely occur over a large area subjected to natural currents and wave action. Under such conditions, it is possible that behavioural choices are less marked than the results obtained here but, given sufficient time for a salinity change to develop, it is expected that behaviours as described will occur.

3.6 Conclusions

In summary, the species tested detected changes in salinity of magnitudes that are comparable to those that may be found in areas affected by brine discharges, such as the Aldbrough brine discharge discussed in Chapter 1. When exposed to high or low salinities the species tested avoided them, even when shelter was available.

Although behavioural responses to salinity change in the existing literature are extremely limited for the species studied and for related species, there was a decreasing tolerance as salinities depart from normal and a clear preference for ambient over hypersalinities once a threshold point is reached. This appears to be consistent with the related species for which information is available. Use of a shelter which is common to all studied species does not override the adverse properties of high salinities.

If the salinity of commercial effluents exceed the behavioural thresholds found here, it is likely that areas of the sea bed affected will become devoid of adult (fishable) specimens as they relocate to more favourable areas. In management terms it is advisable to ensure any hypersaline discharges are limited to the lowest tolerance of all the economically valuable species in the area to avoid loss of revenue in fishery areas.

As there is a notably little information on the salinity preferences of the species studied here, this work adds to our knowledge about these species. This aids a better understanding of their range in the wild, potential issues for lab husbandry and their importance in their community assemblages.

Chapter 4

The effects of hypersalinity on mortality of some commercially important crustaceans of the North Sea

4.1 Introduction

A toxicant may be defined as an agent that can produce a significant, adverse response in a biological system. The response itself may be induced damage to the structure and function in the organism or, in extreme cases, its death (Connell et al. 1999). In the present context, the particular toxicant studied is hyposaline seawater and, in particular, the effects that hypersalinity have on some species of crustaceans that feature prominently in the UK commercial shellfish landings.

A well known and straightforward method for assessing the impact of a change to an environmental variable on the inhabitants of an aquatic environment is to predict and calculate the mortality it causes to these animals. One of the simplest ways of doing this is to determine the lethal concentration or intensity of the variable (substance or toxicant) that is required to kill 50% of a population (or collection of test organisms). This value is the LC_{50} or 'median lethal concentration' (Moriarty 1993; Forbes and Forbes 1994; Connell et al. 1999) and examples of its use include those of pesticides: (Whale et al. 1988; Forget et al. 1998), heavy metals: (Gillis et al. 2006; Felten et al. 2008), hydrocarbons (Tatem et al. 1978); salinity changes (Leonard et al. 2011) and other natural substances at unnatural levels (Eklund et al. 2005).

The large majority of natural, aquatic environments have salinity levels which range between 0 (freshwater) and 35 (normal seawater). Hypersalinity may thus refer to the range of salinity above that experienced normally by an animal or above that to which it is acclimated at the time of test. The present studies refer to sublittoral, fully marine species and hypersalinity in this case refers to salinities >35. Such salinities can arise naturally, mainly in areas where supranormal evaporation levels occur *e.g.* in shallow, tropical estuaries and lagoons. Additionally, and of relevance in the current context, they occur also as 'unusual' events such as when hypersaline brine is discharged at sea or as 'inadvertent happenings' in the commercial environment of post-harvest, live crustaceans, again related often to evaporation. Due to the comparative rarity of natural, temperate

marine hypersalinity occurrence, compared with salinity changes within the ‘normal’ environmental salinity range, there is a consequent scarcity of information relating to the effects of hypersalinity on decapod crustaceans. Various ontogenetic stages of a variety of crustacean species have been used as test organisms for the study of the effects of hypersalinity. Examples include the embryos of *Hemigrapsus edwardsii* and *H. crenulatus*, larval stages of *Armases miersii* (Anger 1996), *Ucides cordatus* (Diele and Simith 2006), *Carcinus maenas* (Anger et al. 1998). That which pertains to the effects of hypersaline media, (up to salinity 65) on adult decapods, includes studies on *Cancer irroratus* and *C. borealis* (Charmantier and Charmantier-Daures 1991) and *Penaeus latisulcatus* (Minh Sang and Fotedar 2004).

As with any stressor, environmental salinities above the normal range of a species or population, necessitates the animals being able to resist impairment to their physiological fitness in order to survive, and, should the prevailing conditions persist, they need to be able to develop a new stabilised response, an acclimated response. If the salinity proves to be too high for the animal to reach an acclimated response, then its death is inevitable. Tolerance, however, does not necessarily imply that all of the animals remain unimpaired by the conditions and some important vital functions (*e.g.* growth rate, gonadal maturation or moult behaviour) possibly will become impaired. Furthermore, there may be sub-lethal, ontogenetic effects such as the impairment or delay of normal larval metamorphosis.

Because of the importance of the species studied here to the local environmental and commercial community, these studies were made in response to commercial, brine discharge activities offshore from the East Yorkshire coast. Some of the largest volumes of crustacean shellfish landed in the UK are made at the local ports (Walmsley and Pawson 2007) and the species tested (*Homarus gammarus, Cancer pagurus* and *Necora puber*) comprise major elements of these landings. Because there may be variability in moult-dependent severity of the effects of hypersalinity, the opportunity was taken to include post-moult and intermoult specimens of *H. gammarus* as these were available when the experiments were being made.

4.2 Aims and objectives

The aim of this study was to determine both the median lethal concentration (LC_{50}) of hypersaline sea water (median lethal salinity), and the median lethal time in a given hypersalinity (LT_{50}), required to kill 50% of a sample of *Homarus gammarus, Cancer pagurus* and *Necora puber* under acute hypersaline stress.

4.3 Materials and Methods

Mortality data were used to determine LC_{50} (median lethal concentration) and LT_{50} (median lethal time) figures for each species and moult stage tested. For the purposes of this investigation, hypersalinity is defined as any salinity above, and hyposalinity any salinity below, what the species normally experiences in the wild in the Yorkshire area. In the case of those tested here, that salinity is 35.

Animals were collected and held as described in Chapter 2. Upon experimentation, the crustaceans were subjected to an abrupt salinity change from the normal salinity 35 to a randomly chosen hypersalinity between 40 and 60 (in increments of 5). Eight intermoult *H. gammarus*, eight post-moult *H. gammarus*, ten to fourteen *C. pagurus* (ten crabs were used at salinity 35 and 14 crabs were used at salinities 40, 45 and 50) and ten *N. puber* were used at each salinity level. *H. gammarus,* and *C. pagurus* were held 2 per tank in opaque plastic tanks with 6L water per animal, *N. puber* were held at a stocking density of up to 5 animals per tank and 3 litres of water per crab. At the end of a 96 hour period in the hypersaline brine, mortality and survival figures were used to calculate LC_{50} and LT_{50} values for each species using probit analysis in SPSS. A parallel control test was also run in which the animals were moved from salinity 35 to salinity 35 to determine if any mortalities were caused by the stress of handling rather than salinity.

4.4 Results

4.4.1 Hypersalinity based mortality calculations for *Homarus gammarus*

Data obtained on median lethal times and associated hypersaline concentrations as calculated for intermoult and late postmoult *Homarus gammarus* respectively, exposed to an abrupt change in salinity, is summarised below (Table 4.1 and Table 4.2).

At salinities 35 and 40 all the intermoult specimens survived the 96 h time period but 3 of this group at salinity 45, and 4 at salinity 50 died within the experimental period. The calculated 96 h LC_{50} salinity for adult intermoult lobsters was 48.9, with a time required to kill 50% of the population (in the laboratory) of 111 h at salinity 45 and 98.32 h at salinity 50. The narrowing of the confidence limits with salinity increases indicates a more consistent effect on the lobsters at the higher salinities tested. As no animals died during the trial al salinities 35 and 40, the LT values at these salinities (for both intermoult and late postmoult lobsters) are calculated by the probit analysis from raw data and lethal values provided therein and are purely theoretical in this case. As salinity 35 is the normal environmental salinity for *H. gammarus* what can be explained from these results is that at salinities > 40 there is a positive relationship between salinity and mortality.

At both salinity 35 and salinity 40, only one late postmoult *H. gammarus* had died by the end of the 96 h time period. This contrasted with the findings at salinity 45 and salinity 50, in which all had died well within the 96 h time period. Specifically, at salinity 45 all were dead by 72 h and at salinity 50 all had died by 24 h. These results indicate that there is a critical salinity somewhere between salinity 40 and 45 at which late postmoult lobsters fail to withstand such a hypersaline challenge. This seems not to occur with the intermoult animals. The statistically calculated 96 h LC_{50} value for late-postmoult lobsters at salinity 40.9 was a lower value than that attained for their intermoult counterparts. The statistically predicted time required to kill 50% of the population (in the laboratory) was 235.6 at salinities 35 and 40.

Table 4.1 Median lethal concentrations and times for adult intermoult *Homarus gammarus* acutely exposed to hypersalinity in a laboratory environment. n = 8 per salinity.

Test	Lethal point	Confidence limits
96 hour LC_{50} (50% expected mortality)	Salinity 48.9	45.8 – 61.1
96 hour LC_{10} (10% expected mortality)	Salinity 42.1	23.9 – 45.3
96 hour LC_{90} (90% expected mortality)	Salinity 55.7	50.9 – 93.7
24 hour LC_{50}	Salinity 58.2	51.5 – 74.7
48 hour LC_{50}	Salinity 55.3	50.1 – 68.4
72 hour LC_{50}	Salinity 52.3	48.2 – 62.2
Salinity 35 LT_{50}	297.3hours	-1547.9– 638.1
Salinity 40 LT_{50}	297.3 hours	-1547.9 – 638.1
Salinity 45 LT_{50}	111.5 hours	82.9 – 238.2
Salinity 50 LT_{50}	98.4 hours	72.9 – 199.8

Table 4.2 Median lethal concentrations and times for late postmoult *Homarus gammarus* acutely exposed to hypersalinity in a laboratory environment. n = 8 per salinity.

Test	Lethal point	Confidence limits
96 hour LC_{50} (50% expected mortality)	Salinity 40.9	n/a
96 hour LC_{10} (10% expected mortality)	Salinity 36.8	n/a
96 hour LC_{90} (90% expected mortality)	Salinity 45.0	n/a
24 hour LC_{50}	Salinity 42.2	38.9—45.7
48 hour LC_{50}	Salinity 41.6	38.3—45.1
72 hour LC_{50}	Salinity 40.9	37.6—44.5
96 hour LC_{50}	Salinity 40.9	37.6—44.5
Salinity 35 LT_{50}	235.6 hours	n/a
Salinity 40 LT_{50}	235.6 hours	n/a
Salinity 45 LT_{50}	Not calculable*	n/a
Salinity 50 LT_{50}	Not calculable*	n/a

* All of the animals died at salinity 45 and 50, before the experiment was concluded.

n/a – SPSS was unable to calculate confidence limits in these instances because of the large variability in the data and the excessive mortalities.

4.4.2 Hypersalinity-based mortality calculations for *Cancer pagurus*

Probit analysis was used to calculate the median lethal times and concentrations for *Cancer pagurus* exposed to an abrupt change in salinity and the data obtained are summarised in Table 4.3. These data show that salinities 35 and 45 were not lethal within the 96 h time period but, at salinity 40, two crabs had died by the end of the trial and, at salinity 50, five crabs had died by the end of the trial. The calculated data showed the salinity required to kill 50% of the population after 96 hours was 55.5 (CL: 48.6 – 57.9). Ten percent of the population would be expected to die after 96 h at salinity 42.9 and 90% of the population are expected to die after 96 hours in salinity 68 (Table 4.3). The direct relationship between high mortality and high salinity are supported by the predicted LT_{50} values which show the time taken to kill 50% of the population decreases from 431.7 hours at normal environmental salinity (35 psu) to 121.6 hours at salinity 50. It must be noted however, that salinity 35 is not a lethal salinity for any of the species here, as it is in fact the salinity in which they spend their lives. The lethal LT_{50} value of 431.7 hours is a figure the probit analysis extrapolates from the raw data. With this in mind, it can be understood from the results, that salinities < 40 are not lethal but higher salinities show a trend of increasing lethality, as evidenced by the LT_{50} data.

Table 4.3 Median lethal concentrations and times for *Cancer pagurus* acutely exposed to hypersalinity in a laboratory environment. n = 10-14 per salinity.
(ten crabs were used at salinity 35 and 14 crabs were used at salinities 40, 45 and 50).

Test	Lethal point	Confidence limits
96 hour LC_{50} (50% expected mortality)	Salinity 55.5	48.6 – 57.9
96 hour LC_{10} (10% expected mortality)	Salinity 42.9	n/a
96 hour LC_{90} (90% expected mortality)	Salinity 68.1	n/a
24 hour LC_{50}	Salinity 55.6	51.5 – 64.4
48 hour LC_{50}	Salinity 55.6	51.5 – 64.4
72 hour LC_{50}	Salinity 54.5	50.8 – 62.5
Salinity 35 LT_{50}	431.7 hours	n/a
Salinity 40 LT_{50}	242.8 hours	n/a
Salinity 45 LT_{50}	428.1 hours	n/a
Salinity 50 LT_{50}	121.6 hours	n/a

n/a - SPSS was unable to calculate confidence limits in this instance due to high variability of data

4.4.3 Hypersalinity based mortality calculations for *Necora puber*

As for the other species studied, probit analysis of the mortality results from acute exposure trials with *Necora puber* exposed to an abrupt salinity change was used to calculate median lethal times and concentrations. The resulting data are summarised in Table 4.4 and reveal that a 50% mortality after 96 h can be expected at a salinity of 41.9. Theoretically, 10% of the population would be expected to die after 96 h at salinity 32.3 and 90% to die at a salinity of 51.5 after 96 h. The time taken to kill 50% of the population decreases with increasing salinity from 126.1 hours at salinity 35 to 12.3 hours at salinity 60.

Table 4.4 Median lethal concentrations and times for *Necora puber* acutely exposed to hypersalinity in a laboratory environment. n = 10 per salinity.

Test	Lethal point	Confidence limits
96 hour LC_{50} (50% expected mortality)	Salinity 41.9	32.6 — 48.1
96 hour LC_{10} (10% expected mortality)	Salinity 32.3	n/a
96 hour LC_{90} (90% expected mortality)	Salinity 51.5	n/a
24 hour LC_{50}	Salinity 61.4	52.9 — 73.9
48 hour LC_{50}	Salinity 49.5	42.5 — 57.2
72 hour LC_{50}	Salinity 44.3	36.6 — 51.2
Salinity 35 LT_{50}	126.1 hours	99.4 — 163.4
Salinity 40 LT_{50}	71.8 hours	56.3 — 89.4
Salinity 45 LT_{50}	99.9 hours	79.5 — 121.2
Salinity 50 LT_{50}	42.8 hours	23.0 — 59.7
Salinity 55 LT_{50}	45.2 hours	27.2 — 61.1
Salinity 60 LT_{50}	12.3 hours	21.7 — 39.4

n/a – SPSS could not calculate confidence limits in this instance due to high variability in the raw data

4.5 Discussion

These studies show clearly that there is interspecific variability of hypersaline sensitivity amongst the species studied. On the evidence of calculated 96 h LC_{50} data, *Cancer pagurus* is the most tolerant and *Necora puber* the least tolerant species. The data also suggest that there is a moult-stage-dependence of hypersaline tolerance as intermoult *Homarus gammarus* was much more tolerant than late intermoult specimens with 96 h LC_{50} values of 48.9 and 40.9 salinity respectively The susceptibility of the postmoult specimens (with a 96 h LC_{50} salinity only 5 salinity units above ambient) probably is indicative of the osmotic-ionic-regulatory problems moulting species have in maintaining homeostasis in hypersaline media. This perceived order of tolerance for hypersalinity is supported by the results of chapter 5 where an examination of the blood chemistry of these three species when under both chronic and acute hypersaline stress has shown that *C. pagurus* appeared to be the best regulator, with the fewest haemolymph variables changing in response to hypersalinity and *N. puber* demonstrating a much greater number of variables changing with salinity increases (see chapter 5).

Necora puber yielded a 96 h LC_{50} salinity value of 41.9, only 6 salinity units above that of normal seawater and, if the assumption is made that the value would be substantially lower with postmoult animals as seen in *H. gammarus*, then it is unlikely that populations of this species would be able thrive in a hypersaline environment such as the vicinity of a brine discharge. Contrastingly, intermoult *Cancer pagurus* showed a high tolerance of hypersalinity as evidenced by its 96 h LC_{50} salinity value of 55.5, over 20 units above that of normal seawater. However this value is also the same as its 24 h LC_{50} value. This may be indicative of variation in response within the species (an intra specific difference) where the less robust specimens die very quickly, but the stronger ones then can survive the same conditions for a much longer time.

The results from chapter 3 have shown that all species tested preferentially choose normal salinity over both hyper- and hyposalinities, with this choice becoming more marked as the deviation from normal increases. Clearly there is therefore an optimum salinity range at, or very near to, 35 which these species prefer to inhabit. It is well understood in the literature that, like the effects studied here, deviations from the normal salinity a crustacean inhabits can cause implications for grown and maturation, which in turn have the potential to affect populations. The rock crab (*Cancer irroratus*) also shows higher levels of osmotic stress and increased mortality when experiencing an

acute deviation from normal salinity (in this case in the hyposaline direction) rather than when acclimated to lower salinities (Cantelmo et al. 1975). The effects of acute salinity changes (in the range 5-55 psu) were studied for the larval and first juvenile stages of the tropical crab *Armases miersii*. Extremes of salinity (5 and 45-55) caused prolonged development, and during prolonged exposure effects of severe osmotic stress outweighed any evidence of acclimation, additionally mortality was more frequent at these extremes (Anger 1996). Salinities below normal (20psu) caused delays to the metamorphosis of *Carcinus maenas* larvae, reduced growth and respiration rates and enhanced number of mortalities when compared with higher salinities closer to the norm of salinity 35 (Anger et al. 1998). Hypersalinities and low temperatures can delay development in the larvae of the tropical crucifix crab *Charybdis feriatus* (Baylong and Suzuki 2007). With these results in mind, it is possible that even if an adult crustacean can survive in an anthropgenically produced hypersaline brine plume, it may have reduced potential for successful reproduction and the maturation of any surviving larvae.

These studies were made in response to brine discharge activities in an area of the North Sea that is particularly important in terms of its value as a source of high catches of crustacean shellfish. It would appear that all 3 species studied are tolerant of the range of salinities that occur within the majority of the discharge plume, which has under routine monitoring (year 2006-7) been shown to not increase further than 47.9 psu at 50m from the discharge point and 37.1 psu at 250m, however the discharge consent does allow the raw brine to be discharged at 284 $g.L^{-1}$ which equates to a salinity of 284 (Jacobs 2007). Physiological tolerances, such as those measured here, give some measure of the ability of a species to tolerate and survive a period of emersion in a hypersaline environment but tell little of the long-term health of the populations should the hypersaline conditions persist. Animals must feed, meet and mate and should any behavioural functioning relating to these activities be impaired by the prevailing conditions, then this will impact negatively on the health of those populations.

Deviations from normal environmental salinity can also affect how well crustaceans cope with other environmental changes. For example, the blue crab *Callinectes sapidus* is known to suffer population decline during particularly cold winters, and this mortality was enhanced when environmental salinity was lower (Rome et al. 2005). Similarly, Hardy et al (1994) conducted 96h mortality tests on both hard and soft shelled snow crab (*Chionoectes opilio*) in response to changing salinity and temperature. In contrast to the findings of this thesis, there was no difference

in the salinity tolerance of the crabs when comparing the two different moult stages. However soft shelled crabs did have a very variable osmotic balance when compared to their hard-shelled counterparts and this osmotic concentration was decreased by increases in temperature which was an effect absent from the hard shelled crabs. The overall conclusion of their study was that the interaction of salinity and temperature made the soft shelled crabs more susceptible to high temperatures when salinity was low, when compared to hard shelled crabs.

Changed susceptibility to infection is another possible effect of abnormal salinity exposure. For example, hypersalinities (compared the normal of 0) in combination with temperature changes and increases in ammonic concentration, caused a reduction in the resistance to the pathogen *Lactococcus garvieae* in the freshwater giant prawn *Macrobranchium rosenbergii* (Cheng et al. 2003). The white shrimp (*Litopenaeus vannamei*) was more susceptible to infection and mortality arising from *Vibrio alginolyticus* when reared under hyposaline conditions (Wang and Chen 2005) and tiger shrimp *Penaeus monodon* transferred from salinity 25 to low salinity levels (5 and 15) and high salinity (35) had reduced immune ability and decreased resistance against *Photobacterium damselae* subsp. *damselae* infection (Wang and Chen 2006).

What these examples indicate is that as well as deviations from the normal salinity affecting mortality of crustaceans, they also induce other effects such as reduced resistance to infection, delays to development of larval stages and that salinity can affect the tolerance of other environmental variables such as temperature changes. Therefore as well as addressing purely salinity based mortalities as a quick way of how any brine discharge is potentially affecting a crustacean population, it is also prudent to look at how salinity can work with other environmental parameters, potentially affecting the tolerance of these too.

4.6 Conclusions

In conclusion, these experiments were designed to be preliminary in nature and were intended to indicate whether they should be supported by subsequent, more in-depth studies relating to the complete moult cycle of the animals. The preliminary findings indicate that, in addition to considerable interspecific variability in hypersalinity tolerance, there is evidence that considerable intraspecific, moult-stage-dependent variability also exists. Because population health can be impaired by lack of tolerance shown by any individual stage of the moult cycle, and that deviations from normal salinity are known in other decapod species to affect metamorphosis, growth, maturation and ability to cope with change to other environmental variables, it is considered here that further, in-depth studies of moult-dependent hypersaline stress are needed for these 3 species, including the embryonic, larval and juvenile life stages. It may also be prudent to investigate the effects of hypersalinity on important food items in their diets.

Chapter 5

Acute and chronic effects of hypersaline exposure and consequences for haemolymph condition in the lobster *Homarus gammarus* (L.) and the crabs *Cancer pagurus* (L.) and *Necora puber* (L.)

5.1 Introduction

Homarus gammarus (Linnaeus 1758), *Cancer pagurus* (Linnaeus 1758), and *Necora puber* (Linnaeus 1767) are stenohaline, osmoconforming species. There are few studies made on their salinity tolerances and these relate to their responses to hyposaline conditions with little, if any, knowledge of their responses to hypersaline challenge. In their natural environments, subtidal marine, temperate crustacean species rarely, if ever, experience hypersaline challenges hence the limited number of papers dealing with this subject. The principal focus of studies that have been made on high salinities relate to the effect of desalination plant discharges in hot climates (Meerganz von Medeazza 2005; Raventos et al. 2006; Smith et al. 2007), saltpan and saline lake species which experience high evaporation effects in their environment (Nunes et al. 2006; Clegg and Gajardo 2009) and mangrove crustacean species (Anger and Charmantier 2000; Gillikin et al. 2004).

Salinity is an environmental master factor in control of the reproduction, larval dispersal and recruitment, and geographical distribution of marine crustaceans (Anger 1991; Anger 1996; Spivak and Cuesta 2009) and hence salinity changes are likely to impact on community structure. Salinity also directly affects osmotic and ionic regulation and indirectly affects acid-base balance and various components of the respiratory system including ventilation, gas exchange, perfusion, O_2 transport by the respiratory pigment and utilisation at the tissues (Wheatley 1988). The E. Yorkshire brine diffuser is sited within an area important for commercial fishing of *H. gammarus, C. pagurus,* and *N. puber,* the catches of which contribute significantly to the economy (Walmsley and Pawson 2007). Consequently although hypersalinity is not a common phenomenon for this region, its ecological effects may have a significant potential commercial relevance. This is true in terms of

the success of fishing and post-harvest marketing operations as well as having potential impacts on larval recruitment and stock replenishment.

It has already been discovered that the study species can differentiate between hypersalinities and ambient salinity and that they show a behavioural preference for ambient salinity. The objective of this chapter is to discover whether the internal physiology of crustaceans is affected by hypersalinity, hence potentially causing problems for their survival in the field. Stressors such as aerial exposure (Durand et al. 2000; Danford 2001; Bernasconi and Uglow 2008), exposure to contaminants (Nonotte et al. 1993; Depledge and Lundebye 1996; Vitale et al. 1999), exercise (Wood and Randall 1981) and salinity (Spicer and Taylor 1987; Wheatley 1988) are all represented by haemolymph changes so it is probable that the stressor of hypersalinity induces similar measureable changes. To assess whether such changes occur it is hypothesised here that there are quantifiable changes to a variety of haemolymph variables of *H. gammarus, C. pagurus* and *N. puber* in response to acute and chronic changes to ambient hypersaline media. The objective of testing the haemolymph was to determine whether such brine discharges impact negatively on these commercially important species in terms of sublethal impacts and physiological changes. Lactate, pH and haemocyanin changes are indicative of anaerobiosis, glucose and protein can be indicative of nutritional state, with proteins also being related to stress level in the form of heatshock proteins. Ammonia is a measure of metabolic state and how effectively an organism is discharging waste. The ionic composition of the blood also has the potential to influence the metabolism and physiological processes. Levels of haemolymph sodium, potassium, magnesium and calcium (all constituents of seawater) can also indicate the degree of both ion and osmotic regulation happening, if any, in hypersaline conditions. Copper, as well as being the metal in the respiratory pigment haemocyanin, can in high concentrations be damaging or lethal to aquatic organisms (Flemming and Trevors 1989; Grosell et al. 2007).

Analysis of these parameters shows whether, under stressed conditions, an organism can still feed, respire and metabolise efficiently and ultimately allows predictions to be made on the survival potential of populations facing such stresses. Such studies are important given the imminent increased gas cavern and desalination plant construction projects and the need to determine their potential impacts on international commercial crustacean fisheries.

5.2 Aims and objectives

The aim of this study is to determine whether hypersalinity causes changes in selected properties of decapod crustacean haemolymph (as described above) and if so to what extent? To answer these questions changes to the haemolymph were assessed under two salinity regimes:

1) acclimation to hypersaline conditions (chronic hypersaline exposure). This was done for *H. gammarus* and *N. puber* only.
2) abrupt salinity change from ambient to hypersaline (acute hypersaline exposure). This was carried out on *H. gammarus, N. puber* and *C. pagurus*. *C. pagurus* was used for the acute response trial only due to limited numbers available at the time of study (see section 5.3.2).

5.3 Materials and Methods

5.3.1 Animal husbandry

Creel-caught, intermoult specimens of *Necora puber*, *Homarus gammarus* and *Cancer pagurus* were obtained from commercial landings at Bridlington, East Yorkshire, U.K. and held as described in Chapter 2.

5.3.2 Experimental set up, observation and recording. Chronic hypersaline exposure

For the chronic tests, tanks were set up as follows: *H. gammarus* was held 2 per tank in opaque plastic tanks with 6L water per animal. *N. puber* was given opaque plastic tanks at a stocking density of up to 5 animals per tank and 3 litres of water per crab. At the start of the test, the crabs were in natural seawater (salinity 35) and, after every 96 h period, the seawater was increased by 5 salinity units until eventually all the animals had died. The seawater was obtained from the East Yorkshire coast and the salinity increases effected with the addition of Instant Ocean™ aquarium salts. Instant Ocean™ was used as it is likely that brine discharges from different geographical locations will have different chemical compositions due to the variable nature of natural mineral resources. Hence this compromise of using artificial seawater to raise salinity instead of brine discharge effluent allows the possibility of translating any responses seen to other temperate latitude brine discharges due to the production of generic, salinity-only, responses rather than responses influenced by any other chemicals that may be present in brine collected from a discharge site.

All test specimens were kept under observation during the first hour following a change of medium and were checked twice daily for mortalities. Torpid animals (those that did not respond to direct physical stimulus from a glass rod) were removed and, where possible, a haemolymph sample was collected to test for death as in the two most concentrated media tested (salinities 55 and 60) it was apparent that the animals had weakened and the difficulty/inability to collect a haemolymph sample was used to distinguish between torpid and dead animals. Haemolymph would still flow easily into the syringe in a torpid animal but not a dead one. Microscopic examination showed the blood in dead animals was either extremely clotted or all the cells had lysed (see Chapter 2).

Immediately prior to a scheduled salinity change, 6 *H. gammarus* and 7 *N. puber* were selected randomly and removed from the experimental chambers for haemolymph sampling. *C. pagurus* was not used in this trial due to the poor quality of specimens from the local fishery at the time of experimentation. Crabs from that year (2009) were of very poor meat yield for their size and were not surviving after capture and there was a complete ban on landing *C. pagurus* imposed by the North Eastern Sea Fisheries Committee (NESFC). This led to a total of 30 *H. gammarus*, and 42 *N. puber* specimens sampled. Blood samples (2 ml each) were obtained through the arthrodial membrane at the base of the 4th or 5th periopod on the crabs and through the underside of the second abdominal segment of the lobsters, using a hypodermic needle. Each individual was bled once only and particular care was taken to ensure minimal stress during sampling as this may have affected the blood properties. Each haemolymph sample was subdivided into 7 individual, labelled micro-centrifuge tubes which (depending on the analysis needed, see below) were treated and frozen (-20 °C) until analysed.

To account for potential handling effects a duplicated series of experiments was run in parallel in which the same stepwise setup and method was undertaken but only with ambient salinity 35.

5.3.3 Experimental set up, observation and recording. Acute hypersaline exposure

For the acute response tests, the same strength media were used as in the chronic response tests (salinity 35, 40, 45, 50, 55 and 60) and the same stocking densities and water volumes. *H. gammarus,* and *C. pagurus* were held 2 per tank in opaque plastic tanks with 6L water per animal. *N. puber* were held at a stocking density of up to 5 animals per tank and 3 litres of water per crab. The lobsters and crabs were taken from the holding tanks (salinity 35) and introduced to a randomly chosen higher salinity test medium without prior acclimation. As in the chronic response experiments, the animals were observed for the first hour then checked twice daily for mortalities. The specimens remained in the test medium for 96 h after which a 2 ml haemolymph sample was obtained for subsequent analysis from between 9 and 10 *H. gammarus* (total 39), between 10 and 14 *C. pagurus* (total 52) and 7 *N. puber* (total 42). The control experiment was run in parallel and, again, only one test medium was used (salinity 35) and a haemolymph sample taken from each individual after 96h exposure.

A further trial was carried out on lobsters in the late-post-moult soft-shelled stage. The same set up was used as described above for the acute trial on adult intermoult *H. gammarus* with the aim of assessing whether lobsters in a hypersaline area could survive the moult stages of their life cycle. It is well known that the moult is a dangerous time for all crustaceans and that in the soft stage, tolerances to environmental variables can change (McLeese 1956; Jury et al. 1994b). The lobsters used were not the completely soft-bodied ones directly after the moult but in the late-postmoult stage where the shell was hardening yet still supple under pressure. The only salinities used were 35, 40 and 45, however at salinity 45 all lobsters died before the end of the 96 hour period so were excluded from haemolymph collection and analysis. Seven lobsters were tested at each salinity increment.

In a further trial, the same set up was used as described above for the acute trial on *H. gammarus* but the hypersaline media was made up with brine from a local hypersaline discharge to compare to the generic response produced by artificial aquarium salts. Eleven lobsters were used at salinity 50 only, for 96h before haemolymph samples were analysed.

5.3.4 Haemolymph analysis

Collected haemolymph samples were analysed following Danford (2001) and Bernasconi and Uglow (2008), methods are described in sections i to vii. After haemolymph sampling, animals were not returned to the experiment but were returned to ambient sea water conditions for recovery. Samples were analysed for serum protein, glucose, lactate, haemocyanin, pH and ionic properties. Where technique or assay kit accuracy was available it is given here.

i. HAEMOLYMPH PREPARATION

Analysis for serum (plasma) protein levels required centrifuging of the sample at 3500 rpm for 10 minutes. The supernatant was then drawn off using a Pasteur pipette and the protein levels analysed as described in section iii. Glucose, lactate and haemocyanin analysis required the deproteinisation of each sample. This was to ensure there were no changes in the haemolymph following sampling and that nothing would interfere with haemocyanin at the diagnostic wavelengths in the spectrophotometer. For deproteinisation, samples were diluted 1:1 with chilled 0.6M perchloric acid (PCA) and then centrifuged at 7200 rmp for 5 minutes before the supernatant was drawn off using a Pasteur pipette and stored in a new eppendorf (Danford 2001; Bernasconi and Uglow 2008). Serum protein levels and pH values were calculated immediately on blood collection. Remaining blood was treated if needed using the methods described above then frozen at ≤ 20°C until needed. Ammonia was measured using a flow injection/gas diffusion method (Hunter and Uglow 1993) with the detector output analysed with chart-recording software (Chart5, AD Instruments) on a PC (see section iv below).

ii. PH MEASUREMENT

Readings of haemolymph pH were taken immediately following sampling and at aquarium temperature. Immediate measurement of pH was necessary to minimise any changes in pH that may occur subsequent to sampling. A pH probe (BDH combination micro-electrode) connected to a pH meter (Jencon PHM2) was used for all determinations. This probe had a small diameter rod that was able to reach the base of 1.5 ml eppendorf centrifuge tubes. The probe was calibrated to pH 4 and pH 7 using buffer solutions before each set of measurements, and was calibrated at aquarium

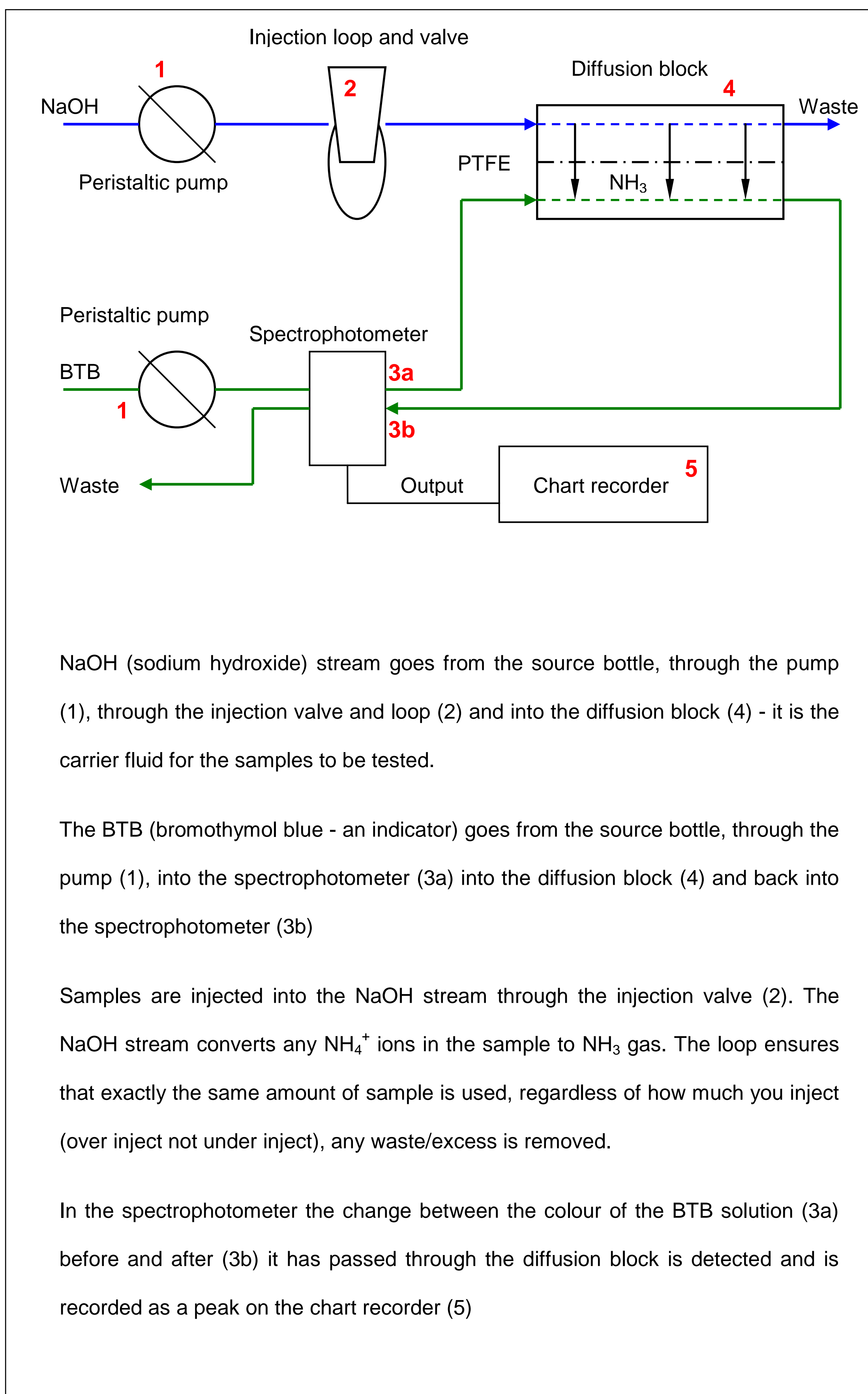
temperature. The probe was dipped in each sample for 20 seconds and the reading shown at 20 seconds was taken as the pH if it had not settled before this time.

iii. SERUM PROTEIN MEASUREMENT

Centrifuged haemolymph supernatant protein concentrations were determined using a hand held clinical protein refractometer (ATAGO Clinical refractometer SUR-NE Cat. No. 2734). Calibration was achieved as per the instructions by adding 200μl distilled water to cover the lower prism slide and adjusting the calibration screw as necessary. For each sample 200μl of haemolymph was added to the slide and the protein concentration (g/100ml) read where the coloured zone changed to white. This method is accurate from 0.0 to 12.0 g/100ml.

iv. TOTAL AMMONIA MEASUREMENTS BY FLOW INJECTION ANALYSIS

Measurement of total ammonia levels (mmol) in the haemolymph was carried out using flow injection analysis (FIA) (Figure 5.1).



NaOH (sodium hydroxide) stream goes from the source bottle, through the pump (1), through the injection valve and loop (2) and into the diffusion block (4) - it is the carrier fluid for the samples to be tested.

The BTB (bromothymol blue - an indicator) goes from the source bottle, through the pump (1), into the spectrophotometer (3a) into the diffusion block (4) and back into the spectrophotometer (3b)

Samples are injected into the NaOH stream through the injection valve (2). The NaOH stream converts any NH_4^+ ions in the sample to NH_3 gas. The loop ensures that exactly the same amount of sample is used, regardless of how much you inject (over inject not under inject), any waste/excess is removed.

In the spectrophotometer the change between the colour of the BTB solution (3a) before and after (3b) it has passed through the diffusion block is detected and is recorded as a peak on the chart recorder (5)

Figure 5.1 – Schematic diagram showing the setup and operation of the Flow Injection Analysis system for ammonia measurement in water and haemolymph samples.
After Hunter and Uglow (1993).

Samples are injected into the NaOH (0.1M) carrier stream causing the conversion of ammonium ions (NH_4^+) to ammonia gas (NH_3). This passes through the spectrophotometer and as it is colourless does not produce an output. The gas then passes through the diffusion block where it joins a new carrier stream of 0.6 gL^{-1} bromothymol blue (BTB) which has a dark green colour. The presence of ammonia causes a change in the colour of the BTB solution towards the more alkaline blue. The solution passes again through the spectrophotometer and this time is recorded as a peak. The heights of the peaks recorded by the FIA system are linearly related to the ammonia concentration. Ammonia standards of 25, 50, 100, 200, 300, 400, 500 μmol/L were made from a stock solution of ammonium sulphate 0.5 mM using Milli-Q™ water. Standards were made prior to the haemolymph analysis and were tested in the FIA apparatus. The peaks of these standards were used to produce a linear calibration curve and the regression equation of this line was used to calculate the amount of ammonia in the haemolymph samples. A set of standards was injected after every 20 haemolymph samples to allow for any change in the sensitivity of the apparatus. This correction was done as precipitates can form along the tubing and on the polytetrafluoroethylene (PTFE) membrane which can impair the transfer of ammonia and result in an underestimation of the concentration. Precipitates were removed by flushing with 10% HCl acid for 1-2 minutes, followed by distilled water and a replacement of the PTFE membrane. The FIA ammonia technique has a lower limit of detection of 0.02 μmol ammonia L^{-1} and a precision in the range of 0.9-3.3% (Hunter and Uglow 1993).

v. HAEMOCYANIN MEASUREMENTS

Haemocyanins have three major absorption maxima in an oxygenated state at 280, 335 and 580 nm. The latter two peaks disappear upon deoxygenation. As haemocyanin is rapidly oxygenated in vitro, the absorption peak can be used as an effective measure of pigment concentration using a 1cm extinction coefficient (ε^{mM}) of 17.26. The ε^{mM} value is calculated from an $\varepsilon^{1\%}$ value of 2.83 given by (Nickerson and Van Holde 1971; Antonini and Brunori 1974)

$$\text{concentration haemocyanin (mM)} = \frac{A_{335} * \text{dilution factor}}{\text{extinction coefficient (17.26)}}$$

Haemolymph samples were diluted 1:49 with distilled water and their absorbancies measured in a cuvette against a distilled water blank at 335 nm on a UV spectrophotometer (Biochrom Libra S11 UV spectrophotometer).

vi. IONIC CONCENTRATION MEASUREMENTS

The haemolymph of *H. gammarus*, *C. pagurus*, and *N. puber* were analysed using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) for their ionic content (Na, Mg, K, Ca, Cu). This was then compared to the same analysis done on water samples. Water samples were either made from a hypersaline brine from a gas cavern discharge diluted to 35, 40, 45, 50, 55 and 60 salinity units using Milli-Q ultra pure water, or from natural sea water at salinity 35 made up to the same units using Instant Ocean™ aquarium salts. Natural sea water and pure hypersaline brine were also analysed for their ionic contents.

Ionic elements of the haemolymph were analysed to help assess the potential influence hypersalinity has on crustacean metabolism and physiological processes. Ionic composition also indicates the degree of regulation happening, if any, in hypersaline conditions.

In ICP-OES, a sample is usually transported into the instrument as a stream of liquid sample. Inside the instrument, the liquid is converted into an aerosol through nebulisation. The sample aerosol is then transported to the plasma where it is desolvated, vaporised, atomised, and excited and/or ionised by the plasma due to temperatures being high enough to cause not only dissociation of any compounds into atoms but to cause significant amounts of collisional excitation (and ionisation) of the atoms to take place. The excited atoms and ions emit their characteristic radiation which is collected by a device that ranks the radiation by wavelength. Detected radiation is turned into electronic signals and converted into concentrations. In OES, the intensity of the light emitted at specific wavelengths is measured and used to determine the concentrations of the elements in the sample. One of the most important advantages of OES results from the excitation properties of the high temperature sources used in OES. These thermal excitation sources can populate a large number of different energy levels for several different elements at the same time. All of the excited

atoms and ions can then emit their characteristic radiation at nearly the same time. This allows the operator to choose from several different emission wavelengths for an element and in the ability to measure emission from several different elements at the same time (Boss and Fredeen 1997).

Samples were analysed using this technique by the Hull University chemistry department for their content of copper (Cu), sodium (Na), potassium (K), magnesium (Mg), and calcium (Ca).

vii. LACTATE MEASUREMENTS

Lactic acid levels in deproteinised, centrifuged haemolymph were determined using a colourimetric kit (Trinity biotech cat no 735). Lactate is converted to pyruvate and hydrogen peroxide (H_2O_2) by lactate oxidase. In the presence of hydrogen peroxide, peroxidase catalyses the oxidative condensation of chromogen precursors to produce a coloured dye with an absorbency at 540 nm. The increase in absorbency is directly proportional to L-lactate concentration in the sample.

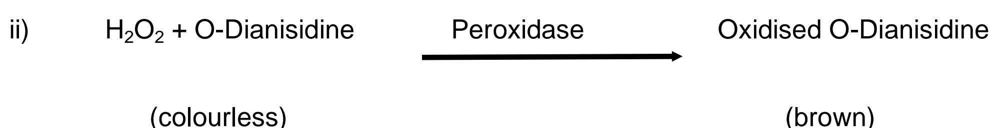
The lactate reagent was prepared by reconstitution with 10ml distilled water. This was then pipetted (1 ml) into numbered/labelled test tubes for each of the samples plus a blank and a standard. To the standard tube 10μl of lactate standard was added and to the sample tubes 10μl of haemolymph supernatant were added. Each tube was carefully mixed by inversion and incubated at room temperature (20 °C ± 2 °C) for 10 minutes. The samples were then read for absorbency at 540 nm against the blank and standard in a Biochrom Libra S11 UV spectrophotometer. Unknown samples were converted to L-lactate concentration using the following equation.

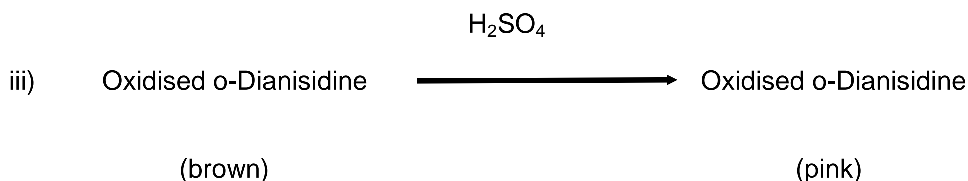
$$\text{Lactate (mg/dl)} = \frac{(A_{540} \text{ of test}) * 40}{A_{540} \text{ of standard}}$$

To convert the results to mmol l^{-1} the result was multiplied by 0.111 as per the kit instructions. The lactate assay precision has a standard deviation of 0.15-0.96 mg dL^{-1} and a lower limit of detection of 2 mg dL-1 = 0.222 mmol l^{-1}.

viii. GLUCOSE MEASUREMENTS

Deproteinised centrifuged haemolymph supernatant samples were taken from the freezer, thawed then tested for their glucose content using the colourimetric Sigma Kit No. GAG0-20. This method of measuring glucose is based upon the following enzymatic reactions:





As per the sigma kit instructions, one capsule of o-Dianisidine reagent was dissolved in 1 ml of distilled water and refrigerated in darkness. 0.8 ml of this reagent was added to 39.2ml of glucose oxidase/peroxidase reagent and again refrigerated in darkness. To measure the glucose concentration of the haemolymph samples, 0.5 ml of sample was added to 1 ml of the assay reagent in a test tube and mixed. Samples were incubated in a water bath at 37 °C for exactly 30 minutes, then the reaction stopped by adding 1 ml of 12M H_2SO_4. This caused the samples to turn from brown to pink in colour. The absorbance of each sample was then read at 540 nm in a spectrophotometer (Biochrom Libra S11 UV spectrophotometer). The absorbance was measured against a distilled water blank and a 0.05 mg glucose standard solution that was treated in the same way as the haemolymph samples. The intensity of the colour at 540 nm is proportional to the original glucose concentration. Glucose concentrations of unknown samples were calculated using the following equation:

$$\text{Glucose (mg)} = \frac{(A_{540} \text{ of test})*(\text{mg glucose in standard})}{A_{540} \text{ of standard}}$$

$$\text{Glucose (mg)} = \frac{(A_{540} \text{ of test})*(0.05)}{A_{540} \text{ of standard}}$$

5.3.5 Activity scoring

Where possible at the end of each trial the crustaceans were scored based on the mobility index given in Chapter 2. This gives a qualitative assessment of how salinity affected the mobility of the animals.

5.3.6 Statistical analyses

Ninety-six hour median lethal concentration and median lethal times (LC_{50} and LT_{50}) were calculated for both testing regimes using probit analysis. Haemolymph chemistry data were analysed using one way analysis of variance (ANOVA) for normally distributed data or a Kruskal-Wallis test for abnormal distribution. *a posteriori* testing was carried out using Tukey, Scheffe's or Games-Howell tests as appropriate depending on homogeneity of sample size and whether parametric or non parametric statistics were used (SPSS v15 to 18). Significant results are described in section 5.4.

Unless specifically mentioned in the text, no significant results were found in the control trials.

Analysis of ammonia using the FIA apparatus was initially done by hand using a paper chart recorder but as the technology became available peaks were analysed using AD Instruments Chart 5 peak analysis software.

5.4 Results: Ionic properties of sea water and crustacean haemolymph.

5.4.1 Ionic properties of sea water

Samples of both natural sea water made up to hypersalinities with artificial aquarium salts (Instant Ocean™) and brine from a gas cavern discharge diluted down to the same hypersalinities using ultra pure Milli-Q water were analysed using the same ICP-OES technique as for the haemolymph samples. Due to budgetary constraints it was not possible to do replicates for this analysis, hence standard deviations and errors were not calculable. However, Table 5.1 gives an indication of how the composition of the water changes with the increasing salinity and hence metallic load of the water. It has also enabled comparisons between the holding water and the haemolymph of the crustaceans to be made to see whether the animals in question are able to osmoregulate at the salinities tested and if so, whether this ability breaks down at any point.

In general the differences in the levels of potassium, calcium and copper were small (Table 5.2). The largest difference in potassium was 9.2 mmol at salinity 70 which is well above the maximum salinity recorded at the E. Yorkshire discharge site near Aldbrough. The same is true for calcium with a maximum difference of 3.7 mmol at 70 psu. The largest difference in copper levels occurred at a salinity of 60 with a difference of 0.0017 mmol between the aquarium salt brine (Aqb) and the gas cavern brine (Gcb).

Larger differences were seen in the levels of sodium and magnesium, which are the most abundant metals in sea water generally between around 450 – 650 mmol (Na) and 30 – 70 mmol (Ca) at the normal salinity for that geographical location (Table 5.3). Sodium showed a maximum of 475.93 mmol difference between the Gcb and the Aqb and again this is at 70 salinity units. This is a difference of almost half a mole. This is a high value compared to the other salinities and is not necessarily indicative of the real difference between the mineral blends as there is a much lower difference in sodium levels at salinities 60 and 65, which is why it would have been preferable if replicate samples could have been analysed. The smallest difference occurs at salinity 45 (63.90 mmol).

Magnesium showed similar values at the lower end of the hypersaline range, at 40 psu there was only a difference of 4.5 between the Aqb and the Gcb at 31.94 mmol and 36.50 mmol respectively.

When comparing the higher hypersalinities there was a greater difference between the magnesium concentrations, but this only reached a difference of 15.9 mmol at a salinity of 70. The largest difference occurred at salinity 50 (32.81 mmol) and the smallest at salinities 40 and 45 (4.5 mmol).

Since, in general, these differences are so small, it is unlikely that they will have any effect on the survivability of crustacean species around the discharge point. This has been confirmed by the lobster brine test (section 5.4.6) which has shown that only pH and haemocyanin levels are affected when comparing the haemolymph of lobsters in salinity 50 Gcb and salinity 50 Aqb.

Table 5.1 Composition of sea water (mmol l^{-1}); comparing sea water collected from Bridlington, commercial artificial sea water for aquarium use and brine from the Aldbrough gas discharge.

Salinity	Source	Na (mmol l^{-1})	Mg (mmol l^{-1})	K (mmol l^{-1})	Ca (mmol l^{-1})	Cu (mmol l^{-1})
25	a	328.44	19.53	3.71	4.26	0.0003
30	a	409.50	25.90	4.77	5.68	0.0005
35	n	477.27	31.60	5.84	6.76	0.0006
40	i	593.43	31.94	6.97	7.37	0.0005
45	i	659.35	47.99	9.51	10.2004	0.0008
50	i	697.61	70.47	14.14	13.28	0.0010
55	i	403.45	65.86	15.66	9.36	0.0006
60	i	890.50	61.43	13.16	10.38	0.0006
65	i	1157.52	82.99	15.58	16.03	0.0007
70	i	1196.86	73.26	15.65	16.61	0.0006
40	b	685.55	36.50	9.70	7.45	0.0018
45	b	723.23	43.47	10.70	8.95	0.0006
50	b	834.48	37.66	11.81	8.55	0.0018
55	b	879.38	58.90	19.34	12.50	0.0016
60	b	991.48	58.77	20.38	12.74	0.0022
65	b	972.24	empty tube	empty tube	empty tube	0.0012
70	b	1417.38	57.37	24.88	12.91	0.0007
Undiluted Gcb		3710.39	90.80	80.91	22.49	0.0004

Key: a = normal sea water diluted with Milli-Q water

n = normal sea water from Bridlington

i = Instant Ocean™ aquarium salts used to make hypersaline water from a natural sw base

b = gas cavern brine used to make concentration, diluted with Milli-Q water

Table 5.2 Differences in concentration (mmol l^{-1}) of metal ions between Gcb and Aqb at each salinity tested. (negative number indicates the aquarium salt had the lower level of the ion).

Salinity	Na difference	Mg difference	K difference	Ca difference	Cu difference
40	-92.117	-4.5559	-2.7173	-0.0835	-0.0013
45	-63.902	4.5202	-1.1857	1.2539	0.0002
50	-136.874	32.8078	2.3359	4.7325	-0.0008
55	-475.93	6.9685	-3.678	-3.1278	-0.001
60	-100.971	2.6552	-7.2196	-2.3645	-0.0016
65	185.2874	empty tube	empty tube	empty tube	-0.0005
70	-220.512	15.8922	-9.2353	3.7054	-0.0001

Table 5.3 Composition of sea water (mmol l^{-1}), comparison of global examples.

Element	Bridlington SW at 35 psu	General SW at 35 psu	New Zealand at 35 psu	General
Na (mmol l^{-1})	477.274	467.69	469.77	456.72
Mg (mmol l^{-1})	31.6032	53.28	53.06	55.54
K (mmol l^{-1})	5.8389	9.98	10.03	9.97
Ca (mmol)	6.7585	10.38	10.26	10.23
Cu (mmol l^{-1})	0.0006	Not available	0.00001	0.00004
Source	Chemistry dept at University of Hull	(Castro and Huber 1992)	(Anthoni 2006)	(Hem 1986)
Element	Typical SW 35 psu	E. Mediterranean sw at 35 psu	Arabian Gulf at Kuwait sw at 35 psu	Red Sea at Jeddah sw at 35 psu
Na (mmol l^{-1})	459.16	513.27	689.44	618.75
Mg (mmol l^{-1})	51.92	57.72	72.62	30.56
K (mmol l^{-1})	9.72	11.84	11.77	5.37
Ca (mmol)	9.98	10.55	12.48	5.61
Cu (mmol l^{-1})	Not available	Not available	Not available	Not available
Source	Modified from Cotruvo (1995)			

5.4.2 Ionic properties of *Homarus gammarus* haemolymph, chronic trial test group

There were significant differences in all the haemolymph ionic components tested in the chronic exposure trial on *H. gammarus*. It appears that K and Ca may be exhibiting a decrease in concentration as salinity is increased. Cu and Mg may be exhibiting an increase with increasing salinity. Na appears not to be showing much of a change with increasing salinity.

Haemolymph sodium (Na) differed significantly during the experiment ($p < 0.001$, $F = 19.414$, df = 4). The sodium concentration at salinity 40 was significantly different to that at all other salinities. There were no other significant differences. The means for each salinity did not differ, except for salinity 40 which was over 100 mmol higher than the others: 35 = 166.39 mmol, 40 = 323.01, 45 = 203.89, 50 = 207.32, 55 = 203.51 (Figure 5.2a), showing no discernible trend in the sodium concentration during acclimation to high salinities. The fact that the higher salinities were not significantly different to salinity 35 suggests that there was no iono-conformation in the lobsters. Evidence of elevated sodium concentration would be present in the haemolymph but this was not the case. This suggests there is a degree of osmoregulation occurring in the lobsters. This is confirmed by the results in section 0, Table 5.1 which indicate a Na level of 477 to 1196 mmol from salinity 35 to 70, whilst the above results have already indicated the lobsters maintaining haemolymph Na levels at around 200 mmol.

There were significant differences in the levels of haemolymph copper (Cu) over the duration of the experiment ($p < 0.001$, $F = 13.530$, df = 4). Post-hoc testing (Scheffe) has shown that as with sodium, the copper concentration in lobster haemolymph at salinity 40 was significantly different to that at all other salinities. There were no other significant differences. All means were similar, except for haemolymph Cu at salinity 40 which was higher than the others: salinity 35 = 0.110 mmol Cu, 40 = 0.14, 45 = 0.11, 50 = 0.12, 55 = 0.13 (Figure 5.2b). Therefore there was no discernible trend in the copper concentration during acclimation to high salinities.

Potassium levels in the blood differed significantly over the range of experimental salinities ($p < 0.001$, df = 4, $\chi^2 = 20.030$). Haemolymph potassium concentration from lobsters at salinity 40 was significantly different to those from salinities 45 and 50, levels at 55 were different to 45 and 50, and 35 was 0.001 units away from being significantly different to salinity 55. The mean mmol potassium for each salinity showed no discernible trend: 35 = 10.73 mmol, 40 = 26.81, 45 = 7.17, 50 = 4.38, 55 = 43.40 (Figure 5.2c).

Significant differences in the levels of haemolymph Magnesium (Mg) were observed ($p < 0.001$, $F = 13.530$, $df = 4$) with the haemolymph magnesium concentration at salinity 55 being significantly higher to that at 35 and 40. There was a trend for increasing haemolymph magnesium levels (mmol) with increasing salinity from 4.04 mmol at salinity 35 to 8.00 mmol at salinity 55 (Figure 5.2e). The significant differences between salinities 35 and 55, and 40 and 55 suggest that at salinity 55 the amount of magnesium in the haemolymph is significantly different to that at ambient 35 psu. It could be at this point the mechanisms for hypo-ionoregulating the Mg below that of the medium (see section 0) break down and the amount of Mg increases significantly. Magnesium in the water samples was between 19 and 82 mmol, so is definitely being hypo-ionoregulated in *H. gammarus* but with the concentration increasing with increasing salinity. It is possible that is contributing to the death of the lobsters in hypersalinities (see discussion, section 5.6).

Haemolymph calcium (Ca) levels showed significant differences ($p = 0.012$, $F = 4.015$, $df = 4$), with the calcium concentration of the blood of the lobsters at salinity 55 being significantly different to that at 40. There were no other significant differences. Mean calcium values indicated a possible trend for decreasing haemolymph calcium with increasing salinity, from 9.05 mmol at salinity 35 to 5.71 mmol at salinity 55 (Figure 5.2d). Values at 40 psu were consistently different throughout the analysis of *H. gammarus* haemolymph ionic data and may be indicative of an error in the analysis by the chemistry dept, or in the mixing of the aquarium salts.

Fig 5.2a

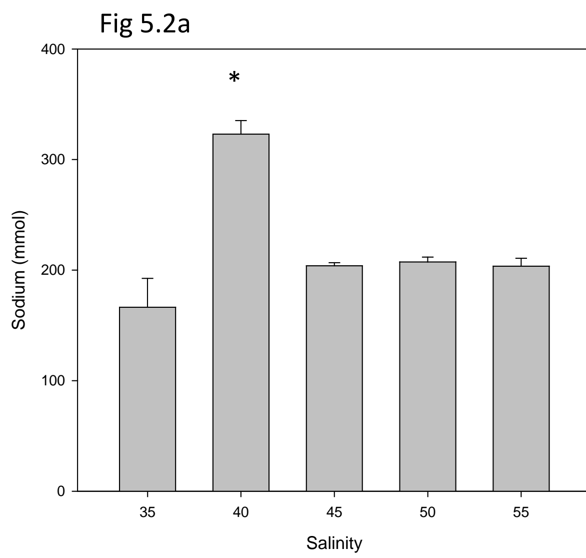


Fig 5.2b

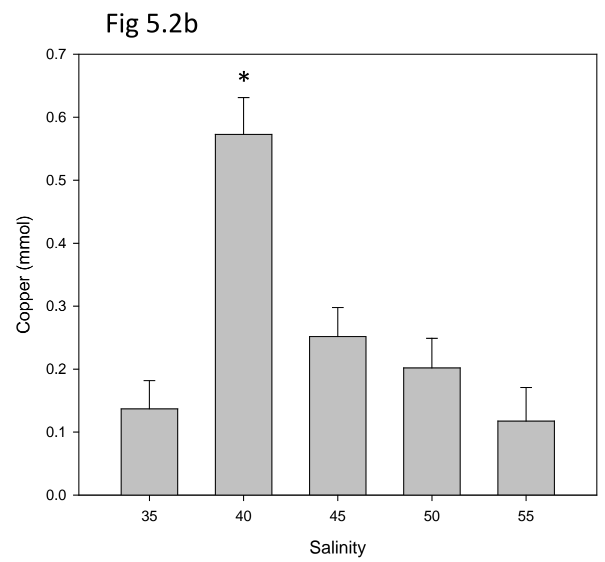


Fig 5.2c

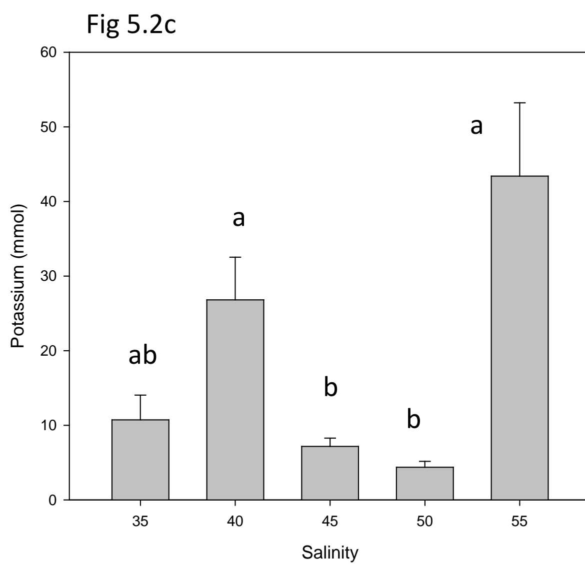


Fig 5.2d

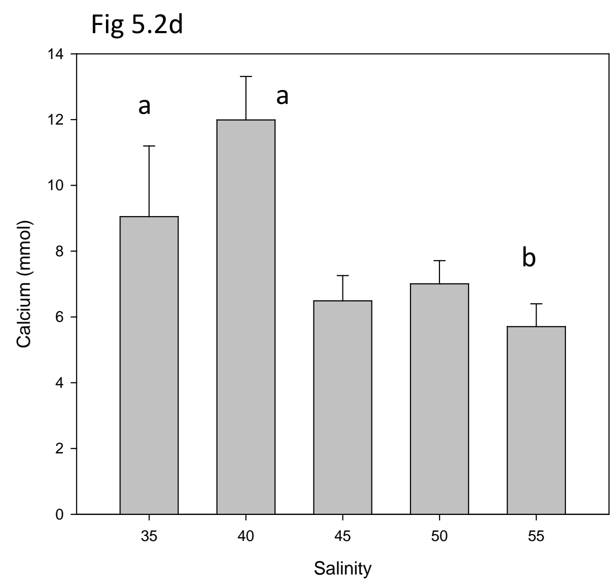


Fig 5.2e

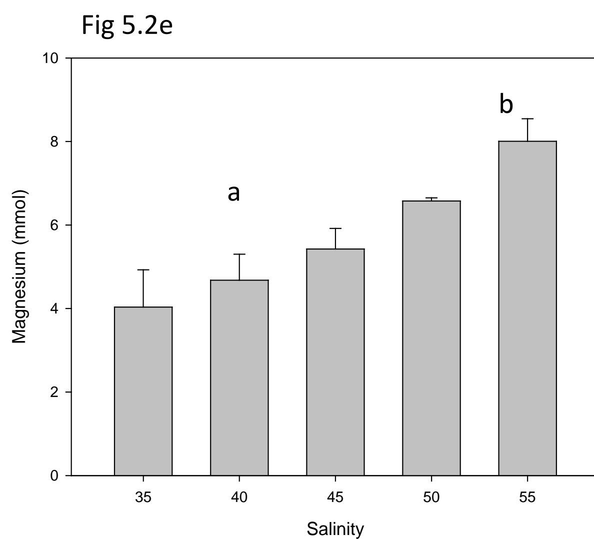


Figure 5.2 Ionic analysis of *H. gammarus* haemolymph in response to hypersalinities. Chronic exposure test group. Only parameters where significant changes occurred are displayed on the figure. Means (+/- SE). n = 30 total. Only haemolymph magnesium shows a consistent trend with hypersalinity.
*** indicates different levels in haemolymph to all other salinities.**
a,b,c indicates significant differences between differing letters.

5.4.3 Ionic properties of *Homarus gammarus* haemolymph, chronic trial control group

For the control portion of the chronic exposure trial, sodium (Na), potassium (K), magnesium (Mg) and calcium (Ca) showed no significant changes in the levels in the haemolymph over the duration of the experiment. Therefore for these elements, handling and water change regimes did not affect the blood chemistry of the animals, any significant changes found in the test portion of the experiment were caused by salinity alone.

The only ionic property of the blood to show significant changes over the duration of the experiment was copper (Cu) ($p = 0.033$, $df = 4$, $\chi^2 = 10.495$). Post hoc testing (Games Howell) has shown that four days (4 days) in salinity 35 was significantly different to twenty days (20 days) in salinity 35 ($p = 0.023$) in terms of Cu content of the haemolymph (Figure 5.9). There were no other significant differences. The means of the data for each time period, show that 4 days = 0.14 mmol Cu, 8 days = 0.57 mmol, 12 days = 0.50 mmol, 16 days = 0.63 mmol, 20 days = 0.51 mmol. It is not obvious why this difference occurs here as although 4 days has the lowest Cu level, 20 days does not have the highest. This difference may be due to large differences in standard error, however the error bars in Figure 5.9 suggest that there is no real trend or change over the experiment with the exception of day 4 and therefore this may be an experimentally anomalous result.

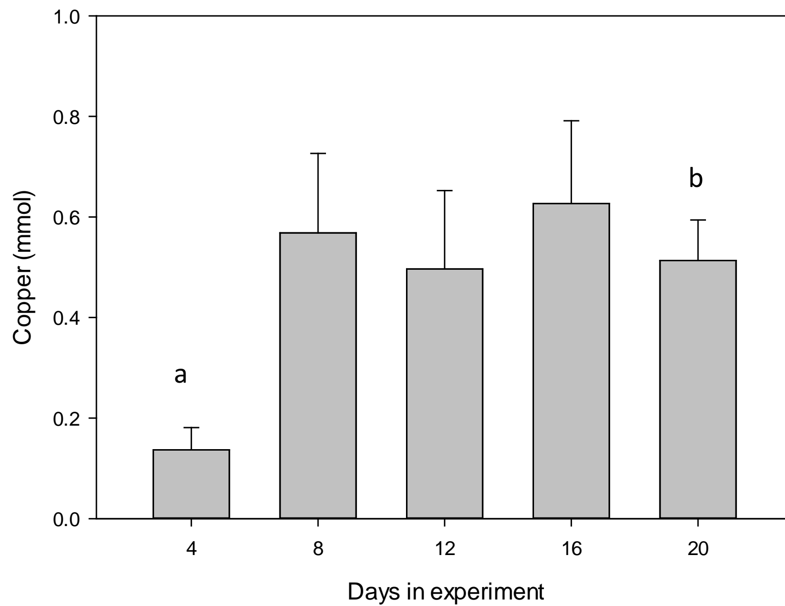


Figure 5.3 Effect of experiment duration on copper levels in *Homarus gammarus* haemolymph during hypersaline acclimation. Control group. Means ±SE. n = 30 total.
Post hoc test revealed that only day 4 and day 20 were significantly different to each other in terms of haemolymph copper level (a, b) n = 39 total.

5.4.4 Ionic properties of *Homarus gammarus* haemolymph, acute trial test group

There were significant differences in the haemolymph sodium (Na) levels at different salinities ($p < 0.001$, df = 3, $\chi^2 = 18.585$). Post hoc testing (Games Howell) has shown that haemolymph sodium from lobsters at salinity 35 (normal sea water) was not significantly different to that at 40, 45 or 50 (probably due to the high standard error at salinity 35), (see Figure 5.4a), however salinity 40 was significantly different to salinity 45 and 50 with regards to haemolymph Na and salinity 45 was different to 50. Overall the trend shown was for increasing levels of Na in the haemolymph as salinity increased, from a minimum of 166.4 mmol at salinity 35 to a maximum of 237.9 mmol at salinity 50. There were no other significant differences. This sodium increase is due to higher sodium levels in the water. This is in contrast to the chronic exposure trial, where there was evidence of osmoregulation happening due to no significant change in Na when the lobsters were able to acclimate to hypersalinity.

There were significant differences in the haemolymph potassium levels at different salinities in the acute exposure trial on adult intermoult *H. gammarus* ($p = 0.003$, df = 3, $\chi^2 = 14.304$) (Figure 5.4b), with the significant difference in potassium occurring between salinity 40 and salinity 50 only. Mean haemolymph potassium levels for each salinity showed that the standard deviation ranged from 0.47 to 8.91 over the salinities so this may account for the lack of difference. At salinity 40 the SD was the lowest at 0.47 and at 50 the SD is the highest in potassium at 8.91. This may account for the significant difference found.

There was a significant difference in the magnesium level of the haemolymph at different salinities ($p < 0.001$, df = 3, $\chi^2 = 22.923$). These significant differences in haemolymph Mg occurred between lobsters from salinities 40 and 45, and 40 and 50. There were no other significant differences. Mean haemolymph magnesium levels for each salinity showed an increase with acute exposure to the higher salinities. 35 = 4.04 mmol, 40 = 2.33 mmol, 45 = 5.32 mmol and 50 = 6.49 mmol (Figure 5.4c).

Haemolymph copper (Cu) also showed significantly different levels between different salinities ($p < 0.001$, $F = 13.227$, df = 3). A post hoc Scheffe test has shown that haemolymph Cu from lobsters in salinity 35 (normal sea water) was significantly different to that at salinities 40, 45 and 50 in terms of Cu level, and that haemolymph Cu at salinity 45 was also significantly different to that at salinity 50. There were no other significant differences. A homogeneous subsets analysis has shown that the salinities can be split into 3 groups: 1 = 35, 2 = 40 and 50, 3 = 40 and 45. Mean copper levels (mmol) for each salinity indicated an increase in the concentration of copper in the haemolymph as the salinity increased (Figure 5.4d). This increase does not appear to be related to copper levels in the water as there is no real increase in copper with hypersalinity in the seawater samples tested (see section 0) Calcium showed no salinity dependent haemolymph response in the acute exposure experiment.

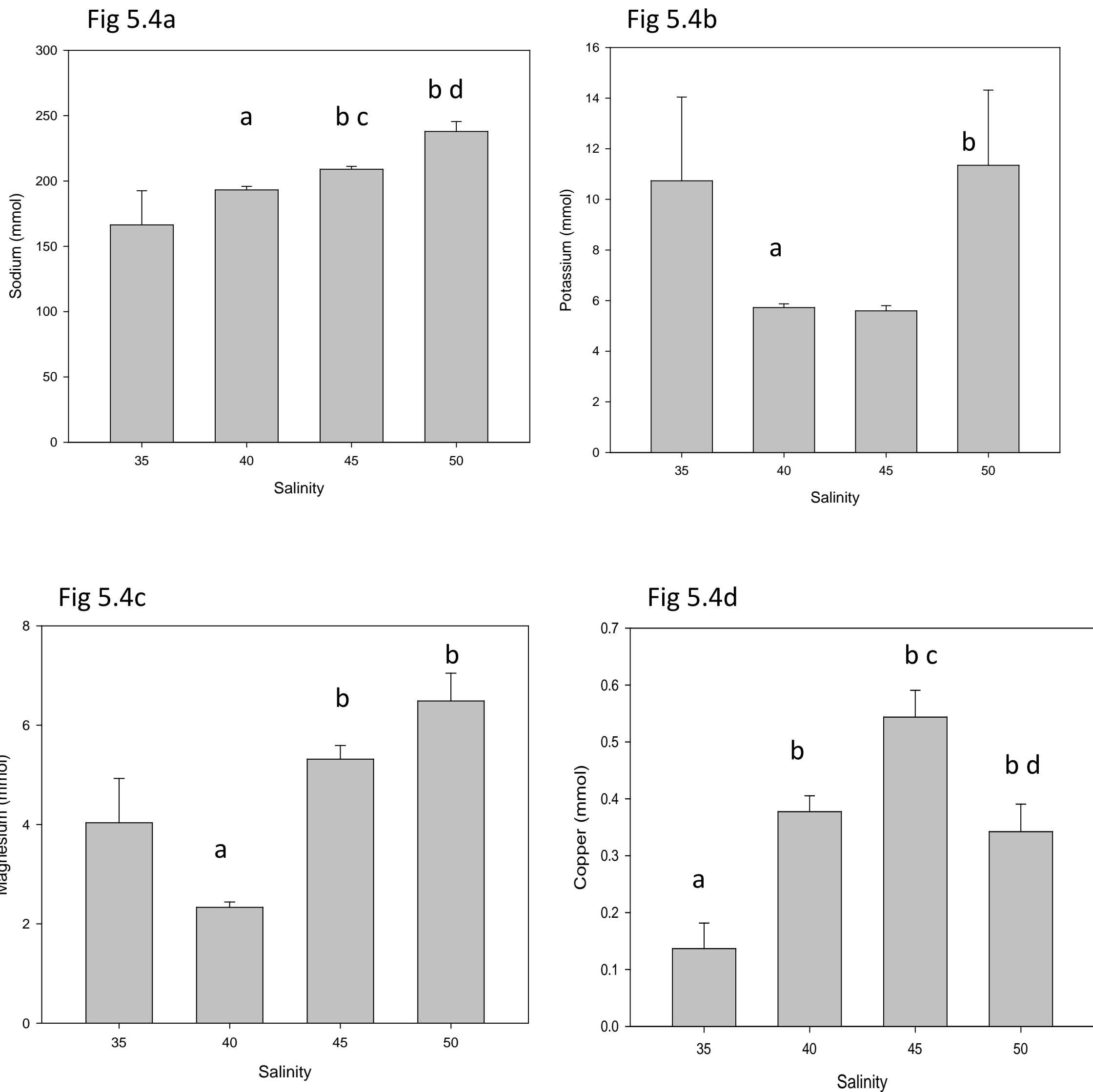


Figure 5.4 ***Homarus gammarus*** **acute trial ionic results. (Means ± SE). n = 39 total.**
Post hoc analysis has indicated that some salinities differ from the others in terms of blood parameter concentration, but not all. These significant differences have been shown in superscripts where: a is different to b, and c is different to d. Bars without superscripts have no significant differences to the other bars.

5.4.5 Ionic properties of *Homarus gammarus* haemolymph, postmoult trial

Lobsters in the late-postmoult stage of the moult cycle were used to assess if the moult stage had an impact on the animals' tolerance to hypersaline conditions. Only salinities 35 to 40 were comparable due to the soft nature of the shells meaning all test animals died at salinity 45. T-tests were used for analysis as data was normally distributed and variances were homogeneous.

Independent samples t-tests on haemolymph of late-postmoult lobsters under a 96h acute hypersaline exposure showed significant increases in the sodium levels and calcium levels (p <

0.01) and potassium and magnesium levels ($p < 0.05$) as salinity increased from 35 to 40. The increases are shown by the mean values in Table 5.4. Copper levels in late-postmoult lobsters showed no significant difference between these salinities.

In summary there were significant differences in sodium (Na), potassium (K), magnesium (Mg) and calcium (Ca) between salinities 35 and 40 in soft (late post moult) lobsters. There were no significant differences in copper (Cu) levels. Since all the soft lobsters in this 96h shock test died before the end of the 96h when tested at 45 psu, no blood could be obtained to be analysed at that salinity. However as the late post moult soft lobsters died at a lower salinity than intermoult lobsters under the same testing regime, it can be assumed that newly moulted lobsters would have an even lower tolerance to the higher salinities.

Table 5.4 Mean values for ionic properties of late-postmoult *Homarus gammarus* haemolymph, under acute hypersaline stress. Only parameters showing significant differences (indicated by *) between the two salinity regimes are displayed. n = 30

Element	Mean level (mmol) at salinity 35	Standard error of the mean at salinity 35	Mean level (mmol) at salinity 40	Standard error of the mean at salinity 40
Na*	178.57	0.95	211.44	2.74
K*	4.00	0.03	5.00	0.23
Mg*	3.10	0.22	4.82	0.56
Ca*	6.04	0.15	7.13	0.12

5.4.6 Ionic properties of *Homarus gammarus* haemolymph, gas cavern discharge to aquarium salt comparison

Independent samples t-tests on haemolymph properties of intermoult adult lobsters at the end of a 96h acute exposure test (one group at 50 psu created by Instant Ocean™ aquarium salts, one group at 50 psu created with brine from a gas cavern discharge), showed significant differences in the haemolymph sodium, magnesium, calcium and potassium levels ($p < 0.001$). Copper however showed no significant difference dependent on the origin of the brine. The haemolymph collected

from lobsters tested in brine from the gas cavern discharge had consistently lower levels of all of the metals (except copper) than that of lobsters housed in brine created from artificial aquarium salts (Table 5.5).

Table 5.5 Mean values for ionic properties of *Homarus gammarus* under hypersaline stress. Only parameters showing significant differences (indicated by *) between the two salinity regimes are displayed. n = 30.

Parameter	Gas cavern brine at salinity 50 (mmol)	Standard error of the mean for gas cavern brine at salinity 50	Aquarium salts at salinity 50 (mmol)	Standard error of the mean for aquarium salts at salinity 50
Sodium (Na)*	184.10	3.85	237.87	7.61
Potassium (K)*	3.72	0.25	6.49	2.97
Magnesium (Mg)*	6.30	0.56	8.68	0.56
Calcium (Ca)*	4.20	0.22	11.35	0.60

5.4.7 Ionic properties of *Homarus gammarus* haemolymph, normal sea water to gas cavern discharge comparison

When comparing the ionic constituents of the haemolymph of adult intermoult *H. gammarus* under acute exposure to either natural sea water (salinity 35) or a hypersaline brine from a gas cavern dissolution project (salinity 50), there were a number of differences found.

Significant differences occurred between sodium, potassium and copper concentration at the different salinities tested ($p < 0.05$). Mean values showed this difference was an increase in concentration with increasing salinity for Na and Cu, and a decrease for K (Table 5.6). There were no significant differences in the haemolymph magnesium or calcium levels.

Table 5.6 Mean concentration of haemolymph ions in *Homarus gammarus* at salinities 35 (normal sea water) and 50 (hypersaline brine effluent). Only parameters showing significant differences (indicated by *) between the two salinity regimes are displayed. n = 30.

Parameter	Concentration (mmol) in normal sea water (salinity 35)	Standard error of the mean for normal sea water at salinity 35	Concentration (mmol) in brine discharge effluent (salinity 50)	Standard error of the mean for brine effluent at salinity 50
Sodium (Na)*	166.39	26.12	184.10	3.85
Potassium (K)*	10.73	3.31	4.20	0.25
Copper (Cu)*	0.14	0.044	0.27	0.24

The lower potassium level in salinity 50 compared to salinity 35 is unexpected but the 50 psu effluent brine also had a lower potassium level than the 50 psu aquarium salt hypersaline water so may be due to the mineral balance the goes together to make the overall salinity.

5.4.8 Ionic properties of *Cancer pagurus* haemolymph

Haemolymph sodium levels in adult intermoult *Cancer pagurus* under acute hypersaline exposure showed significant salinity-dependent differences ($p < 0.001$, ANOVA $F = 20.068$, df = 4). A post hoc Scheffe's test was performed and showed that the sodium level in the haemolymph of the crabs at salinities 35 was significantly different to that at salinity 45, 50 and 55. Haemolymph Na at salinity 40 was different to 45 and 55, salinity 45 was different to 50 and salinity 50 was different to 55, overall indicating increasing haemolymph Na with increasing salinity. Homogeneous subsets analysis has shown that the salinity data can be separated into 3 groups: group 1 = 35 and 40, group 2 = 40 and 45, group 3 = 50 and 55, with both this and the means of the data confirming an increase in the sodium level in the haemolymph as the salinity increases from 201.01 mmol at salinity 35, to 243.45 mmol at salinity 55 (Figure 5.5a).

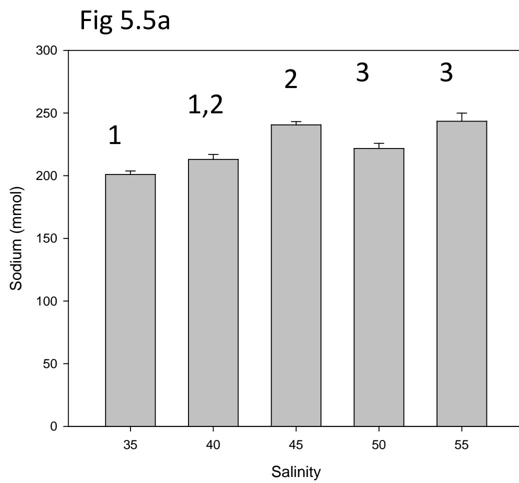
Significant salinity-dependent differences occurred for copper concentration ($p = 0.049$, df = 4, $\chi^2 = 9.533$). Haemolymph copper levels at salinity 35 were significantly different to levels at salinity 45 (Figure 5.5b). There were no other significant differences. Copper is the pigment in haemocyanin and changes in the haemolymph levels may be representative of changes in the levels of this respiratory pigment and therefore blood cells. The significance level of 0.049 is extremely close to

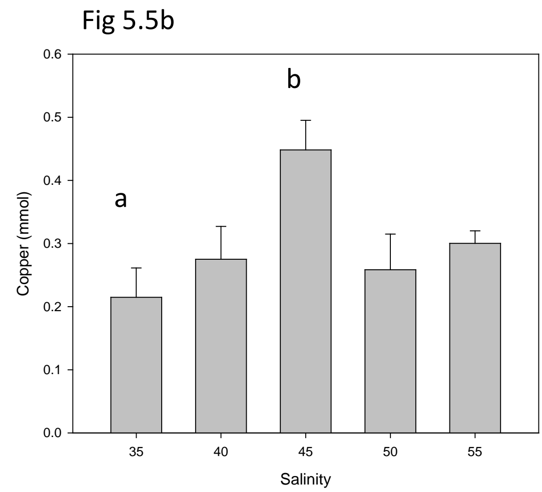
the threshold of significance of 0.05, therefore it is possible the there is no real difference in the Cu levels at all.

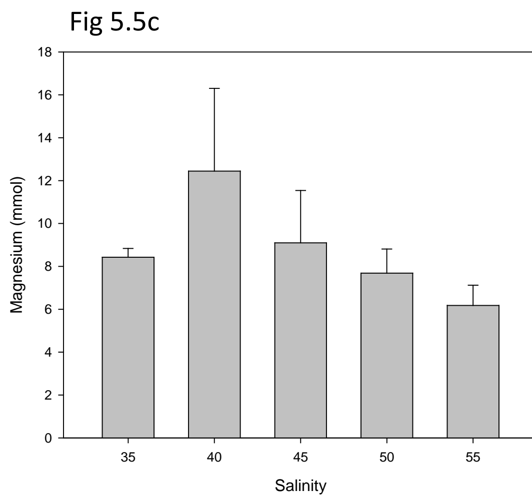
Haemolymph potassium also showed significant salinity dependent differences ($p < 0.001$, $F = 8.340$, $df = 4$). Haemolymph potassium concentration at salinities 35 and 40 was significantly different to 45 (Figure 5.5d). There were no other significant differences.

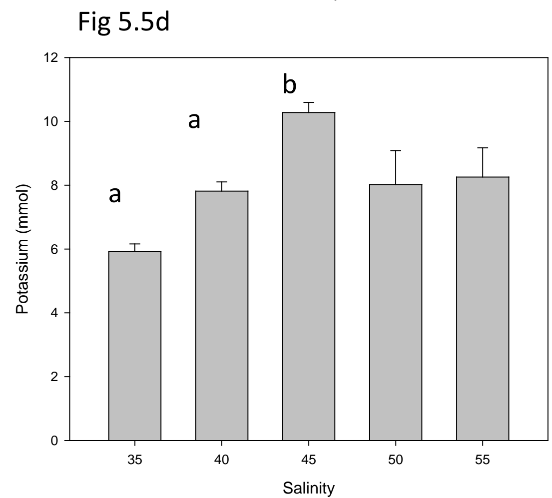
There were significant differences in the magnesium levels of the crabs' haemolymph at different salinities ($p = 0.038$, $df = 4$, $\chi^2 = 10.139$) (Figure 5.5c), however a post hoc Games Howell test has failed to find where these differences occur. Despite attempting a number of transformations to normalise the spread of the data, all post hoc tests attempted failed to find at which salinities the significant differences in magnesium occurred.

A one way ANOVA on $\log_{10}$ transformed calcium levels (to normalise the data spread) in *C. pagurus* haemolymph after a 96h hypersaline shock showed significant differences in the haemolymph calcium (Ca) levels at different salinities ($p = 0.003$, $F = 4.709$, $df = 4$). Calcium concentration at salinities 35 and 40 was significantly different to 45 (Figure 5.5e). There were no other significant differences. Of note is that despite the ANOVA indicating significant differences in Ca, both the normal and transformed data homogeneous subsets tables calculated as part of the post-hoc analysis grouped all salinities as one, suggesting that possibly there were no real differences in the calcium levels during the acute test.









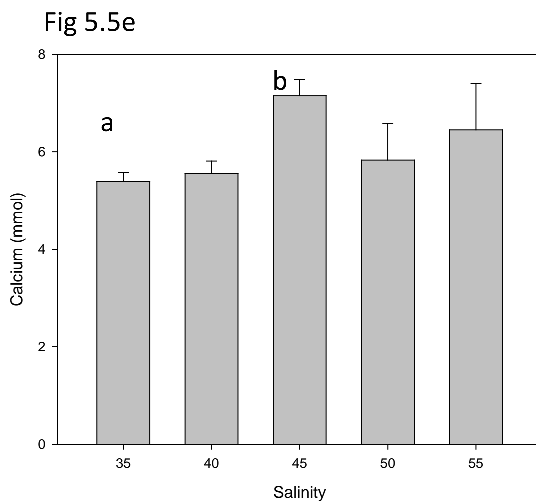


Figure 5.5 ***Cancer pagurus*** **acute hypersaline exposure test group. Ionic parameters where significant changes occurred. Mean ±SE. n = 52 total.**
1,2,3 = homogeneous subsets.
a,b = significant difference in haemolymph level of ion between the points.
Magnesium post-hoc testing could not point out the significant differences hence no annotation on figure.

5.4.9 Ionic properties of *Necora puber* haemolymph, chronic trial test and control groups

In *Necora puber* acclimated to hypersalinities (from 35 to 60), significant salinity-dependent differences in magnesium (Mg) occurred ($p = 0.001$, df = 5, $\chi^2 = 20.074$), with several salinities differing in terms of haemolymph Mg level: salinity 35 was significantly different to 45 and 60. Salinity 40 was significantly different to 60. Salinity 45 was significantly different to 55 (and 35 as said before). 50 showed no significant differences at all. 55 was significantly different to 60 (and 45 as said before). 60 was significantly different to (35, 40 and 55 as said before). Hence there may be some increase with increasing salinity, specifically at salinity 50 (Figure 5.6b), although from the figure there is no consistent linear trend indicated.

Potassium (Figure 5.6a) and sodium (Figure 5.6d) data from the *N. puber* chronic exposure test group showed that both haemolymph potassium ($p < 0.001$, df = 5, $\chi^2 = 27.211$) and sodium ($p = 0.001$, df = 5, $\chi^2 = 20.384$) had significant differences in their concentrations over the salinities tested. In the case of potassium (K), salinity 35 was significantly different to salinity 55 and 60. There were no other significant differences. The control group showed no significant salinity dependent change in K level with the duration of the experiment, so the changes in the test group are due to the salinity alone. In the case of haemolymph sodium (Na), salinity 35 was different to 45, 55 and 60 psu, but not to salinities 40 or 50. There were no other differences. However since salinity 35 and salinity 60 are not significantly different to each other it appears that there was an initial increase in sodium concentration which then levelled off as salinity increased further.

Significant differences in the haemolymph calcium concentration also occurred as indicated by the Kruskal Wallis analysis ($p = 0.032$, df = 5, $\chi^2 = 12.212$) (Figure 5.6c). However, post hoc testing (Games Howell) has shown could not find the source of these significant differences in the data. The differences found by the Kruskal Wallis test could be due to the wide ranging standard errors indicated for Ca in Figure 5.6c. A further post hoc test was performed (Least Significant Difference) and showed significant differences in haemolymph calcium between crabs at salinity 50 and 40, 45, 55. There were no other significant differences suggesting that salinity 50 may be an anomaly of sorts. Two t-tests were performed to assist in the confirmation of the suspected differences picked out by the LSD test. They showed that salinities 35 and 50 had no significant differences, as also seen in the LSD. 50 and 55 did have significant differences ($p = 0.025$, $t = 2.466$, df = 16). Further

t-testing was avoided as it is inappropriate to perform multiple t -tests as a way of doing post hoc analysis. Transformation of the Ca data via Lg_{10}, Ln, 1/X, sqrt etc had no influence on the KW test. Overall the analysis of the Ca data indicated no trends related to salinity in terms of haemolymph Ca level in the way that was seen for K and Na.

There were no significant salinity-dependent differences in copper levels in the haemolymph.

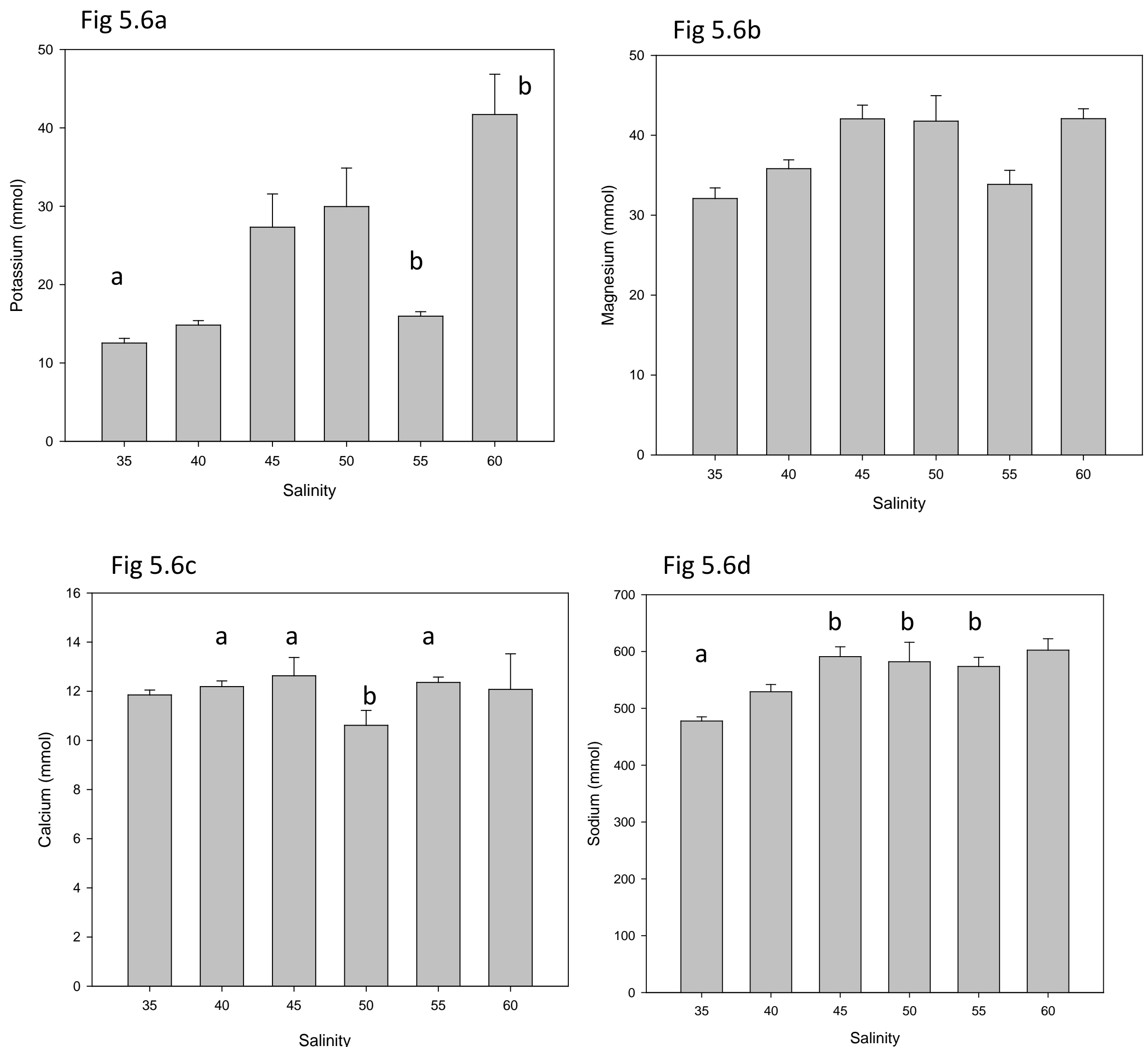


Figure 5.6 ***Necora puber*** **hypersaline chronic exposure test group. Ionic parameters where significant changes occurred. Mean (+/- SE). n = 42 total.**
a,b = statistically significant concentration of haemolymph parameter between the two letters/salinities. Please see section 4.4.9 for which salinities differ specifically with regards to magnesium as the differences are too numerous and complex to show on the figure.

In the control portion of the study, there were significant differences in calcium (Ca) levels over the duration of the experiment ($p < 0.001$, $F = 7.286$, $df = 5$), with the difference occurring between 4

days and 16 days only (Figure 5.7a). This is the equivalent of 35 and 50 psu in the test group. Since no other changes occurred it is unlikely that this change is due to the duration of the experiment. In the test group, salinity 50 was lower than the rest and may be something of an anomaly.

Haemolymph magnesium (Mg) levels in the control test showed significant differences over the duration of the experiment ($p = 0.001$, $df = 5$, $\chi^2 = 20.330$), with these significant differences occurring between 4 days duration and all other days duration (Figure 5.7b). It is therefore unlikely that the experiment duration is having an effect on the crabs as the latter portions of the trial show no other differences. The changes seen in the test group are likely due to salinity alone. Copper, sodium and potassium showed no significant time dependent changes over the duration of the experiment.

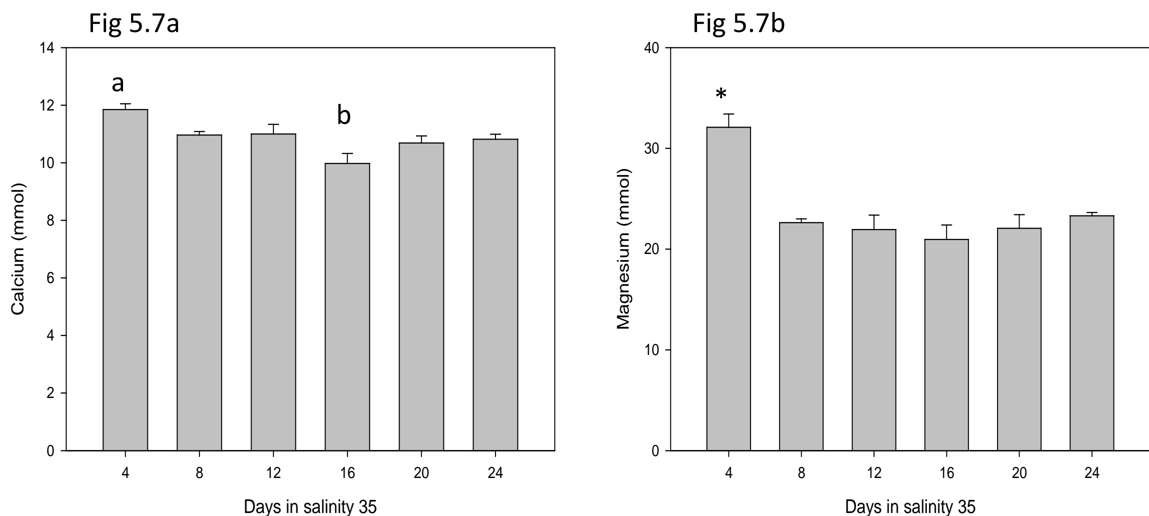


Figure 5.7 ***Necora puber*** **chronic exposure control group. Ionic parameters where significant changes occurred. Mean (+/- SE). n = 42 total.**
*** denotes significant difference from others. a,b = significant difference between the two letters/days.**

5.4.10 Ionic properties of *Necora puber* haemolymph, acute trial test and control groups

No salinity dependent differences were found for the salinities tested (35 to 50) for any of the metals in the acute trial ($p > 0.05$ in all cases).

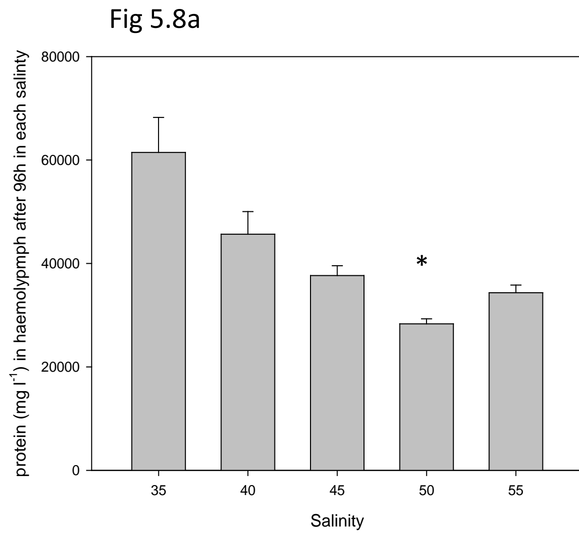
5.5 Results: Effects of salinity on haemolymph variables of crustaceans

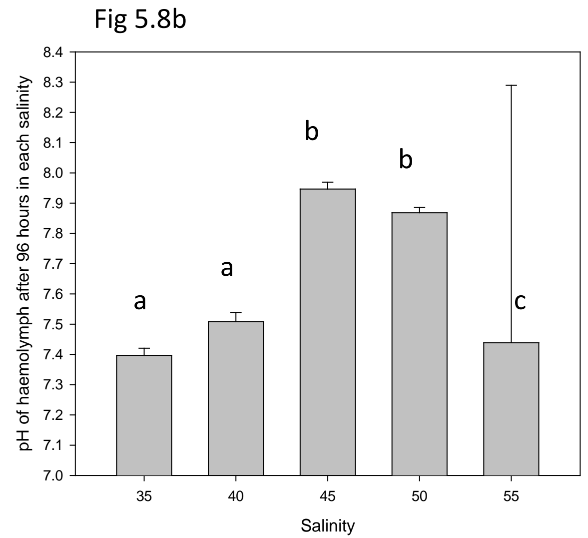
5.5.1 Acute and chronic hypersaline exposure in intermoult *Homarus gammarus*

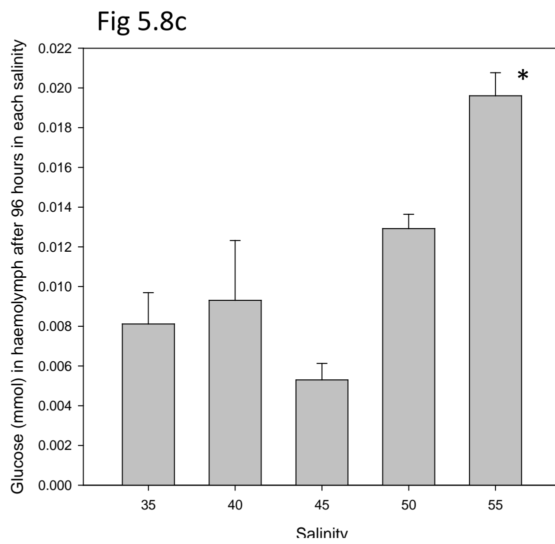
Significant differences in the levels of haemolymph serum protein occurred at the salinities used in the chronic trial ($p < 0.001$, $F = 11.433$, df =4) (Figure 5.8a). A post hoc Scheffe test has shown that salinities 50 and 55 are not different to each other in terms of haemolymph protein level, but 50 has significantly lower protein levels than all below, suggesting a change in protein occurs at salinity 50. In the acute exposure trial no salinity-dependent significant difference in the haemolymph protein of intermoult adult lobsters was found, this was the same for the control acute group. In the chronic exposure control group, the amount of time the lobsters spent in the experiment had some significant effect on the protein levels in the blood ($p = 0.020$, df = 4, $F = 3.550$) (Figure 5.8a), however post hoc analysis (Scheffe) indicated a difference between 20 days and 12 days only.

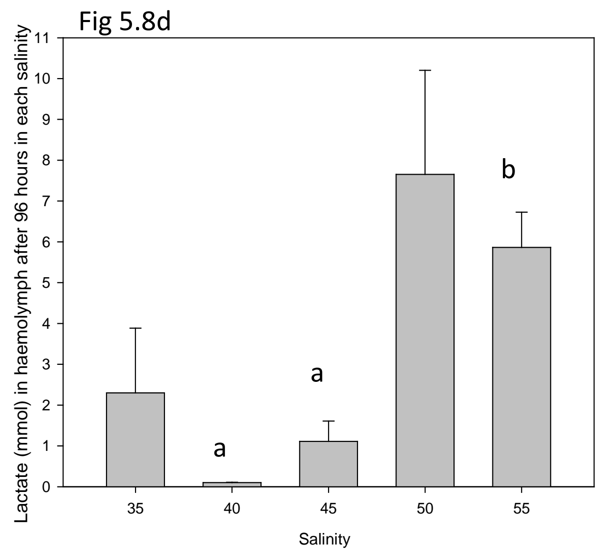
Haemolymph pH changed significantly throughout the chronic exposure experiment ($p < 0.001$, $F = 34.357$, df = 4). Post hoc testing (Tukey) has shown that blood pH at salinities 35 and 40 was significantly different from 45 and 50. Salinities 45 and 40 are also different from 55 (Figure 5.8b). This is more clearly indicated by Figure 5.10 which shows the clear division between groups. Two outliers were present in this analysis at salinity 55 which otherwise showed a very close range of pH values.

In the acute exposure trial significant differences in haemolymph pH with regard to salinity were also found ($p < 0.001$, $\chi^2 = 29.253$, df = 3). A post hoc Games Howell test showed that salinity 35 is different to 40 and 50 in terms of haemolymph pH level. pH at salinity 40 is different to all salinities. As the controls of both the chronic and acute trials showed significant differences in pH it indicates that salinity alone may not be the determining factor in changes to haemolymph pH.









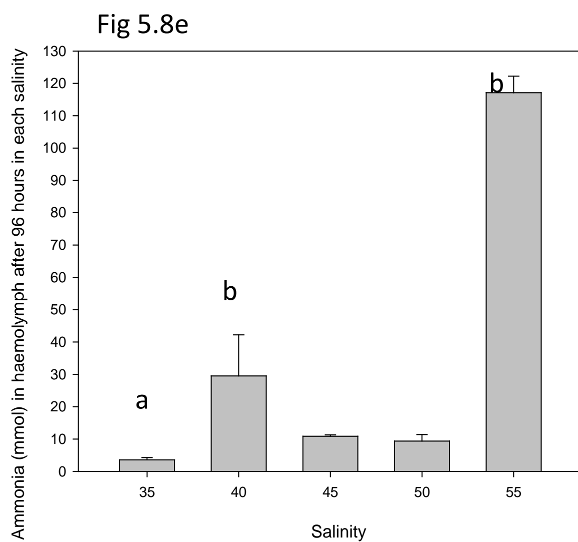


Figure 5.8 Haemolymph parameters exhibiting significant differences in the chronic exposure trial for *Homarus gammarus.* Mean (+/- SE), n = 30 total.

Lobsters spent 96h in each salinity before being stepped up to the next increment. * indicates significantly different haemolymph levels to all lower salinities. Where differences were not clear cut, significant differences in haemolymph levels of each parameter are indicated as occurring between differing letters.

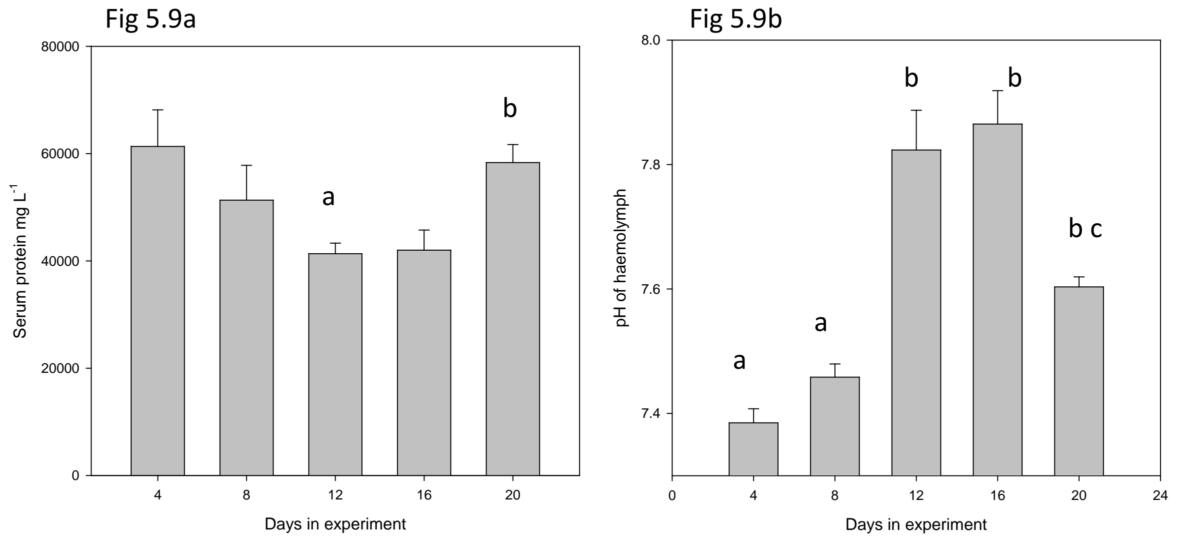


Figure 5.9 Haemolymph parameters that showed significant differences in the chronic exposure trial (control group) f or *Homarus gammarus.* Mean (+/- SE), n = 30 total.
Significant differences in haemolymph levels of each parameter are indicated as occurring between differing letters.

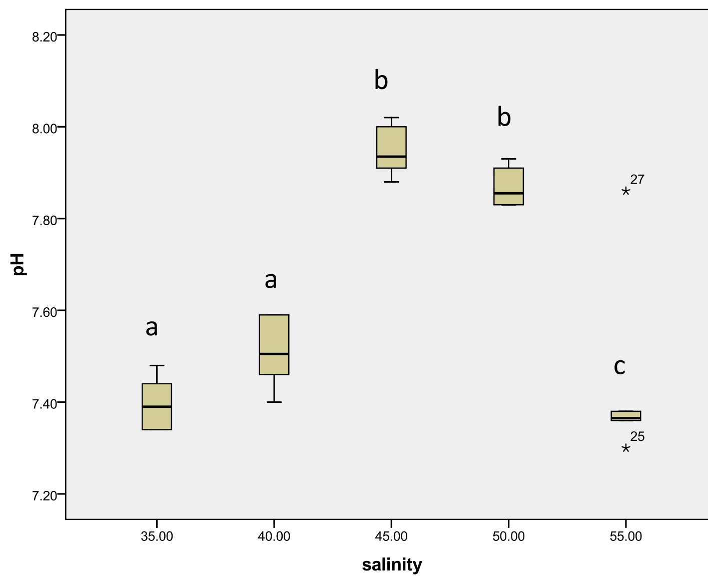


Figure 5.10 Significant changes in pH chronic exposure to hypersalinity experiment on adult intermoult *Homarus gammarus.* Three distinct subsets are indicated by the results displayed in the box plot (a, b, c). n = 30 total.

Glucose increased significantly in concentration in the haemolymph of test group chronic exposure *H. gammarus* ($p < 0.000$, $F = 10.749$, df = 4), with the haemolymph glucose level at salinity 55 differing to all those below except for salinity 40. Glucose at salinity 40 is not different to any other salinity (Figure 5.8c). No differences in glucose levels were observed in the control portion of the chronic trial. In the acute exposure to hypersalinity trial there are differences in the glucose of the haemolymph dependent on salinity ($p = 0.003$, $F = 5.570$, df = 3), with blood glucose at salinity 35 being significantly different to 50, suggesting that once the salinity of the surrounding media reaches 50 psu, there will be a change in glucose levels in the haemolymph of intermoult adult lobsters. The mean glucose for each salinity shows an increase as salinity increases (salinity 35 = mean 0.675 mmol glucose, 40 = 0.941 mmol, 45 = 1.049 mmol, 50 = 1.324 mmol), with this increase becoming statistically significant at salinity 50.

There were significant salinity related differences in the levels of lactate in the haemolymph of the lobsters ($p = 0.003$, $F = 5.186$, df = 4) in the chronically exposed group. Only haemolymph lactate from lobsters at salinity 55 is significantly different to those at salinities 45 and 40 (Figure 5.8d). The control group also show some salinity dependent significant differences in lactate levels ($p < 0.001$, $F = 34.103$, df = 4) with post hoc testing (Scheffe) indicating that these differences occur throughout the duration of the experiment with all days different to all other days except day 8 with the exception of day 20, which is different only to day 8 in terms of lactate concentration in the blood. There is no clear trend of change in lactate level during the control trial for except for a notable increase in lactate concentration after 20 days in salinity 35 are reached (Figure 5.11).

Salinity had no significant effect on lactate levels in the acute trial or acute control trial.

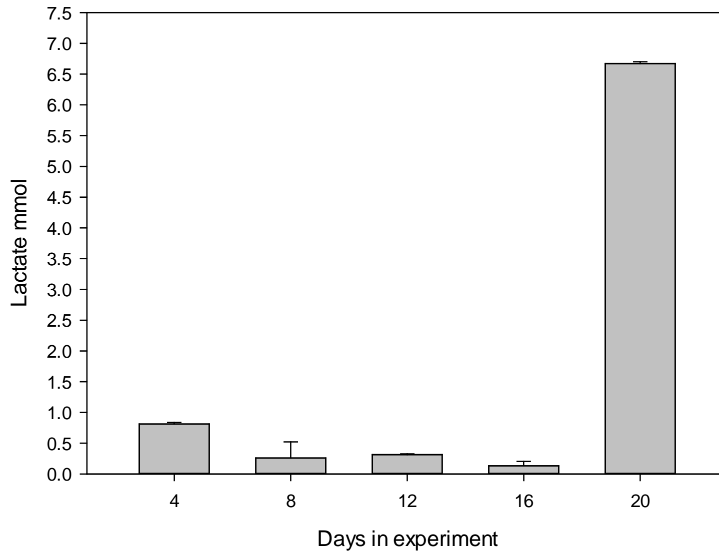


Figure 5.11 Effects of experiment duration (chronic control trial) on lactate levels in the haemolymph of *Homarus gammarus* (n = 39 total). Control group. Mean (+/- SE). n = 30 total.

Significant differences occur throughout the duration of the experiment with all days different to all other days except day 8. The exception is day 20, which is different only to day 8.

Haemolymph ammonia levels increased significantly in the chronic exposure test group ($p = 0.001$, $F = 7.200$, $df = 4$) during the trial, with blood ammonia at salinity 35 being significantly different to salinity 40 and salinity 55 (Figure 5.8e). For the acute trial, significant differences in haemolymph ammonia with regards to salinity were observed ($p < 0.001$, $\chi^2 = 17.962$, $df = 3$) and showed that ammonia concentration at salinity 35 is different to 40 and 45.

No significant changes to haemolymph haemocyanin levels were noted for either the test or control portions of the chronic exposure trial. However when acutely exposed, significant salinity dependent differences were observed ($p < 0.001$, $\chi^2 = 27.607$, $df = 3$) that showed a general increase in haemocyanin with increasing salinity. Salinity 35 differed from 40 and 50 in terms of haemocyanin concentration. Haemocyanin levels in the blood from lobsters at salinity 40 were also significantly different to those at 45 and 50. The means of the original data sets for each salinity show that there was a general increase in haemolymph haemocyanin as the salinity increased: salinity 35 = 11.767 mmol, 40 = 37.713 mmol, 45 = 151.025 mmol), 50 = 95.026 mmol.

The activity level of the lobsters was also scored from 1-5 (with 1 being active and 5 not responsive to stimulus) and analysed as a quantitative assessment of how salinity affects mobility. Activity is dependent on the salinity ($p = 0.004$, $df = 3$, $\chi^2 = 13.113$). Post hoc Games Howell testing revealed that salinity 35 was significantly different from 45 and 50 with activity significantly decreasing as salinity increases.

During the acute trials, significant behavioural changes were observed that were not seen in the chronic exposure test: many lobsters that were introduced to salinities of 55 and above immediately became quiescent with no movement of legs, eyestalks, antennae or mouthparts. Even after 96h was complete they were still exhibiting this response and had not moved from where initially placed in the tank, failing to respond to direct stimulus. It was also noted that when animals that had been weak but alive at salinities of 55 and above at the end of the trial were returned to normal conditions after the experiment, there was no recovery and death followed within a short time (< 24 hours).

5.5.2 Acute and chronic hypersaline exposure in late-postmoult *Homarus gammarus*

As the 96h LC_{50} test indicated that late-postmoult lobsters could not survive for 96 hours in salinity 45 (all died by 72 hours, see Chapter 4), the blood chemistry testing was only attempted up to salinity 40, hence salinity 35 and 40 were the only salinities comparable. There were no significant differences in protein, pH, lactate, ammonia or haemocyanin concentrations in the haemolymph of late postmoult *H. gammarus* at salinity 40 when compared with the normal salinity 35 (using either an independent samples t-test or a Mann Whitney U test depending on the normality of the data distribution). Haemolymph glucose concentration was significantly different between salinity 35 and salinity 40 ($p = 0.08$, $t = -3.16$, $df = 12$). Mean glucose levels for each salinity (1.087 mmol at salinity 35 and 1.287 mmol at salinity 40) suggest that haemolymph glucose increased as salinity increased. As none of the test lobsters survived the 96h trial at the next stepwise salinity (salinity 45), this trend could not be investigated further.

5.5.3 Haemolymph effects of hypersaline exposure in intermoult *Homarus gammarus* using brine from a discharge site

As the testing above has indicated that salinity 50 is where most of the changes in the haemolymph of *H. gammarus* became significantly different and where 50% of the test population died in the LC_{50} trial (although the probit analysis gave the salinity point 48.9 [45.8– 61.1]), it was decided to test the haemolymph condition of lobsters housed in brine from a gas cavern discharge site at this salinity against artificial aquarium salt at this salinity as well as at normal sea water at salinity 35. This would then indicate if gas cavern discharge had a different effect on the physical condition of the lobsters to generic hypersalinity with regards to differences in specific ionic composition between brine and artificial sea water.

i. SALINITY 50 (BRINE) TO SALINITY 50 (AQUARIUM SALT) HAEMOLYMPH COMPARISON IN *H. GAMMARUS*

The only parameters to have significant differences between lobsters tested in seawater with the salinity increased via aquarium salt or with brine effluent from the Aldbrough gas caverns, were pH ($p < 0.001$, $z = -3.582$) and haemocyanin ($p = 0.015$, $z = -2.428$). The mean values for each salinity type show that the pH of the haemolymph was higher when the lobster was in brine effluent (pH = 7.721) than in "Instant Ocean" (pH = 7.392). Haemocyanin levels in the haemolymph were much higher in lobsters from brine effluent (HCY = 245.187) than from "Instant Ocean" (HCY = 95.025). All the haemolymph HCY values for lobsters housed in brine effluent were proportionately higher than the values for lobsters housed in sea water made with Instant Ocean™. In contrast, the haemolymph metals (as indicated by section 5.4.6) all are lower in concentration in the effluent brine than in the equivalent aquarium salt based media.

ii. SALINITY 50 (BRINE) TO SALINITY 35 (NORMAL SEA WATER) HAEMOLYMPH COMPARISON IN *H. GAMMARUS*

An independent samples t-test showed no significant differences between lobsters acutely exposed to 50 psu brine created with effluent from the gas caverns and lobsters held in normal 35 psu sea water for glucose, lactate, protein and pH ($p>0.05$). Haemocyanin and ammonia showed significantly higher levels in lobsters from brine effluent ($p = 0.002$, $df = 8$, $t = -4.667$ for haemocyanin, $p = 0.008$, $df = 17$, $t = -3.002$ for ammonia). The activity of the lobsters was not significantly different between brine effluent and aquarium salts.

Haemolymph haemocyanin had a much higher mean value (245.2 mmol) at 50 psu brine effluent, than in normal seawater at 35 psu under otherwise identical conditions (11.8 mmol), ammonia also showed higher concentration in haemolymph of lobsters exposed to brine effluent 50 psu (6.5 mmol) than in normal seawater at 35 psu under otherwise identical conditions (3.2 mmol).

When comparing the results of artificial aquarium salt at 50 psu to artificial aquarium salt at 35 psu, in lobster haemolymph from artificial aquarium salt at 50 psu there was no significant difference between ammonia at 50 psu and 35 psu ($p > 0.05$). Under artificial aquarium salt only there was a significant difference in haemocyanin at 35 and 50 psu ($p < 0.001$). The mean haemocyanin level in the haemolymph from 50 psu was 95.0 mmol. This concentration is considerably less than that found in lobster haemolymph when effluent brine was used (245.2 mmol). The difference at 50 psu between artificial and effluent brine is statistically significant ($p = 0.05$).

5.5.4 Effects of hypersalinity on lobster haemolymph. Intermoult *H. gammarus* to late postmoult *H. gammarus* comparison

As described previously in Chapter 4, the late-postmoult lobsters only survived the trial up to salinity 40. So here, the properties of their haemolymph at this salinity were compared to those of intermoult lobsters at the same salinity to see if the moult stage may be having an effect on their physiology's response to supranormal salinities. For example, if the lobster can survive in the intermoult stage at *e.g.* salinity 40 or 45, but cannot survive this when it enters the moult, it is useful to know what changes may have prompted the reduction in survivability. Hypersaline solution was made using Instant Ocean™ aquarium salts.

Protein ($p = 0.001$, $z = -3.354$), haemocyanin ($p = 0.047$, t =t 2.177, $df = 14$), ammonia ($p = 0.001$, $z = -3.334$), and pH ($p < 0.001$, $t = -8.864$, $df = 14$) and mobility of the lobsters ($p = 0.037$, $t = 2.301$, $df = 14$) all showed significant differences between different stages of the moult cycle at the same salinity (salinity 50). Glucose and lactate showed no significant differences between intermoult and late-postmoult status. Mean values for these parameters indicate higher concentrations of haemocyanin and ammonia in late-postmoult lobsters, with protein, pH and activity levels all higher in the intermoult lobsters (Table 5.7).

Table 5.7 Mean haemolymph concentrations of significantly different parameters (indicated by*) between intermoult and postmoult lobsters at salinity 40. n = 39 total.
Mean values indicate higher concentrations of haemocyanin and ammonia in late-postmoult lobsters, with protein, pH and activity levels all higher in the intermoult lobsters. SE = standard error of the mean.

Parameters showing significant differences between moult stage	Mean level in intermoult lobsters	SE for intermoult lobsters	Mean level in postmoult lobsters	SE for postmoult lobsters
Protein*	9.24 mg l^{-1}	0.47	5.514 mg l^{-1}	0.28
Haemocyanin*	28.38 mmol	8.46	53.63 mmol	7.34
Ammonia*	5.42 mmol	0.50	13.41 mmol	1.55
pH*	7.814	0.02	7.56	0.02
Activity level* (1 high activity, 5 no activity)	1.66	0.29	2.71	0.36

5.5.5 Acute hypersaline exposure in intermoult *Cancer pagurus*

Only acute hypersalinity testing was carried out for *C. pagurus* due to limited availability of crabs as previously explained. Significant salinity-dependent differences were found in the glucose levels of the blood at different salinities ($p = 0.003$, $df = 3$, $\chi^2 = 13.69$). Post hoc testing (Games Howell) revealed that salinity 35 was significantly different to salinity 45 in terms of haemolymph glucose level. Means of the haemolymph glucose levels for each salinity did not indicate an obvious linear change as salinity increased from the normal 35 psu through the hypersalinities (Table 5.8)

There were significant salinity dependent differences in the blood ammonia ($p < 0.001$, $df = 3$, $\chi^2 = 39.46$) of *C. pagurus*, with ammonia concentration from lobsters at salinity 35 being significantly different from those at salinity 45 and 50. Haemolymph ammonia concentration at salinity 40 was also significantly different to that at salinity 50. Mean blood ammonia values at each salinity level indicated a decrease in ammonia as salinity increased (Table 5.8). Therefore it can be concluded that when exposed to hypersaline shock of salinity 45 and above (from the normal of 35), *C. pagurus* produces significantly less ammonia than normal.

The activity level of the crabs was also scored from 1-5 (with 1 being active and 5 not responsive to stimulus) and analysed as a quantitative assessment of how salinity affects mobility. Activity is dependent on the salinity ($p < 0.001$, $df = 3$, $\chi^2 = 27.77$). Post hoc Games Howell testing revealed that the activity level of crabs at salinity 50 was significantly different from those below and that the activity level at salinity 35 is also significantly different from that at salinity 45. Mean activity values indicated that activity decreased as salinity increased (at salinity 50 all crabs died before the 96h experiment was completed) (Table 5.8).

Haemocyanin, protein, pH and lactate showed no salinity-dependent differences in *Cancer pagurus* acutely exposed to hypersalinity.

Table 5.8 Mean values and standard erros of the mean for haemolymph parameters that showed significant (indicated by *) salinity dependent differences in intermoult *C. pagurus*, n =52 total.

Parameters showing salinity dependent differences	Mean level:							
	Salinity 35	SE at 35	Salinity 40	SE at 40	Salinity 45	SE at 45	Salinity 50	SE at 50
Glucose (mmol)*	0.59	0.03	0.26	0.12	0.27	0.08	0.50	0.16
Ammonia (mmol)*	311.14	50.34	381.22	119.57	61.12	6.21	16.93	2.75
Activity level* (1 high activity, 5 no activity)	1.20	0.13	1.71	0.38	2.79	0.24	5.00	0.22

5.5.6 Acute and chronic hypersaline exposure in intermoult *Necora puber*

Haemolymph protein levels showed a significant change in the chronic exposure trial for *Necora puber* ($p < 0.001$) (Figure 5.12a) and was the only parameter to show a significant change in the acute exposure trial ($p = 0.002$). In the chronic exposure trial the significant increase occurred once the salinity reached 55. In the acute exposure trial salinities 45 and 50 are significantly higher than 35 in terms of haemolymph protein ($p = 0.002$). These changes were not observed for either of the control groups.

Haemolymph pH decreased significantly in the chronic exposure trial in crabs at salinity 55 ($p < 0.001$) (Figure 5.12b) and also in the chronic exposure trial control group, where haemolymph pH after 4 days had a significantly lower pH than after 16 days ($p = 0.001$) (Figure 5.13a). Unlike the test animals which showed a change at a salinity of 55, the control results show differences in pH between 16 days and 4 days alone, hence suggesting that it is salinity rather than the time spent in the experiment that is causing the change in the blood pH seen in the test group. The acute trial showed no significant change in pH for both the test and control groups.

Glucose was significantly higher in the haemolymph ($p < 0.001$) at salinity 55 for the chronic exposure test group than in blood from lobsters in salinities lower than this (Figure 5.12c), when compared to haemolymph glucose levels in crabs from lower salinities. In the control group glucose levels were significantly higher after 4 days exposure than those recorded after 24 days (Figure 5.13b). This suggests differences in the haemolymph glucose are caused by the salinity change alone and not the experimental procedure or handling activities. The acute exposure test group and control showed no significant changes in glucose levels.

For the chronic exposure group, haemolymph lactate increased significantly at salinity 55 ($p < 0.001$) (Figure 5.12d) and no significant changes occurred in the control group. In the acute exposure test and control groups there were no significant changes in lactate.

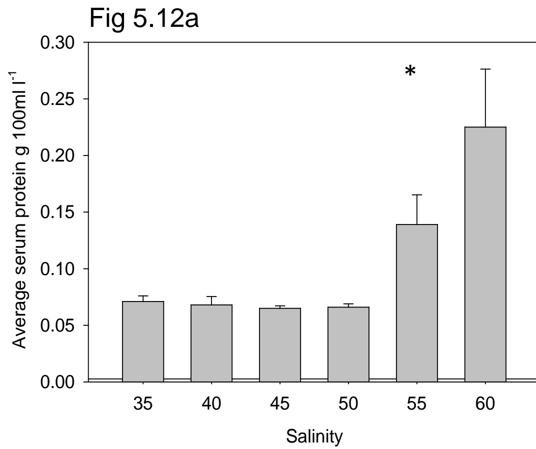
Haemolymph ammonia levels increased significantly ($p < 0.001$) in the chronic exposure test group once the salinity reached 55 (Figure 5.12e). This change was not seen in the control group or in the acute exposure trial or control.

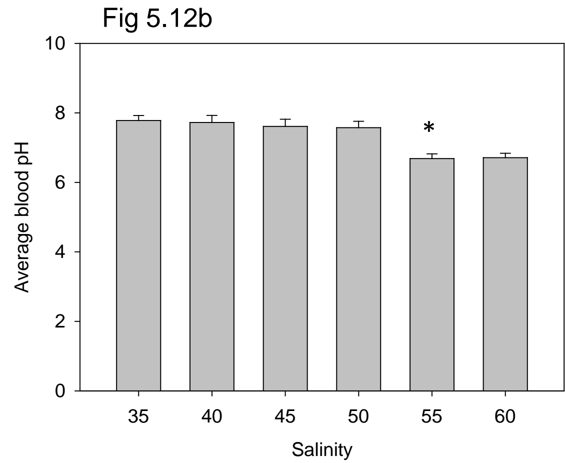
Haemocyanin (HCY) levels exhibited a significant decrease in the chronic exposure trial ($p = 0.040$) with haemolymph HCY concentration in *N. puber* from salinity 60 being significantly different to the

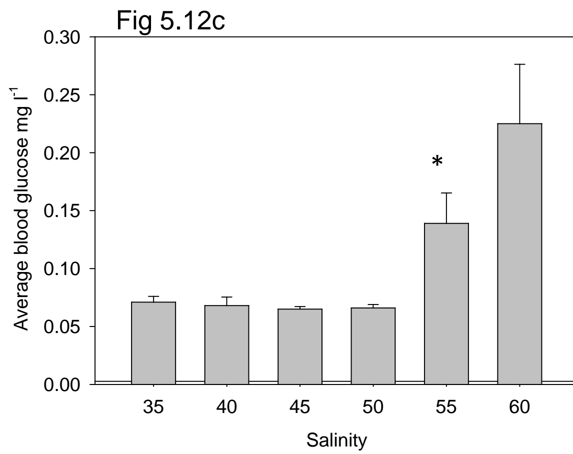
others (Figure 5.12f). No significant differences in HCY were observed in the chronic control, or in the acute exposure test and control groups.

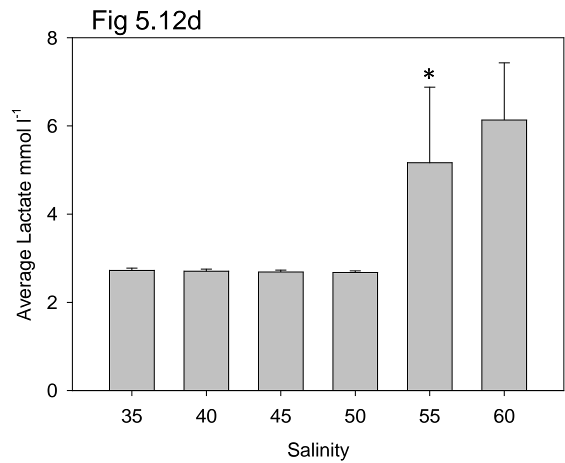
In the chronic exposure test group there were no significant changes between the mean haemolymph values at salinity 55 and 60 for any of the variables measured with the exception of HCY which exhibited a continued significant decrease between salinities 55 and 60 ($p = 0.040$).

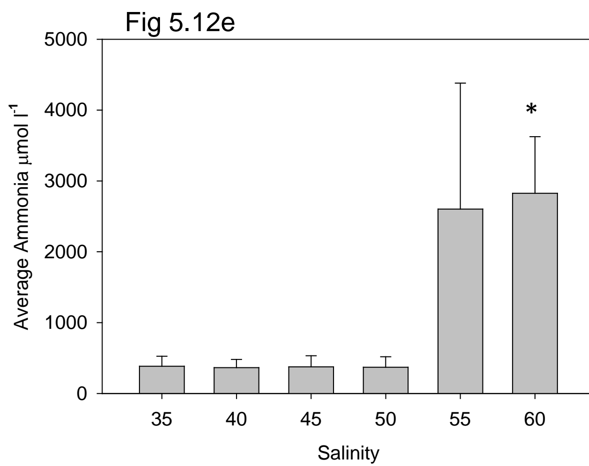
No crabs died during the chronic tests but, in the acute tests, animals at salinity 55 and 60 died before the end of the experiment, hence haemolymph changes were not observed as the crabs died quickly in these salinities before any blood could be drawn. During the acute test, significant behavioural changes were observed that were not seen in the chronic exposure test. Crabs that were introduced to salinities of 50 and above immediately drew their legs in tightly underneath the abdomen and appeared to cease all visually observable activity completely with no movement of legs, eyestalks, antennae or mouthparts. Even after several hours they were still exhibiting this closure response and had not moved from where initially placed in the tank, failing to respond to direct stimulus. It was also noted that animals that had been weak but alive at salinities of 55 and above were returned to ambient conditions after the experiment, there was no recovery and death followed within a short time (< 24 hours).











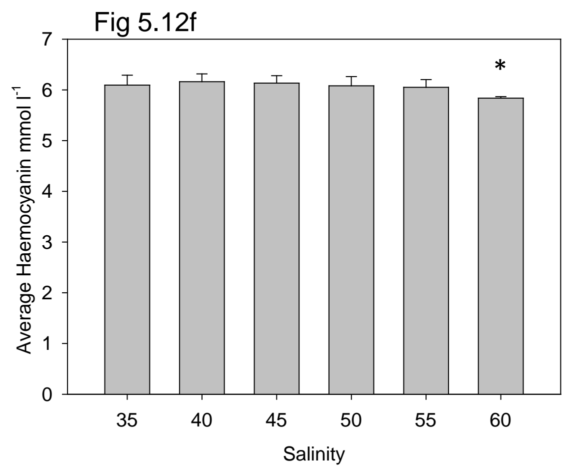


Figure 5.12 ***Necora puber*****, chronic hypersaline exposure test group. Showing haemolymph parameters where significant, salinity-dependent changes occurred. Means (+/- SE). n = 42 total.**
*** indicates the haemolymph variable had significantly different levels when compared with levels from crabs kept at all salinities below (significance is at the 0.01 level).**

Fig 5.13a

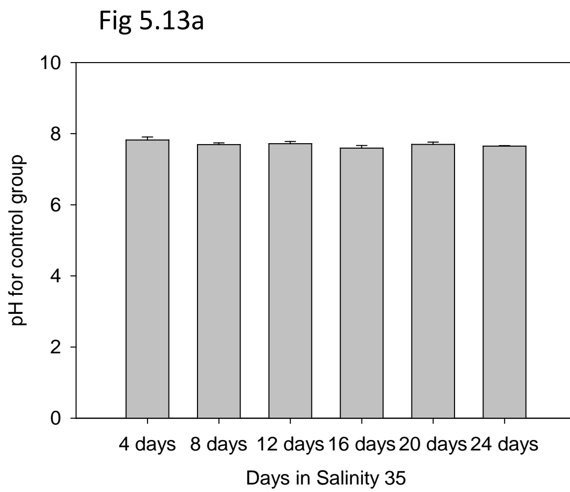


Fig 5.13b

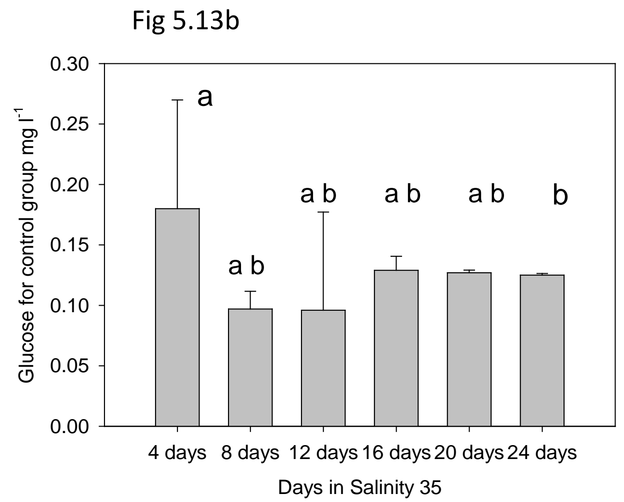


Figure 5.13 *Necora puber*, chronic exposure control group. Showing haemolymph parameters where significant, time-dependent changes occurred. Means (+/- SE). n = 42 total.

Fig 4.7a – 16 days is significantly different to 4 days (p = 0.001)

Fig 4.7b – post hoc testing indicated two subgroups (a and b) with only 4 days and 24 days being significantly different with no overlap between the groups (p = 0.002)

All animals died in salinities 55 and 60 in the acute exposure group so these salinities were not included in the statistical analysis as no blood could be sampled from dead animals.

5.6 Discussion

5.6.1 Summary of findings

The crustacean species tested all showed significant changes to haemolymph constituents when salinity exceeded 40. *Necora puber* was the least sensitive in terms of haemolymph changes with significant changes in haemolymph properties occurring at salinities 55 and 60. *Cancer pagurus* was also was insensitive to hypersalinity in terms of haemolymph constituents with significant changes observed only in ammonia production and their overall activity level.

The poorer ability to cope with hypersaline conditions when recently moulted was further highlighted by the difference in the blood chemistry demonstrated when comparing the intermoult lobsters to the late postmoult lobsters at salinity 40. The softer shelled animals had statistically lower activity levels, haemolymph pH and protein levels, and statistically higher haemolymph ammonia and haemocyanin levels than the intermoult lobsters under identical testing regimes. When comparing the effects of Gcb to Aqb at the same salinity, both pH and haemocyanin were significantly higher in the blood of lobsters situated in the Gcb than in brine created from aquarium Aqb. However all the metallic ions tested for were lower in the Gcb.

As all of the *Necora puber* in the acute exposure trial died before the end of the salinity 55 and 60 sections (leading to an LC_{50} of salinity 41.9, see chapter 4), there were minimal changes observed in the haemolymph parameters, with only protein showing a significant response at the lower salinities as salinities 55 and 60 could not be included in the statistical analysis. All of the haemolymph parameters tested for the chronic exposure group showed a significant change, with most of the haemolymph levels showing an increase with increasing salinity. As these changes were not replicated in the controls, any significant differences seen at higher salinities in the test group are considered to be caused by the salt not the handling.

Of the three species tested, *Cancer pagurus* had the highest tolerance to hypersalinity, with an LC_{50} under acute hypersaline stress of salinity 55.5. Like the other crustaceans, *C. pagurus* also experienced salinity dependent changes to the blood chemistry, although only ammonia and the mobility/activity of the crabs were affected with significant decreases in both of these parameters once salinity reaches 45, indicating that this is the point at which the crabs begin to be affected by the hypersaline conditions.

Of note is that the analysis of the seawater performed by the University of Hull's chemistry department has produced values for Mg, K and Ca that are approximately 30%-50% lower than in the other global examples shown in Table 5.3. This may be purely down to the lack of repetition resulting in means not being able to be calculated for any of the elements, but may also be indicative of an error with the ICP-OES machine or the dilutions prepared by the operator. Statistical calculations were performed correctly with the data provided from the ICP analysis. Nevertheless, what was being compared in this study was how these elements in the haemolymph of crustaceans change with changing salinity, hence where crustaceans appear to show a conformer or regulator type of pattern for the blood composition, because the same machine and the same technique was used for all analysis within this thesis, the general trends are therefore valid. These lower figures for Mg, K and Ca must be borne in mind however by anyone using the information herein to directly compare these elements to their own results for crustacean haemolymph or water analysis.

5.6.2 Ionic haemolymph

In the adult *Necora puber* intermoult chronic trial sodium (Na) appears to show no change with increasing salinity. In the chronic trial and the acute trials on *Homarus gammarus*, late postmoult *H. gammarus* and *Cancer pagurus* sodium in the haemolymph increases significantly with increasing salinity suggesting that when acutely exposed these species do not have the same ability to regulate that is seen when given time to acclimate (as seen in the chronic trial for *H. gammarus*). *N. puber* appears to maintain the haemolymph levels of Na a little lower than the external concentration. The major contributor to blood osmolality in euryhaline crabs is sodium-chloride, and thus the regulation of the fluxes and permeability of these two ions is central to the animals' ability to tolerate salinity gradients (Towle 1997). The magnitude of Na^+ gradients appears to be the key parameter influencing relative sensitivity to copper in osmoregulating organisms (Grosell et al. 2007). Several transport systems have been suggested for the transport of sodium across the gill epithelium, each possibly participating in the transepithelial uptake of sodium ions into the haemolymph (Figure 5.14) (Towle 1997).

Of note is that the levels of approximately 200 mmol $^{l-1}$ found in the chronic trial for haemolymph Na in *H. gammarus* are much lower than those observed in *Homarus americanus* in normal

seawater conditions: approximately 470 mmol l^{-1} (Taylor and Whiteley 1989) or in *H. gammarus* at approximately 490 mmol l^{-1} (Lucu and Devesconi 1999). Whether the low figure found here in the chronic trial is an error in the ICP-OES analysis, or due to a factor such as starvation is unknown. However, sodium was noted as decreasing in *Gammarus duebeni* under hyposaline stress, and under hypersaline stress the same species showed a lower level of sodium in the body when starved when compared to fed animals (Sutcliffe 1971) so the results herein may not be unusual.

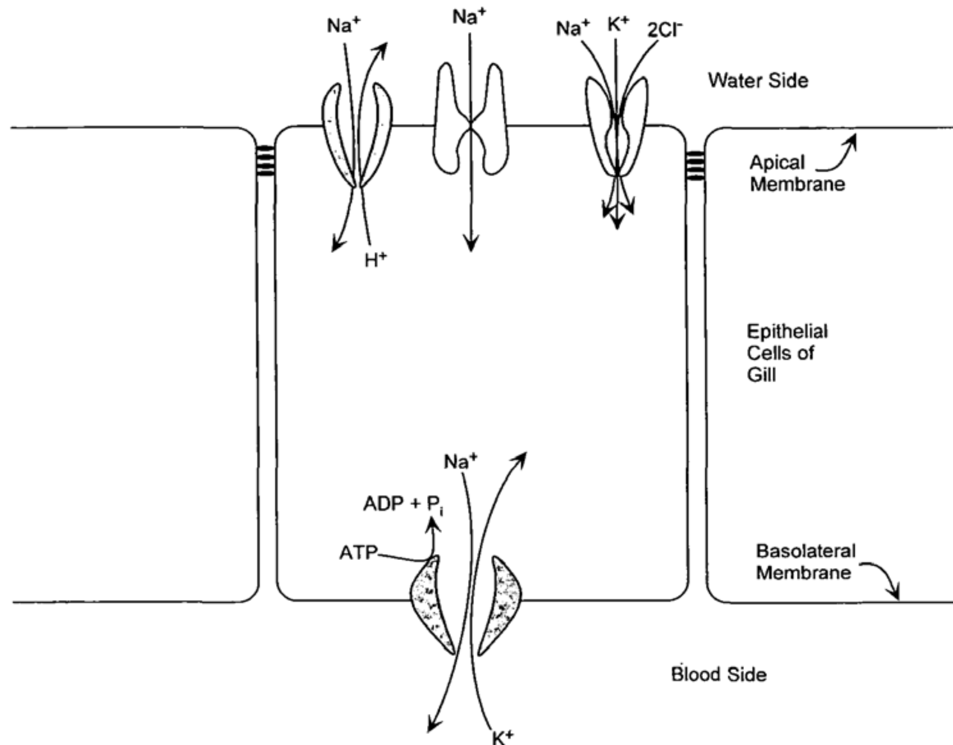


Figure 5.14 Candidate transport systems involved in Na+ uptake across epithelial cells of euryhaline crab gill. The basolateral sodium pump is believed to couple the hydrolysis of ATP to NA+ extrusion into the blood in exchange for K+ or NH4+. Suggested apical uptake systems include (left to right) the Na+/H+ antiporter, the epithelial Na+ channel, and the Na+/K+/2C1~ cotransporter. *After* Towle (1997).

In the adult lobster intermoult chronic trial magnesium (Mg) increased with increasing salinity that was not observed in the control. The same increase was seen in the acute trial and in late postmoult *H. gammarus,* as well as in the chronic trial for *N. puber*. These results suggest there is a lack of a firm regulatory mechanism for this element in these species however, the levels although increasing with increasing salinity, are still maintained lower than those found externally. In *C. pagurus,* magnesium was significantly different at different salinities, but there was no similar trend in the holding water, so for *C. pagurus* there was no clear pattern of hypo/hyper–ionoregulation or ionocomformation. Hyporegulation of magnesium, as seen in the present study, is the most common feature of ionic regulation in crustacean blood (Brown and Terwilliger 1992). All crustaceans tested in the present study showed decreased activity levels with increasing salinity and the anaesthetic effects of Mg are therefore likely to be a factor in the quiescence. In the crab *Pachygrapsus crassipes* the blood magnesium concentration is lowered when the crab is immersed in a medium absent of Mg and raised when the medium Mg is abnormally high (Gross and Marshall 1960). Regulation of magnesium is necessary to facilitate neuromuscular transmission and is a characteristic feature of ionoregulation in active decapod crustacea (Dehnel 1964). The role played by Mg in neuromuscular transmission is shown by low haemolymph magnesium levels being often associated with high levels of activity or a greater degree of terrestrial behaviour in crustaceans (Brown and Terwilliger 1992) and high magnesium levels are often used to anaesthetise marine invertebrates for laboratory analysis.

In the adult lobster intermoult chronic trial it appears that calcium (Ca) may be exhibiting a decrease in concentration as external salinity is increased that was not replicated in the control or the acute trial. In late postmoult *H. gammarus* Ca increased in correspondence with increasing environmental salinity. No change was seen in *N. puber* in either the acute or chronic trial and in *C. pagurus* although significant differences in concentration were observed there was no clear trend and hence no evidence of iono-regulation or conformation. Ca has different effects on different crustaceans, in the Australian yabbie *Cherax destructor*, apparent water permeability (AWP) is not affected by either salinity change or Ca levels, however in the crayfish *Astacus astacus*, salinity affects AWP but Ca on its own does not. *Carcinus maenas* lowers its AWP with decreasing salinity, especially when Ca levels are low, but the ability is lost somewhat when Ca levels are higher (Rasmussen and Bjerregaard 1995). This suggests that Ca may play a role in osmotic regulation in some crustaceans. *C. destructor* and *A. astacus* are freshwater species and therefore the lack of

Ca in their natural environment may explain why the presence of it in laboratory trials has little effect on AWP when compared to the marine *C. maenas*. In fresh water, where the external media is likely to be at a lower osmotic concentration than the body, it may be more energetically advantageous to maintain a constant low AWP and instead regulate cell volume through mechanisms such as increased urine production.

Potassium (K) did not consistently increase with increasing salinity as shown by the other elements in all three species tested. In the adult lobster intermoult chronic trial it appears that K may be exhibiting a decrease in concentration as external salinity is increased that was not replicated in the control. In the acute trial on *H. gammarus*, haemolymph potassium shows peak levels at salinities 35 and 50 with lower concentrations in between, however the error bars at the extreme ends are large and indicate high intraspecific differences in response. Salinity 40 and salinity 50 were where the only significant differences occurred indicating no salinity-dependent trend. In late postmoult *H. gammarus* K increased in correspondence with increasing environmental salinity. There was no change in the acute trials on *C. pagurus* or *N. puber* with increasing salinity. In the chronic trial on *N. puber*, K increased with increasing salinity with haemolymph levels always at a higher concentration than the external media. Blood K concentrations are affected by varying concentrations of Mg in the external medium of both dilute and concentrated salinities but with no definite trend (Gross and Marshall 1960).

In the adult lobster intermoult chronic trial haemolymph copper (Cu) may increase with increasing salinity. In the control, copper was significantly higher but only by the end of the experiment (4 days to 20 days) and therefore may have more to do with the starvation than with any handling and water change effects. In the acute trial on *H. gammarus* haemolymph copper shows a peak in concentration at salinity 45. In *C. pagurus* although significant differences in Cu concentration were observed there was no clear trend and hence no evidence of iono-regulation or conformation. None of the other trials showed significant changes in Cu. Copper (Cu) is an essential micronutrient and acts as a co-factor in multiple enzymatic processes, however it is potentially toxic to aquatic organisms in higher concentrations (Grosell et al. 2007). In freshwater animals, disruption to respiration or osmoregulatory disturbance is often the cause of mortality when high concentrations of copper are experienced (Grosell et al. 2002).

When comparing the responses of Gcb to artificial Aqb, both at salinity 50, there are significant differences in the sodium levels, magnesium levels, calcium levels and potassium levels which reflects the degree of osmoregulation/osmoconformation happening in the crustaceans. For the *H. gammarus* chronic trial, it appears that the lobsters are conforming with regards to Mg, hypo-ionoregulating K and Ca and also regulating Na below the external levels. The increases in the ions in the late postmoult lobsters in line with salinity suggest that when acutely exposed, at this stage of the moult cycle, there are no physiological methods in place in *H. gammarus* for regulating Na, Mg, K and Ca. When in the intermoult stage it may be that the hard shell acts as a barrier protecting the animal, to a certain extent, from any environmental changes. When in the postmoult stage, before the shell is fully hardened, this physical barrier is not in place and may help to explain why mortality occurs at a lower salinity in softer specimens (as seen in chapter 4). The *N. puber* the chronic trial was the only trial to show any ionic change with increasing salinity and only Ca and Mg show change in the control group and do not follow any trend in the water. Hence the changes seen in the experiment are likely to be caused by the salinity challenge and not by the handling and water change regime. Osmoregulatory crustaceans maintain their haemolymph hyperosmotic to the external water by having an exoskeleton, which resists osmotic swelling and has a reduced permeability to ions to prevent ion leakage (Lignon and Péqueux, 1990 in Whiteley et al 2001), and by active and passive uptake mechanisms for specific ions. During the moult and postmoult stages tolerances to environmental variables can change (McLeese 1956; Jury et al. 1994b). Post moult (stages A and B) *Crangon crangon* were more susceptible to mortality caused by Cu and Zn exposure than intermoult stages (Price and Uglow 1979).

The ionic analysis on the water samples clearly shows that there is an increase in the water of sodium (Na), magnesium (Mg), potassium (K) and calcium (Ca) as the salinity increases. Copper (Cu) is the only element not to show an increase. All three species of crustacean tested showed salinity dependent changes in some or all of these parameters, but the majority of the time, even though the haemolymph concentrations increased in line with the external increases, the levels were consistently maintained somewhat lower than externally available. This increase, but in a controlled way, suggests that there is both an element of hypo-iono-conformation and iono-regulation happening. If there was pure conformation, internal concentrations would match the external ones, and if there was full iono-regulation happening, levels would be constant. At the

salinities tested, the responses suggest that the crustaceans are affected by the hypersaline media, but are not at a point where they are overwhelmed by it.

5.6.3 Osmotic control of internal fluids

Whereas in *N. puber* most of the haemolymph parameters increased significantly with increasing salinity and were not observed in the control trials, the results for *H. gammarus* are less clear, with the control trials also showing some significant changes.

The low variability (shown by the error bars in Figure 5.12 for *N. puber*) at salinities 35 to 50, then the sudden increase in standard error at 55 and 60, especially for glucose, lactate and ammonia indicates the high ability of *N. puber* to regulate its internal blood chemistry at the lower hypersalinities. However once the test media reaches salinity 55 the mechanisms for regulation begin to fail and will occur at different rates in different specimens hence the sudden increase in inherent variability in the data. This is in contrast with the findings of Dorgelo (1979) who found that *N. puber* is an osmoconforming species, although this was at a lower salinity range than used here. As an osmoconforming species, in the more hypersaline test media used here, it is expected to lose water to the external medium and gain salt, (therefore maintaining a similar internal fluid osmolarity to the external) thereby increasing concentrations of haemolymph metabolites unless these were strictly regulated at such times.

Some metabolites are not carried naturally in the blood for example lactate, which only occurs with anaerobic respiration, so the increases seen here suggest that a physiological stress on the crabs caused by hypersalinity is effecting a change in the composition of the haemolymph in addition to purely osmotic changes. *N. puber* may therefore be an osmoconformer in the salinities around ambient, but an efficient osmoregulator in hypersalinities, up to 55, at which point the regulatory mechanisms fail. A similar phenomenon appears to be happening for *H. gammarus* although not to such a clear extent as seen in *N. puber*. The larvae of *H. gammarus* are known to be osmoconformers, whilst the adults are (in low salinity) osmoregulators (Charmantier et al. 2001). The less clear responses to high salinity in *H. gammarus* than for *N. puber* suggests that whilst the lobsters are able to regulate in hypersaline media, that this ability is less than for the velvet crab and this may be due to a higher intra-species variability in responses (*cf* error bars in Figure 5.8 and Figure 5.9 for example, which are greater throughout the whole trial than those for *N. puber*

under the same conditions (e.g. Figure 5.12). Variability may itself be an indicator of stress as animals do not respond in a uniform way to environmental changes, and so varying responses can indicate that whilst some individuals of a species or population are coping with stressors, others are not. As occurred for the species studied here, the concentration of organic molecules (such as proteins and sugars) in the haemolymph of the crab *Carcinus maenas* has also been shown to increase at high salinities, mainly by net supply from the tissues. This suggests that organic molecules play a role in regulation of the permeability for salts (Spaargaren 1975).

As *C. pagurus* was only used in the acute trial and only activity/mobility and ammonia showed a salinity dependent response, it is possible that the other parameters that showed no response are being well regulated at the salinities in the test. This is in contrast with the results of previous studies indicating that *C. pagurus* is (in low salinities) an osmoconforming crab with limited ability to regulate cell volume, but can survive due to being able to tolerate osmotic cell size change (Wanson et al. 1983). The significant decrease in ammonia seen in the current trial may indicate a shift to another pathway of nitrogen metabolism. This adaptation is seen in *C. pagurus* under emersion stress, where aerial exposure causes a decrease in ammonia production and an increase in glutamate production, with the nitrogenous waste being stored in the tissues of the cheliped (Regnault 1992).

Activity/mobility of the lobsters was also shown to decrease significantly as salinity increased. This may be as a result of putting more energy into regulating and coping with the external salinity. Hypersalinity is effectively a toxicant, in that it is a factor at levels that are not normally present in the environment. Hypersalinities of 45 psu retard limb regeneration in the fiddler crab *Uca pugilator* (Weis 1976) and changes to the physico-chemical proeperties of seawater are known to affect metabolism, growth, moult rate/stages and ultimately survival (Staples 1991; Chen et al. 1995). Contamination by zinc, 3,4-dichloroaniline, oxygen and ammonia stress has been shown to reduce the scope for growth in the freshwater shrimp *Gammarus pulex* (Maltby et al. 1990) and in the Baltic Sea amphipod *Gammarus oceanicus,* increasing salinity stress lead to a reduced metabolic rate, a reduced feeding rate, reduced faeces production and reduced ammonia excretion (Normant and Lamprecht 2006). Salinity is also known to affect the toxicity of other substances in seawater. In the fiddler crab *Uca pugilator* low salinity seawater increases the susceptibility of the crabs to cadmium poisoning (O'Hara 1973) and in various estuarine and marine isopods the toxicity of

mercury is increased by both lower than normal salinities and higher than normal temperatures (Jones 1973). Additionally, toxicants that have little or no effect on crustaceans in unstressed conditions can, under stressed conditions, affect their ability to cope and adapt to a changing environment and so affecting the environmental range and decreasing their chance for survival (Bamber and Depledge 1997).

5.6.4 Acid base balance

After exercise or periods of hypoxia, lactate accumulates in the tissues and haemolymph of crustaceans, seen here as a significant increase in lactate in both the *N. puber* and *H. gammarus* chronic exposure groups at salinity 55. Lactate is produced under these conditions as the main end product of anaerobiosis (Fincham and Rainbow 1988; Sneddon et al. 1998) leading to an acidosis of the blood evidenced by the significant decrease in pH seen in the chronic test group of *N. puber*. These changes at the higher salinities were not replicated in the controls, suggesting for *N. puber*, that the experimental set up and protocol was not responsible for the differences. This is consistent with Wyman et al (1985) who also found that handling and bleeding procedures did not cause elevated blood glucose and lactate levels in captive *N. puber*. The chronic control portion of the trial for *H. gammarus* also showed significant changes in the lactate levels over time however there was no evident linear trend, except for a massive increase on the final day of the trial. This increase in the control group may again reflect starvation or hypoxic effects rather than any handling or disturbance effects as in general in the control group, lactate levels ranged between 0 and 1 mmol and in the salinity stressed group ranged between 1 and 7 mmol.

N. puber has a high aerobic demand with the highest circulating oxygen levels and oxygen carrying capacities when compared to other sublittoral crabs such as *C. pagurus* and *Maja squinado* (Watt et al. 1999). Of these three species, haemolymph PCO_2 values and lactate levels were also lowest in *N. puber*, indicating high ventilation rates and a lower anaerobic component to the metabolism (Watt et al. 1999). This high aerobic demand is likely to be the reason why *N. puber* (and also the other two species used in this study) cease all observable activities in the highest salinities tested here. A build up of acidic metabolites is confirmed by the significant increase in lactate levels in the blood. The lactate accumulation accompanied by the significant reduction in blood pH found here as well as the additional high oxygen demand found by Watt et al (1999), suggest that *N. puber* is

poorly adapted to respire anaerobically, with the switch to anaerobic respiration occurring in the range of salinity 50 – 55.

The pH of *H. gammarus* haemolymph showed the opposite response to that seen in *N. puber*, with significant increases in pH with increasing salinity, whereas *N. puber* showed significant decreases. In both the chronic test and control groups, the pH showed an increase with increasing salinity/time in the trial, then on the last day (day 24), a decrease in pH. Haemolymph pH is affected by acid metabolites such as lactic acid and carbon dioxide. Whereas in *N. puber* the significant acidification of the blood was as expected due to increases in lactate levels associated with hypoxia, the alkalisation of the blood of the lobsters is more difficult to explain when they also showed a significant increase in lactate levels which would be expected to prompt a drop in pH.

The Bohr Effect is a property of blood pigments such as haemocyanin where in the presence of CO_2 and/or a decrease in pH, the oxygen affinity of the pigment decreases meaning that it binds to oxygen with less affinity (Riggs 1988). As a product of the Bohr Effect the significant decrease in pH seen in this study for *N. puber* effected by the significant increase of L-Lactate in the blood, caused haemocyanin to carry oxygen to the tissues less effectively, increasing further anaerobiosis, and further decreasing the effectiveness of the haemocyanin pigment. The significant decrease of haemocyanin pigment found here for *N. puber* relates to a decrease in the oxygen bound haemocyanin rather than the total haemocyanin. This decrease in the O_2 bound portion is consistent with the lactic acidosis and anaerobic metabolism that has been shown above. The decrease in pH may also be the result of a build up of carbon dioxide in the tissues and bloodstream due to the closure effect exhibited by the crabs in the highest two salinities (whereby the crustaceans stop all observable behaviours and curl their legs beneath themselves, a behaviour suggested by Curtis et al (2007) that has the effect of preventing exchange across the gills), further enhancing the Bohr shift. When given time to acclimate to hypersalinity (as in the chronic exposure trial) there were no significant changes observed for haemocyanin levels in *H. gammarus*. However, when acutely exposed to an abrupt increase to ambient salinity, significant increases were observed in HCY levels with increasing salinity, which is in contrast to the findings for *N.puber* as is the significant increase in pH. Therefore it appears that salinity is having the opposite effect on *H. gammarus* as it does on *N. puber*. *N. puber* also appears to react differently in hypersaline conditions *cf* hyposaline. The results here suggest *N. puber* is in fact an effective regulator up to salinity 55 which is in contrast to the findings of Whiteley et al (2001) who

suggested that *N. puber* lacks ion and osmoregulatory mechanisms and this is why thirty percent seawater (10 psu) had no effect on haemolymph acid–base adjustments.

Some crustaceans can increase the alkalinity of their blood to counter the Bohr shift (Taylor 1982; Hagerman and Uglow 1985; Truchot 1993) mainly through hyperventilation under hypoxic conditions. This may be an adaptation designed to counter the potential reduction in the oxygen affinity of haemocyanin caused by changes in the ionic concentration of the blood when exposed to changing salinities, therefore ensuring that under endurable salinity changes the O_2 transport of the blood can be maintained at least for a limited time. This adaptation may, under normal field conditions such as during a tidal cycle, be sufficient to help the animal survive environmental salinity fluctuations. However this adaptation was not seen here in *N. puber* suggesting that *N. puber* is poorly adapted to long term hypersaline exposure. It may be possible that the increases in pH seen for *H. gammarus* are indicative of a mechanism like this for compensating for acidosis. Under aerial exposure 75% of the buffering capacity of *H. gammarus* against haemolymph acidosis was accounted for by bicarbonate ions at 10 °C (Whiteley and Taylor 1990). In *Crangon crangon* haemocyanin production was increased in mild hypoxic conditions but under starvation, haemolymph haemocyanin levels decreased (Hagerman 1986), the same was found for starved *H. gammarus* (Hagerman 1983). These findings may explain the significant decrease in haemocyanin seen in *N. puber*, as in addition to acidosis of the blood causing a drop in the O_2 bound portion of haemocyanin, animals that survived to the end of the chronic experiment were not fed for 24 days (no changes in haemocyanin were found for *H. gammarus* and *C. pagurus*). However, if high environmental salinity is experienced in the field, inducing the closure response observed here in all three test species, then starvation would be likely to occur as the crabs do not move and so quickly die, there is no adaptation to salinities of 55 and above even after 96h have elapsed. This closure response induced by hypersaline exposure means there will be no aerobic respiration and no feeding; leading to a lower O_2 bound portion of haemocyanin and less haemocyanin production.

5.6.5 Glucose metabolism

The significant decrease in glucose (hypoglycaemia) seen in the *N. puber* chronic control group between the first 4 days and the final 4 days is caused by the long period of starvation (24 days) experienced during this test, as the control group had an absence of any other stressors (such as

the hypersaline media). The additional stress caused by the hypersalinity experienced in the test group had a stronger effect on the crabs than starvation, effectively overriding the hypoglycaemia caused by starvation with a resultant significant increase in glucose (hyperglycaemia) in the group exposed to the hypersalinity. In both the chronic and acute trials for *H. gammarus*, glucose also showed significant increases with increasing salinity, which were not shown in the control animals, therefore as with *N. puber*, salinity had an effect on the glucose concentration in the lobsters. In the late postmoult trial, glucose was the only parameter of the haemolymph tested which showed any salinity dependent changes, showing a significant increase between salinity 35 and 40.

As all *C. pagurus* in the 55 psu 96h test died before the 96 hours were completed no blood results could be gained from them. However the fact that 100% mortality occurred at salinity 55, and the significant decrease in activity as salinity increases, suggests that should they be exposed to such a high hypersaline shock in their natural environments for more than a short period it may cause high mortality of specimens around the minimum landing size. This could be potentially very detrimental to commercial fisheries.

Hyperglycaemia, as seen in the test crabs and lobsters as a significant increase in glucose at salinities of 55 and above in the chronic trial, is recognised as an indicator of stress in crabs (Lorenzon 2005). Starvation is a common cause of hypoglycaemia in crustaceans. In a similar experimental timescale, Hervant et al (1999) found that 28 day nutritional stress caused hypoglycaemia in the amphipod *Gammarus fossarum.* A strong hyperglycaemic condition developed in the first 12 h of emersion of *Maja squinado* during a switch to anaerobic metabolism (Durand et al. 1999). In *Libinia emarginata* asphyxia caused a significant degree of hyperglycaemia, thus further supporting the suggestion in this study that hypersalinity causes an internal hypoxia in *N. puber.* It is also known that emersion stress resulted in hyperglycaemia and increased lactate levels in *C. pagurus* with an associated significant increase in crustacean hyperglycaemic hormone (CHH) (Webster 1999). It is thought that hyperglycaemia in crustaceans under stress is caused by the release of glycogen from stored polysaccharides (mainly in the hepatopancreas) which in turn is converted to glucose (Hall and van Ham 1998).

5.6.6 Ammonia metabolism

Ammonia is the principal nitrogenous waste product in aquatic crustaceans and is excreted via the gills. In *H. gammarus* haemolymph ammonia showed significant increases in both the acute and chronic trials that were not replicated in the controls. Both showed that the normal salinity 35 was significantly different to those above indicating that the hypersaline challenge is having an effect on the ammonia production in this species. Ammonia has also been shown in this study to significantly increase at salinity 55 in *N. puber* chronically exposed to hypersalinity. This change was not observed in the control group. The increases in ammonia in *H. gammarus* and *N. puber* are in contrast to what was found for *C. pagurus* which shows a significant decrease with increasing salinity. Significant increases of ammonia suggest that it is being retained in the haemolymph rather than being excreted via the gills. High salinity stress has been shown to decrease the rate of ammonia excretion in the shrimps *Metapenaeus monaceros* (Pillai and Diwan 2002) and *Penaeus mondon* (Chen et al. 1994).

In addition to the changes in glucose discussed above, changes in ammonia excretion rates or haemolymph levels are also widely recognised as indicators of stress in crustaceans (Aarset and Aunaas 1990; Schmitt and Uglow 1997; Bergmann et al. 2001). In several decapod crustaceans, ammonia excretion tends to increase when animals are hyperosmoregulating and decrease when they are hypoosmoregulating (Lee and Chen 2003).

The retention of ammonia in the haemolymph of chronically exposed *N. puber* and *H. gammarus* is a product of a decrease in cardioventilatory activity (see chapter 6) and the closure response observed in the test specimens (explained in section 5.6.4) and suggests that the crabs are trying to maintain an internal osmolarity that is lower than that of the external media by stopping the fluxes across the gills. With the gills and heart not functioning effectively there will be a build up of ammonia in the haemolymph and the significant increases in the bloodstream seen here suggests that the crab is inadequately discharging waste. From the responses of *C. pagurus* it appears that these crabs are not reacting in response to hypersalinity with either aerobic or anaerobic metabolism. The metabolism has slowed down to such an extent that there is no change in the parameters. The crabs are evidently reducing production of ammonia as it is not present in the haemolymph. It is possible that when faced with hypersaline challenge, a metabolic switch happens and ammonia stops being the end product of nitrogen metabolism. The crabs could be

instead producing urea or uric acid as the salinity increases. Under emersion stress the spider crab *Maja squinado* reduced ammonia excretion and stored ammonia in both the blood and tissues. It also increased urate production, indirectly decreasing ammonia production (Durand et al. 2000), and under similar conditions *C. pagurus* decreased ammonia production and increased glutamate production, again storing nitrogenous metabolic products in the tissues (Regnault 1992).

5.6.7 Haemolymph protein

In the chronic exposure group, significant increases in serum protein levels at salinity 55 were observed for *N. puber* that were not seen in the control. In the chronic exposure trial of *H. gammarus* intermoult adults the opposite was seen, with protein showing a decrease with increasing salinity which became significant at salinity 50. These changes were not seen in the acute trial.

The significant increases in blood protein seen in both the acute and chronic exposure tests for *N. puber* could be indicative of internal dehydration of the tissues caused by increased external>internal osmotic gradient at the higher salinities. However it may also be indicative of an increase in the stress proteins (heat shock proteins). Environmental stressors such as changes in temperature and oxygen levels can induce the production of stress proteins which act to prevent protein aggregation and to maintain functionality of the organism (Chang et al. 1999). Salt stress has been shown to induce the production of stress proteins (heat shock proteins) and metallothionein-like proteins in a range of organisms including the crustaceans *Eurytemora affinis* (an estuarine copepod) (Gonzalez and Bradley 1994; 1995) *Homarus americanus* (Chang 2005) and *Callinectes sapidus* (De Martinez Gaspar Martins and Bianchini 2009). In *C. sapidus* it was found that stress protein production was a branchial response induced by the calcium concentration in the environment. It is not clear why the protein decreases significantly in lobsters under the same hypersaline challenge as *N. puber*.

In *C. sapidus*, increased gill metallothionein like protein concentration in low salinity is an adaptive response to hypo-osmotic stress. This response is mediated, at least in part, by the calcium concentration in the gill bath medium (De Martinez Gaspar Martins and Bianchini 2009). In the larvae of many decapod crustaceans, when under severe nutritional stress, there is usually a preferential degradation of lipids. Under moderate malnutrition internal lipid reserves may partially

be invested to complement insufficient nutrients available for growth and morphogenesis (Anger 1998). The regulation of intracellular osmotic effectors affects the amino-acid metabolism and hence the protein composition under osmotic stress. Extracellular osmoregulation, is associated with energy expenditure for active ion transport, involving the breakdown of energy-rich compounds such as lipids. These mechanisms produce biochemical changes in terms of lipids and proteins in response to salinity variation (Torres et al. 2002). In the decapod shrimp *Penaeus setiferus,* in unfed animals ammonia excretion diminished in direct proportion to the decrease of dissolved oxygen (DO), whilst fed animals were ammonia-regulators. In low salinity the animals maintained proteins as their energy substrate at all levels of DO, while in the case of full seawater (salinity 35) the shrimp changed the metabolic substrate from lipids-proteins to proteins suggesting that juveniles are capable of changing their energy substrate in response to salinity and DO changes and that a pool of free amino acids, whether of muscular or nutritional origin, are the key to this strategy (Rosas et al. 1999).

Serum protein levels are decreased by starvation and can be affected by temperature. It can also be an indicator of body weight and quality of diet (for review see Lynch and Webb 1973). In the amphipod *Orchestia gammarellus* there was an inverse relationship between haemolymph protein and acclimation salinity (Spicer and Taylor 1987). Lynch and Webb (1973) found that in female specimens of estuarine *Callinectes sapidus* there was a positive correlation between salinity and total serum protein levels attributed to increased synthesis of intracellular amino acids related to the spawning cycle. As *N. puber* is a marine rather than estuarine species, it is unlikely that this is the case here, and that the increases in protein are due to another biological mechanism. As long as hypersaline discharges are limited to within the tolerance range of *N. puber* there should not be any variation in survival between winter and summer months with regard to the parameters tested here, as long as any heat present in the discharge from the mining activity dissipates quickly. Dorgelo (1979) found that in *N. puber* temperature does not influence the blood osmolarity within the non-lethal salinity range.

5.6.8 Final points

The blood parameters tested for here cannot be considered separately, there are many studies showing that there are links between them and that alterations in one parameter, both organic and inorganic can lead to changes in another. For instance, carbonic anhydrase is an enzyme in aquatic invertebrates that facilitates rapid equilibration between molecular CO_2 and HCO_3^- and serves in gas exchange and acid–base balance regulation (reflected in pH and lactate values). In the crabs *Chasmagnathus granulata* and *Callinectes sapidus* this enzyme is sensitive to copper (Vitale et al. 1999; Skaggs and Henry 2002) suggesting that it may play a role in copper induced disturbances to the acid–base balance. HCO_3^- and H^+ not only affect acid–base equilibria but also act as counterions in the transfer of Cl^- and Na^+ across plasma membranes via electroneutral ion transporters between the extracellular space and either the ambient water or the intracellular compartment (Whiteley et al. 2001). Brown and Terwilliger (1992) hypothesised that in *Cancer magister*, Ca and Mg may be involved in modulating the oxygen binding properties of haemocyanin as these elements have already been implicated in affecting the oxygen affinity of haemocyanin from a number of crustacean species (Larimer and Riggs 1964; Truchot 1975). In *Carcinus maenas* both ions decrease the O_2 partial pressure at 50% saturation, and Mg^{2+} increases the Bohr factor (Truchot 1975). In osmoregulating crabs, transfer from marine to low salinity results in a metabolic alkalosis in the haemolymph that can be transient or persistent (Whiteley et al. 2001). For some animals living in hypersaline waters, what appears to be the upper limit of salinity tolerance may in fact be the lower limit of dissolved oxygen tolerance as the solubility of oxygen decreases with increasing salinity (Bayley 1972).

The differences found in *C. pagurus* may be due to high intraspecific variability in this species, or rather the fact that there are few significant differences in *C. pagurus* (and none in the *N. puber* acute trial) may also suggest that there is a strong degree of regulation happening as nothing changes significantly with salinity. This is likely to be the case for *C. pagurus* as it has a high 96h LC_{50} (salinity 55.5) despite being the acute trial, however in the case of the acute *N. puber* trial, the 96h LC_{50} (salinity 41.9) was low and the reason there are no significant changes in the haemolymph parameters may be due to death occurring so quickly that the changes could not be seen. It may be therefore that is not the changes in the blood chemistry in this case that led to the death of the organism (at least those blood chemistry changes that were recorded) but rather

something else happening *e.g.* the cessation of respiration/cardioventilatory behaviour, although it would be expected that this would prompt some sort of change to the haemolymph.

The salinities where 100% mortality occurred (as evidenced in chapter 4) indicate that at this point, regardless of what is happening in the body, the animals are unable to handle hypersalinities. This is especially true for the late-postmoult lobsters which could not survive hypersalinity at all past salinity 40. It is envisaged from these results and those of the intermoult adult lobsters (which could tolerate higher salinities than the soft) that early-postmoult lobsters would be even less tolerant of changes to environmental salinity, due to having no hard shell to act as a barrier and the implications of this finding for population survival in areas of brine discharge is a concern.

5.7 Conclusions

Elevated salinity is toxic, both lethal and sublethal, to the species studied. When given sufficient time to acclimate, significant changes in protein, pH, glucose, ammonia and haemocyanin levels occur in *Homarus gammarus* and *Necora puber* which are characteristic of those found during periods of hypoxia in other decapod species. In general, the responses seen here indicate that when in higher than normal salinities, *H. gammarus* is a weak regulator, with the ionic composition of the blood increasing with the external increases in ion concentration, although maintaining the haemolymph at a slightly lower level than externally. Lobsters in the late-postmoult stage have been shown to be less tolerant of salinity change than their intermoult counterparts, and even when the carapace is approaching full hardness the animals cannot tolerate salinities over 40. *Cancer pagurus* has the highest tolerance of the three species studied at salinity 55.5 (96h LC_{50}). The fewer indicators of haemolymph change in this species when compared with the other test species suggests a stronger degree of osmo and iono regulation in *C. pagurus* which is supported by the highest mortality point of the species tested. When exposed to a hypersaline environment this study has shown that *N. puber* is able to strictly regulate the haemolymph variables within the salinity range 35 – 50 units. Subsequent changes require a more prolonged acclimation period otherwise they are lethal. The inability of *N. puber* to survive at salinities of 55 and 60 in the acute trial indicates that they cannot cope with the sudden changes that this induces.

The changes shown in haemolymph variables may be eventually lethal or, given additional time may be restored to normal (normoxic values/normal salinity values). Ultimately for the purposes of keeping commercial fisheries sustainable, brine discharges should be limited at tolerance level of the species with the lowest tolerance, in this case *N. puber,* hence by limiting the discharge to keep this species alive in the affected area, it therefore keeps the other commercially important species which have higher tolerance levels alive too.

Chapter 6

Behavioural assays, concealed behavioural responses of crustaceans to hypersaline exposure. Heart and scaphognathite activity changes.

6.1 Introduction

The quantitative and qualitative cardioventilatory beat behaviours of various species of decapod crustaceans have been used in many studies of their physiological responses to changes in the intensity of environmental variables (Ansell 1973; Cumberlidge and Uglow 1977a; Walters and Uglow 1981). It is understood that one of the ways in establishing if stress has occurred is a change in the rate of a physiological process (Bayne 1980), *e.g.* cardioventilation. Consequently, tests which involved recording the cardio-ventilatory activities of the test species were made whilst they were under hypersaline challenge.

Heart and ventilation responses are often coupled, although they are to some degree subject to independent control or can change in opposite directions in response to the same stimulus (Hume and Berlind 1976). Cardioventilatory behaviour is closely associated with the fluxes of oxygen and carbon dioxide into and out of the body across the branchial wall. An altered oxygen demand such as occurs at the onset or cessation of movement or following a change in the intensity of an environmental stressor (*e.g.* salinity) may evoke an altered beat frequency or some other aspect of beat behaviour. Not all such changes are related to the fluxes of gases as the exchanges of many ions are also mediated via branchial transepithelial fluxes. Flux itself is dependent on the rate of flow of the fluids external (branchial chamber flow) to and internal (blood flow) to the permeable branchial epithelium (Spaargaren 1974; Spaargaren 1976; McDonald et al. 1980).

The variability of flux that such a system confers is indicative of a sensitive means of fine tuning flux in step with changes to environmental variables. This variability in cardiac activity has been described as an indicator of the Darwinian fitness of decapods (Depledge and Lundebye 1996), in terms of their physical ability to cope with changing environmental gradients.

The validity of using cardioventilatory behaviour data in the present context is dependent on the techniques used to obtain them not being a stressor to the animals. Historically, methods of collecting such data have been destructive *e.g.* Wilkens & McMahon (1972), Pilkington & Simmers (1973) or intrusive *e.g.* Cumberlidge and Uglow (1977a, 1977b), Paterson & Thorne (1995), Dufort et al (2001). Heart rates were commonly collected using impedance pneumography, initially using paired electrodes (Cumberlidge & Uglow, 1977a, 1977b) and subsequently using a single electrode (Dyer and Uglow 1977). With these techniques, organ rate measurement usually depended on electrode(s) being inserted through the carapace in the cardiac region of the dorsal carapace or in the region of the scaphognathite on each side of the animal. Macruran decapod anatomy is such that scaphognathite activity could be measured with electrodes hooked around the branchiostegite margin and anchored to the dorsal carapace (*i.e.* without carapace puncture). The present data were collected using infra red sensors which do not require puncturing of the carapace or intrusion into the body.

It has been suggested that a non-lethal physiological approach to toxicity testing is advantageous as unlike the common LD_{50} and LC_{50} tests, the physiological approach generates data at the sublethal level, (impacts can be detected early, before death occurs). In this way it can be seen that toxicants that have little or no effect on crustaceans in unstressed conditions may then affect their ability to cope and adapt to a changing environment and so affecting the environmental range and decreasing their chance for survival (Bamber and Depledge 1997). Responses such as this have potential to be used as an early indicator of change and could be useful for management of environments and fisheries. In *Homarus americanus* there is an almost linear increase in oxygen consumption, heart and scaphognathite rates in animals exposed to reduced salinity seawater, with an almost double increase in metabolic rate when animals were moved from salinity 20 to 15 to 10 (Jury et al. 1994a). The effects of parameters other than salinity can also affect the animals in a similar way, for instance Depledge (1984b) showed that in shore crabs both circulatory and respiratory activity was disrupted when crabs were exposed to selected trace metals.

Preliminary tolerance tests at 8 °C in the laboratory revealed that the lobster, *Homarus gammarus* and the velvet crab, *Necora puber* were both tolerant of hypersaline conditions to give upper LC_{50} values of salinity 48.9 and 41.9 respectively (see chapter 4). The laboratory tests also revealed that as salinities approached lethal values both species became immobile but gave no other observable, quantifiable indication of possible physiological impairment. This behaviour has

prompted an examination of cardioventilatory beat activities to determine whether such concealed behaviour (behaviour not observable to the naked eye in the way a movement of the legs for example would be) may be associated with the blood chemistry changes already discovered to occur at such times and therefore help explain the mortality of these animals when challenged by a hypersaline environment. The causes of death in extreme low or high salinity appear to be primarily related to a number of physiological factors, this chapter will focus on indirect damages caused by lowering of metabolic rate or activity, leading to a failure of ability to escape an unfavourable situation or failure to pump/filter effectively (Kinne 1966).

6.2 Aims, objectives and hypotheses

The main aim of this study was to determine whether sub-lethal effects of hypersaline exposure could be detected in *Necora puber* and *Homarus gammarus* in terms of changes to the activity of the heart and scaphognathites when the crustaceans were subjected to environmentally based physiological challenges (hypersalinity) in the laboratory. A number of null hypotheses were posed to test these unknowns in these studies:-

1. hypersalinity does not cause any change to heart beat behaviour in the crustacean species tested;
2. hypersalinity does not cause any change to scaphognathite beat behaviour in the crustacean species tested;
3. the crustaceans tested cannot distinguish between normal and hypersalinity;

Hypersalinity is defined here as any salinity above what the species' normally experience in the natural environment (in the case of those tested here, that salinity is 35). The species studied are mainly sublittoral in nature and so would rarely experience any change to ambient salinity. Responses of *Cancer pagurus* were not studied due to the ban on landing this species at the time of research (as previously explained).

6.3 Materials and Methods

6.3.1 Animal husbandry

Locally creel caught individuals of *Homarus gammarus* (minimum landing size ±3 mm) and *Necora puber* (carapace width 65-75mm) were kept for five days before experimentation in a filtered recirculation system at salinity 35 and at 8°C ± 1°C. They were not fed during this time. Decreases in salinity and dissolved oxygen along with increases in temperature caused a lower survival rate of soft shelled lobsters (McLeese 1956) and Jury et al (1994b) only used intermoult individuals for this reason. As the same may be true for high salinities, these hypersalinity trials were conducted on intermoult lobsters and crabs only.

6.3.2 Experimental procedure

Organ beat behaviours were detected using non-invasive near infra red sensors (VISHAY semiconductors No CNY07) attached to the carapace adjacent to the organ under study (heart, left scaphognathite and right scaphognathite) with cyanoacrylate glue (Figure 6.1). Both scaphognathites and the heart were recorded simultaneously in all specimens, hence 3 sensors were used per animal. Heart and scaphognathite rates of intermoult adult *H. gammarus* (n = 8) and *N. puber* (n = 5) were measured initially on exposure to a new salinity regime (in increasing increments of 5 psu from the normal of 35) and subsequently following acclimation for 24 hours (tank size 30 cm * 30 cm * 10 cm). As the shape of these organs changes with each beat, the intensity of light reflected back to the detector fluctuates. The detected signal was amplified and fed to a Power Lab data acquisition unit (PowerLab/8SP, ADInstruments Pty Ltd, Castle Hill, Australia), the digitised outputs of which of which were displayed, recorded and subsequently analysed using ‘Chart 5’ software (AdInstruments) on a laptop PC (Figure 6.2). The animals were introduced to ambient salinity (salinity 35) then every 24 hours the salinity was increased by 5 until death occurred. Beat activity was recorded for three alternative thirty minute sessions following first introduction to a new salinity, and for a further three thirty minute periods after 24 hours had elapsed. The salinity was then increased again by another 5 units and the procedure repeated.

Fig 6.1a

Fig 6.1b

Figure 6.1 Cardioventilatory organ beat recording. Sensor positioned over heart (6.1a) and scaphognathite (6.1b) with cyanoacrylate glue before immersion.

In animals where beats were interspersed with periods of inactivity, the average beats per minute (bpm) during the active time was calculated and the % of time when the activity occurred was calculated as a qualifier. This was in addition to the total bpm over the 30 minutes including the inactive periods. A control was included where the animals were fitted with the same sensors and attached to the same recording apparatus, but only at salinity 35, the salinity of the natural environment. This accounted for the effects that the handling procedure may have had on the beat regime at higher salinities. Both left and right scaphognathite rates were recorded, then later a combined beat rate was calculated for each individual by adding the rates for the left and right together and dividing by two for each salinity tested.

Crabs were tested in a closed room with the signal wires passing through a door to the adjacent room so that there was no visual disturbance that may affect the beat rate. In general the beat rates showed that there was no evidence of stress caused by handling after 15 minutes. This time period was not included in the analysis.

The semiconductor sensor was cubic (7 mm) and fitted to a 2.5 mm diameter wire. Animals were removed from the water, the carapace dried and the sensor fitted. The maximum amount of time the animals were out of the water for was five minutes whilst the cyanoacrylate glue dried. Wires were loosely suspended above the tanks where the animals were individually housed and did not

appear to impair movement at all, however both the lobsters and crabs had to have their chelae banded so as to prevent removal of the sensors placed over the scaphognathites.

The sensors were used to record beat frequency only. Signal amplitude which can be used as an indicator of the beat strength was not used as slight mispositioning of a sensor between specimens could result in a severe change in the strength of the signal whilst still producing the same beat frequency.

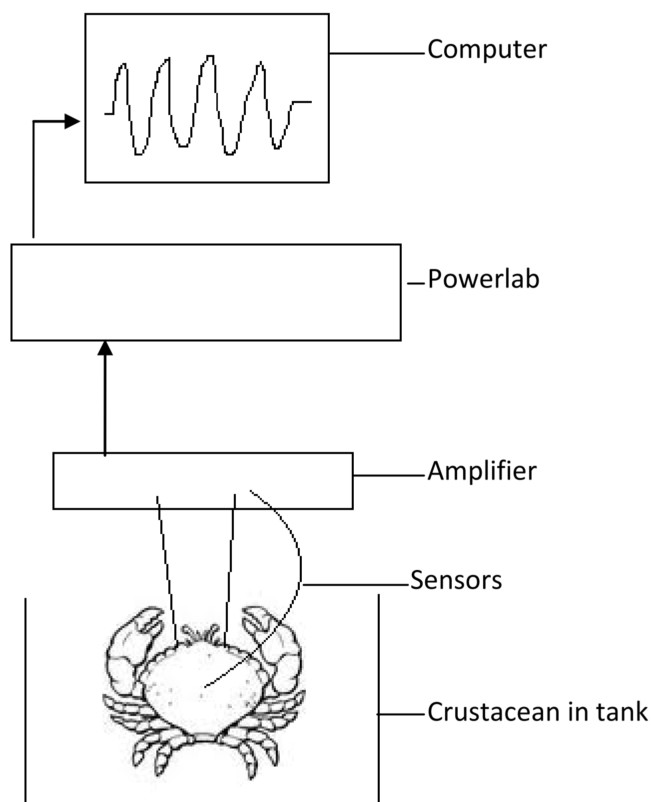


Figure 6.2 Schematic diagram of the experimental set up for recording heart and scaphognathite rates.

6.3.3 Statistical analyses

Differences in beat activity between salinities were analysed using a one way ANOVA or a Kruskal Wallis test depending on normality of the data, followed by a post hoc Scheffe or Games Howell preferably, or if not possible (due to the Scheffe or Games Howell finding no differences despite the Kruskal Wallis or ANOVA indicating there were), a Least Significant Difference test. Correlations between organ activity were analysed with either Pearson or Spearman Rank correlation depending on normality of distribution.

The heart and scaphognathite activities of each animal were recorded continuously for 30 minutes at each recording session. Beat rates have been expressed as number of beats per minute but it should be noted that, at the higher salinities particularly, the 30 minute mean values were inclusive of periods when the beat rate was considerably higher or lower than the session mean rate. Data relating to T = 0 h and 24 h beats were analysed to gain an overall view of the organ behaviour responses to hypersalinity. In addition, where possible, the organ beat activities at T = 24 h was compared with those that prevailed shortly after the initial exposure to a new salinity. This was possible for *H. gammarus* only as the high mortality of *N. puber* with these treatments resulted in very few valid data being collected at the higher salinities tested and this precluded the same statistical analysis being made.

6.4 Results

6.4.1 *Homarus gammarus*

Example data selections taken over a two minute period are given to highlight the difference in beat rates. Heart beat rate at salinity 50 is slower than at salinity 35 as indicated by the greater distance between peaks (Figure 6.3). The difference in beat activity is further highlighted in Figure 6.4 which shows that the scaphognathites show periods of inactivity between periods of beating (salinities 45 and 60 in Figure 6.4) and show variation not only in the duration of each beat, but in the beat frequency.

The relationships between *Homarus gammarus* beat behaviour and prevailing salinity, in terms of mean beat frequency and the mean relative period of beating activity showed a general decline in both beat rate and the % of time spent beating for both the heart and scaphognathites (Table 6.1). Clearly, there is some variability associated with each mean (shown by the standard error (SE)), and for the scaphognathites, expressing the data purely as a simplistic mean beat rate obscures the change in beating from constant to arrhythmic over the salinities. Table 6.1 therefore gives the period of relative (%) activity per unit time for each group at each test salinity.

The mean heart rate of *H. gammarus* decreased from a maximum of 36.8 bpm at the normal salinity 35, to a minimum of 12.6 bpm at salinity 60 during active periods (Table 6.1). When the bpm was calculated over the whole recording period, thus inclusive of inactive periods, there was still a noticeable decrease, from a high of 36.6 bpm at salinity 35 to a low of 10.7 bpm at salinity 60. In general between salinities 35 to 55 the heart activity of *H. gammarus* was relatively constant, actively beating for 90% - 100% of the time, however once salinity reached 60, the heart was active for only 55.5% of the time. The associated standard error of this figure (±20.115) is very large (*c.f.* the lower salinities) and is indicative of the large intra-species variation once salinity reaches this point, suggesting the conditions have induced a marked bradycardia in some of, but not the entire test group.

The scaphognathite behaviour was also altered in the test salinities and showed a decrease in the mean bpm as salinity increased, although the trend does not appear to be as strong as that for the heart rate as there was a slight increase in bpm before the rate decreases again. The highest scaphognathite rate occurred at salinity 40 where when active, the scaphognathites beat at an

average of 80.9 bpm and when averaged over the whole recording period they beat at 78.7 bpm. The lowest bpm occurred at salinity 45, averaging 46.3 bpm when active and 27.9 when accounting for the periods of inactivity. The percentage of time the scaphognathites beat varied from a maximum of almost 100% at salinity 35 to a minimum of 72% at salinity 45, coincidently where the lowest bpm occurred.

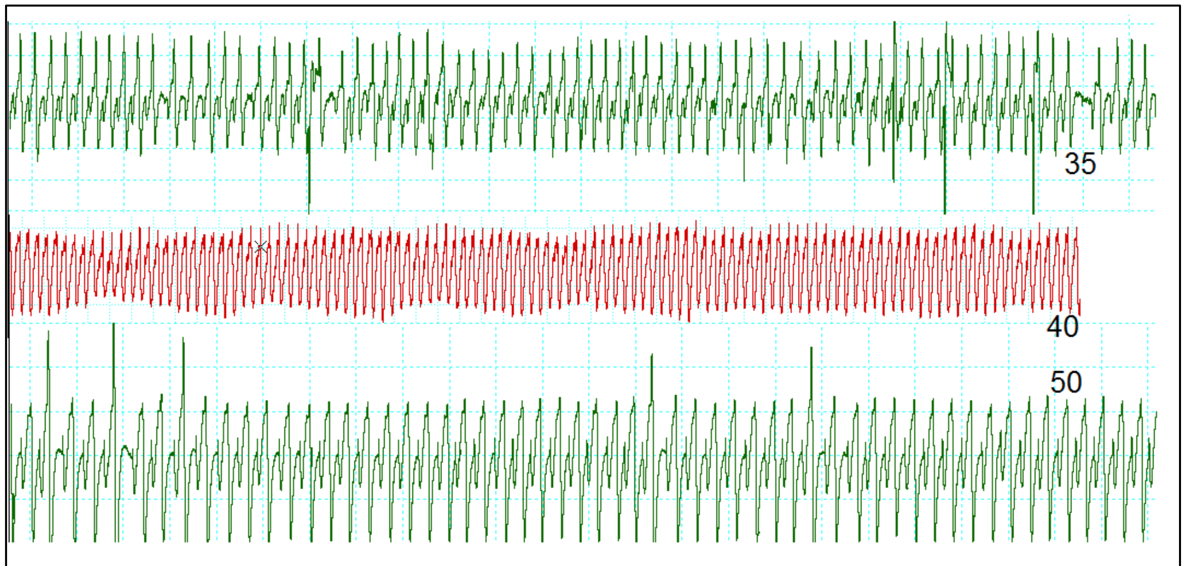


Figure 6.3 Example heart rate traces from *Homarus gammarus* at different salinities (35, 40 and 50 salinity units). Traces are taken over a two minute period. Spikes are indicative of a stronger beat, however only beat frequency and not the amplitude was included in the analysis

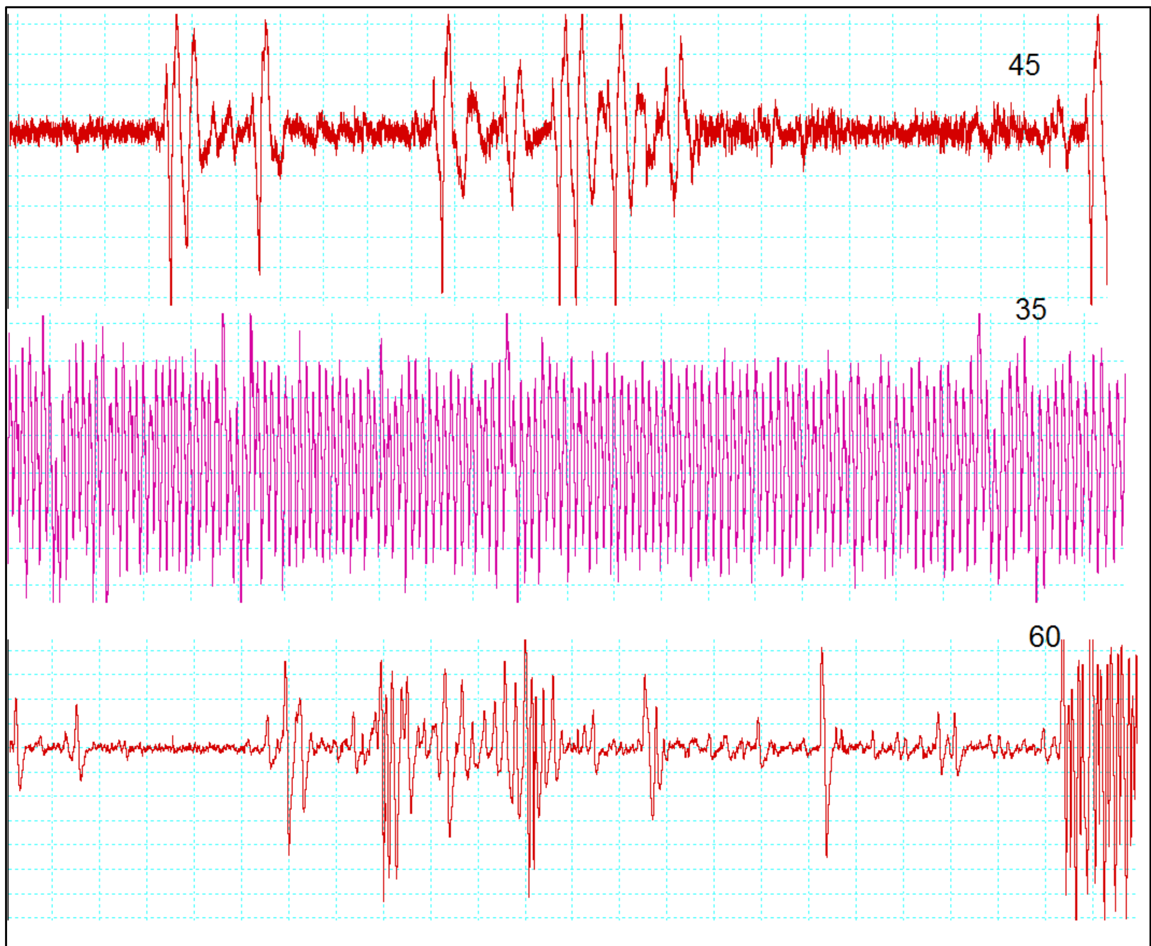


Figure 6.4 Example scaphognathite rate traces from *Homarus gammarus* at different salinities (45, 35 and 60 salinity units). Traces are taken over a two minute period. Spikes are indicative of a stronger beat, however only beat frequency and not the amplitude was included in the analysis.

Table 6.1 Mean heart and scaphognathite beat rates for *Homarus gammarus* per salinity tested (n=8).

Salinity	Mean heart BPM when beating	SE of mean heart BPM when beating	% time heart beating	SE of % time heart beating	bpm heart incl inactivity	SE of bpm heart incl inactivity
35	36.762	4.536	99.750	0.250	36.608	4.418
40	30.882	2.507	96.250	3.750	29.550	2.690
45	27.978	1.955	92.159	5.610	25.873	2.516
50	22.739	1.722	100.000	0.000	18.701	2.780
55	24.296	1.927	90.000	6.831	21.757	2.379
60	12.551	3.820	55.453	20.115	10.679	4.417
Salinity	Mean scaph BPM when beating	SE of mean scaph BPM when beating	% time scaph beating	SE of % time scaph beating	bpm scaph incl inactivity	SE of bpm scaph incl inactivity
35	63.139	5.377	99.773	0.227	63.786	5.867
40	80.984	11.279	95.000	4.510	78.657	12.147
45	46.281	4.340	71.971	12.730	27.943	7.815
50	53.263	4.351	76.939	8.126	43.518	6.426
55	59.661	9.325	85.417	10.133	55.057	10.815
60	56.740	5.628	81.156	10.835	48.527	8.667

The results of a series of statistical tests on the heart and scaphognathite activity of *H. gammarus* are summarised in Table 6.2 and explained below. Both the mean bpm over the recording period were analysed as well as the bpm only for the periods the organs were beating (with the % time actively beating included for qualification).

There were significant differences in the heart bpm between different salinities in *H. gammarus* (Table 6.2). Salinity 60 was different to those below in terms of BPM and 35 differed from 50. When the bpm was averaged out over the whole recording period there was still a significant difference in the heart rate with regards to salinity. These significant differences in BPM were found between salinities 35 – 50, 35 – 60, 40 – 60. The means of the data for each salinity show that there was an almost linear decrease in the heart rate of the lobster as salinity is increased (Figure 6.5 and Figure 6.6). With the periods of heart inactivity taken into account, the linear relationship (r^2) becomes stronger by 0.062 but the error bars increase somewhat (Figure 6.6), however there is still the same marked decrease in the heart rate as salinity increases.

Although close to the 95% point, there was no significant difference in the percentage of time the heart was active for over the salinities tested ($p = 0.054$) (Figure 6.7), meaning that salinity has no effect on the amount of time the heart beats for (within the range tested). *H. gammarus* can regulate the heart's activity well in the salinity range 35-50, and after this point there is a decrease prompted by the high salinity (Figure 6.7). The increase in SE at this point is indicative of high intra-specific differences, suggesting that some individuals are still maintaining a relatively constant activity whilst some are experiencing marked periods of inactivity.

Although when inactive periods were excluded there was no correlation between the percentage time the heart is actively beating for and the average heart bpm, when the bpm was calculated to include inactivity there was a significant correlation between the heart rate and the percentage of time it beats ($r_s = 0.470$, $p < 0.001$, $n = 64$) (Table 5.2).

When inactive periods were excluded there was no significant difference in the bpm of the total combined scaphognathites (left and right analysed together) of *H. gammarus* in relation to salinity change, however when the bpm was calculated over the whole recording time and therefore included inactivity, significant differences were found (Table 6.2). These differences in beat rate occurred between the following salinities: 35 – 45 and 40 – 45. The lowest mean scaphognathite beat rate occurred at salinity 45 (bpm = 27.9) whereas salinities 35 and 40 had the highest bpm at 78.7 and 63.8 respectively.

The right scaphognathite showed significant differences in the percentage time beating for at different salinities. These occur between salinities: 35 – 60 and 40 – 60 (Table 6.2). The means for each salinity show that there is no real trend in the percentage of time the right scaphognathite is active for with regards to salinity due to the overlap of the SE bars (Figure 6.8).

There was a significant correlation between the total mean scaphognathite bpm and the total mean % time the scaphognathites were active ($r_s = 0.550$, $p < 0.001$, $n = 56$) (Table 6.2). There was also a significant correlation between the average left scaphognathite bpm and the mean % time the left scaphognathite was active ($r_s = 0.597$, $p < 0.001$, $n = 49$), and also the mean right scaphognathite bpm and the mean % time the right scaphognathite was active ($r_s = 0.381$, $p = 0.013$, $n = 42$) (Table 6.2). The same correlations occurred when the scaphognathite bpm took the periods of inactivity into account (Table 6.2). This indicates a definite connection between the activity of the scaphognathite and the beat rate.

Table 6.2 Statistical analysis of the organ beat data for *Homarus gammarus*. n = 8.

Null hypothesis	Test used	Results	Answer	Post hoc differences
There is no change in heart BPM with salinity (excluding inactivity)	One way ANOVA	F = 6.876, df = 5, p < 0.0010	Reject null hypothesis	Scheffe: 35 – 60, 40 – 60, 45 – 60 also 35 – 50
There is no change in heart BPM with salinity (including inactivity)	One way ANOVA	F = 6.400, df = 5, p < 0.0010	Reject null hypothesis	Scheffe: 35 – 50, 35 – 60, 40 – 60
There is no significant difference in the % time the heart was active for dependent on salinity	Kruskal Wallis	χ^2 = 10.559, df = 5, p = 0.054	Accept null hypothesis	n/a
There is no correlation between the % time the heart is actively beating for and the mean heart bpm (excluding inactivity)	Spearman Rank	correlation coefficient = 0.121, p = 0.340, n = 64	Accept null hypothesis	n/a
There is no correlation between the % time the heart is actively beating for and the mean heart bpm (including inactivity)	Spearman Rank	correlation coefficient = 0.470, p < 0.001, n = 64	Reject null hypothesis	n/a
There is no significant difference in the bpm of the left scaphognathite of *Homarus gammarus* in relation to salinity change (excluding inactivity)	One way ANOVA	F = 1.539, df = 6, p = 0.188	Accept null hypothesis	n/a
There is no significant difference in the bpm of the left scaphognathite of *Homarus gammarus* in relation to salinity change (including inactivity)	One way ANOVA	F = 1.945, df = 6, p = 0.091	Accept null hypothesis	n/a
There is no significant difference in the bpm of the right scaphognathite of *Homarus gammarus* in relation to salinity change (excluding inactivity)	One way ANOVA	F = 1.862, df = 6, p = 0.144	Accept null hypothesis	n/a
There is no significant difference in the bpm of the right scaphognathite of *Homarus gammarus* in relation to salinity change (including inactivity)	One way ANOVA	F = 1.292, df = 6, p = 0.277	Accept null hypothesis	n/a
There is no significant difference in the bpm of the total combined mean scaphognathites of *Homarus gammarus* in relation to salinity change (excluding inactivity)	Kruskal Wallis	χ^2 = 8.046, df = 5, p = 0.154	Accept null hypothesis	n/a
There is no significant difference in the bpm of the total combined mean scaphognathites of *Homarus gammarus* in relation to salinity change (including inactivity)	Kruskal Wallis	χ^2 = 13.070, df = 5, p = 0.023	Reject null hypothesis	Games Howell: 35-45, 40-45
There is no significant difference in the percentage of time the left scaphognathite was active for with regards to salinity.	Kruskal Wallis	χ^2 = 8.683, df = 5, p = 0.122	Accept null hypothesis	n/a
There is no significant difference in the percentage of time the right scaphognathite was active for with regards to salinity.	Kruskal Wallis	χ^2 = 12.578, df = 5, p = 0.028	Reject null hypothesis	LSD test*: 35-60, 40-60
There is no significant difference in the percentage of time the total combined scaphognathites were active for with regards to salinity.	One way ANOVA	χ^2 = 10.133, df = 5, p = 0.072	Accept null hypothesis	n/a

Null hypothesis *(continued)*	**Test used**	**Results**	**Answer**	**Post hoc differences**
There is no significant correlation between the mean left scaphognathite bpm (excluding inactivity) and the percentage of time the left scaphognathite was active for.	Spearman Rank	$r_s = 0.597$, $p < 0.001$, $n = 49$	Reject null hypothesis	n/a
There is no significant correlation between the mean left scaphognathite bpm (including inactivity) and the percentage of time the left scaphognathite was active for.	Spearman Rank	$r_s = 0.714$, $p < 0.001$, $n = 49$	Reject null hypothesis	n/a
There is no significant correlation between the mean right scaphognathite bpm (excluding inactivity) and the percentage of time the right scaphognathite was active for.	Spearman Rank	$r_s = 0.381$ $p = 0.013$, $n = 42$	Reject null hypothesis	n/a
There is no significant correlation between the mean right scaphognathite bpm (including inactivity) and the percentage of time the right scaphognathite was active for.	Spearman Rank	$r_s = 0.652$ $p < 0.001$, $n = 43$	Reject null hypothesis	n/a
There is no significant correlation between the mean total scaphognathite bpm (left and right combined) (excluding inactivity) and the percentage of time the scaphognathites were active for.	Spearman Rank	$r_s = 0.550$, $p < 0.001$, $n = 56$	Reject null hypothesis	n/a
There is no significant correlation between the mean total scaphognathite bpm (left and right combined) (including inactivity) and the percentage of time the scaphognathites were active for.	Spearman Rank	$r_s = 0.760$, $p < 0.001$, $n = 56$	Reject null hypothesis	n/a
There is no significant correlation between the mean heart beat of *Homarus gammarus* (excluding inactivity) and the total mean scaphognathite beats (excluding inactivity).	Pearson correlation	$r = 0.123$, $p = 0.476$, $n = 36$	Accept null hypothesis	n/a
There is no significant correlation between the mean heart beat of *Homarus gammarus* (including inactivity) and the total mean scaphognathite beats (including inactivity).	Pearson correlation	$r = 0.209$, $p = 0.220$, $n = 36$	Accept null hypothesis	n/a

*Despite attempting a number of transformations on the data to normalise the spread, the only *posteriori* test that could pick up any of the differences was the Least Significant Difference (LSD) test.

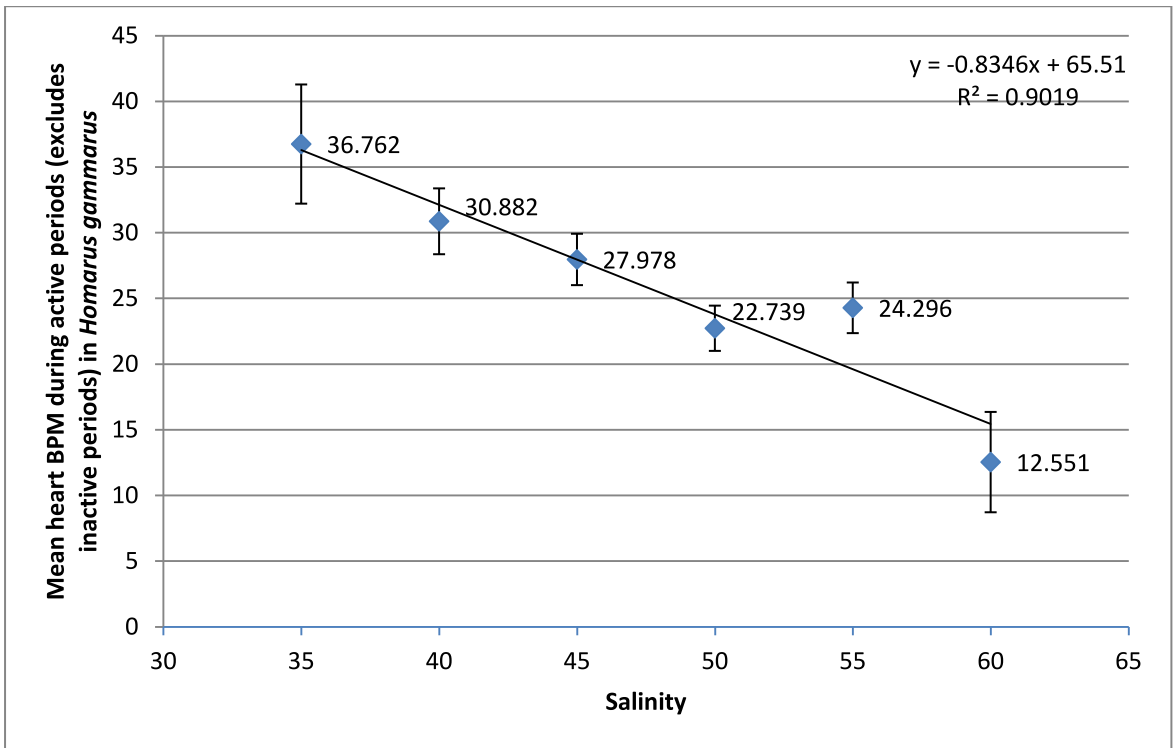


Figure 6.5 **Mean (± SE) heart rate of *Homarus gammarus* (bpm) at different salinities during periods of active beating (excludes periods of inactivity).**
The following pairs of salinities are significantly different in terms of bpm: 35 – 60, 40 – 60, 45 – 60 also 35 – 50.

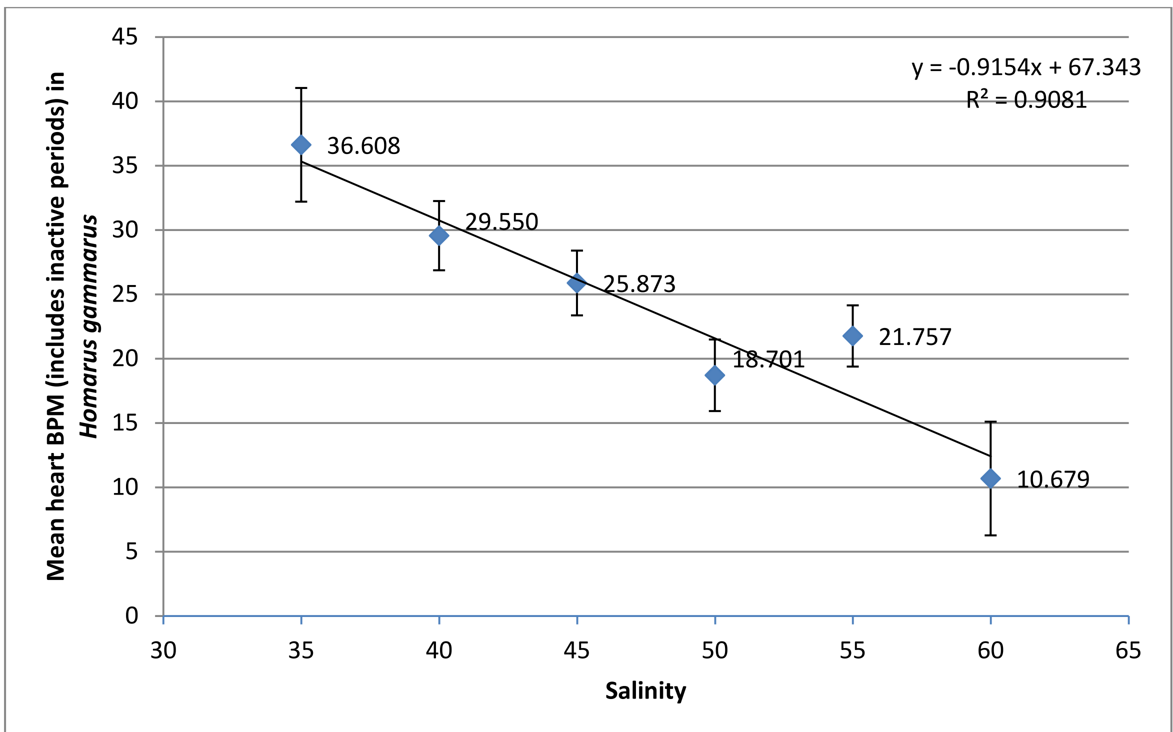


Figure 6.6 **Mean (± SE) heart rate of *Homarus gammarus* (bpm) at different salinities during periods of active beating (includes periods of inactivity).**
The following pairs of salinities are significantly different in terms of bpm: 35 – 50, 35 – 60, 40 – 60.

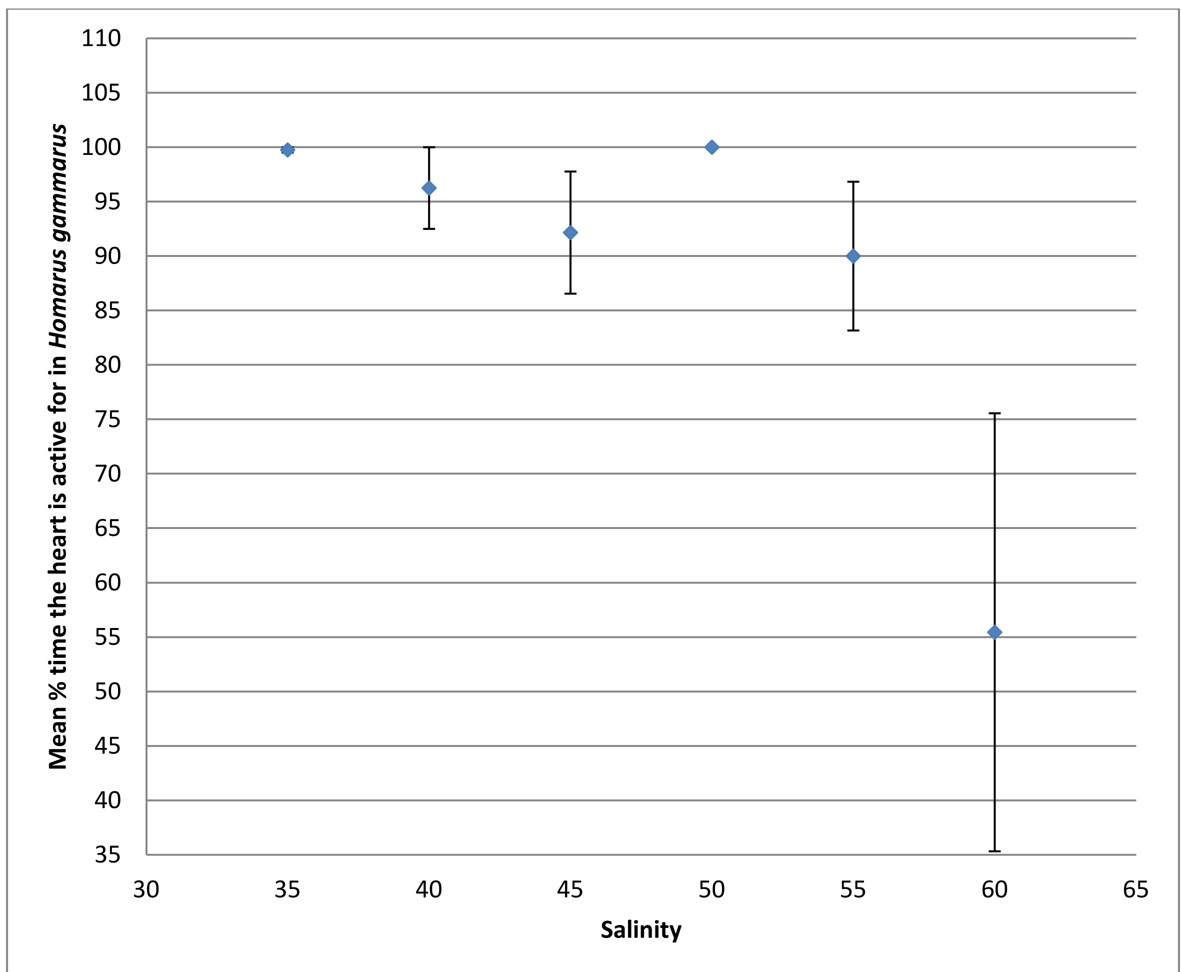


Figure 6.7 Effect of raised salinity on the % of time the heart is actively beating for in *Homarus gammarus.*
Mean % time active (± SE). No significant differences in activity between salinities (p=0.054).

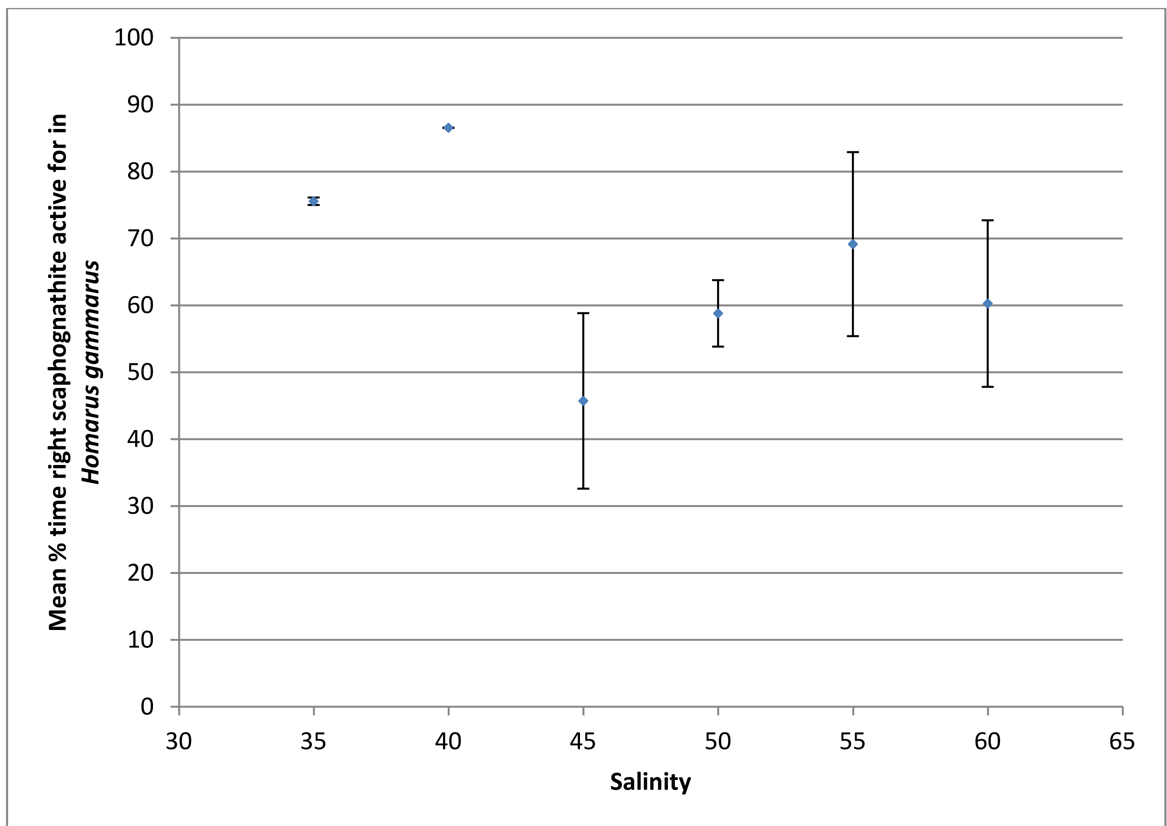


Figure 6.8 The effect of raised salinity on the % of time the right scaphognathite is active for in *Homarus gammarus* (means ± SE).
Significant differences occur between salinities 35-60, 40-60 in terms of activity ($p<0.05$). No consistent trend of salinity dependent change in scaphognathite activity evident.

6.4.2 *Necora puber*

Figure 6.9 and Figure 6.10 show examples of the heart and scaphognathite beats of *Necora puber* at various salinities taken over a typical two minute period. To ascertain if the differences seen in these beat rates was due to the change in salinity, the mean bpm and the % time active were calculated for both the heart beat and scaphognathite beats of *N. puber* (Table 6.3). When compared with *H. gammarus*, clearly there is a larger variability associated with each mean in terms of the standard error (SE). For the scaphognathites, expressing the data purely as a simplistic mean beat rate again obscures the change in arrhythmic beating over the salinities. To account for this, Table 6.3 gives the period of relative (%) activity per unit time for each group at each test salinity. There is an apparent decrease in both the heart and scaphognathite beat rate as salinity increases.

For *N. puber* there is an apparent decrease in the mean heart bpm during the active periods from a high of 43.5 bpm at the salinity 40, to a low of 29.9 bpm at salinity 55. When the bpm is calculated over the whole recording period and therefore takes into account the periods of inactivity, there is still a decrease in mean bpm, from a high of 41.9 bpm at salinity 35 to a low of 27.1 bpm at salinity 55. The difference between the values that exclude and include inactivity only occurs at salinity 55 and is very small. Salinity 55 was the only period when the heart of the crab was not beating for 100% of the time. This may be indicative that the velvet crabs' hearts cope better than the lobsters' during exposure to hypersalinity, though this does not take into account the mortalities.

The scaphognathites show a decrease in the mean bpm as salinity increases and this trend appears to be stronger than for the heart rate, however just as with *H. gammarus,* there is an increase in bpm before the rate decreases again. The highest scaphognathite rate occurs at salinity 45 where when active, the scaphognathites beat at a mean of 116.8 bpm and when calculated over the whole recording period they beat at a mean of 79.2 bpm. The lowest bpm occurs at salinity 60, averaging just 12.9 bpm when active and at salinity 55 when accounting for the periods of inactivity averaging 6.3 bpm. The percentage of time the scaphognathites beat varies from a high of 61% at salinity 35 to a low of 22.5% at salinity 55.

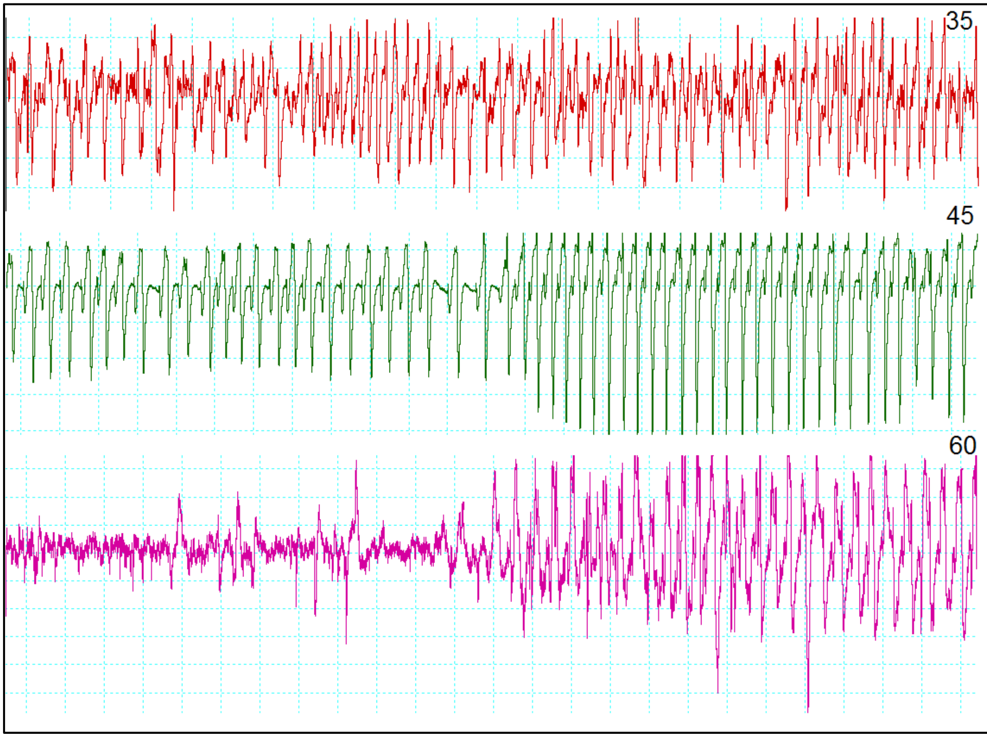


Figure 6.9 Example heart rate traces from *Necora puber* at different salinities (35, 45 and 60 salinity units). The example traces shown are taken over a two minute period.

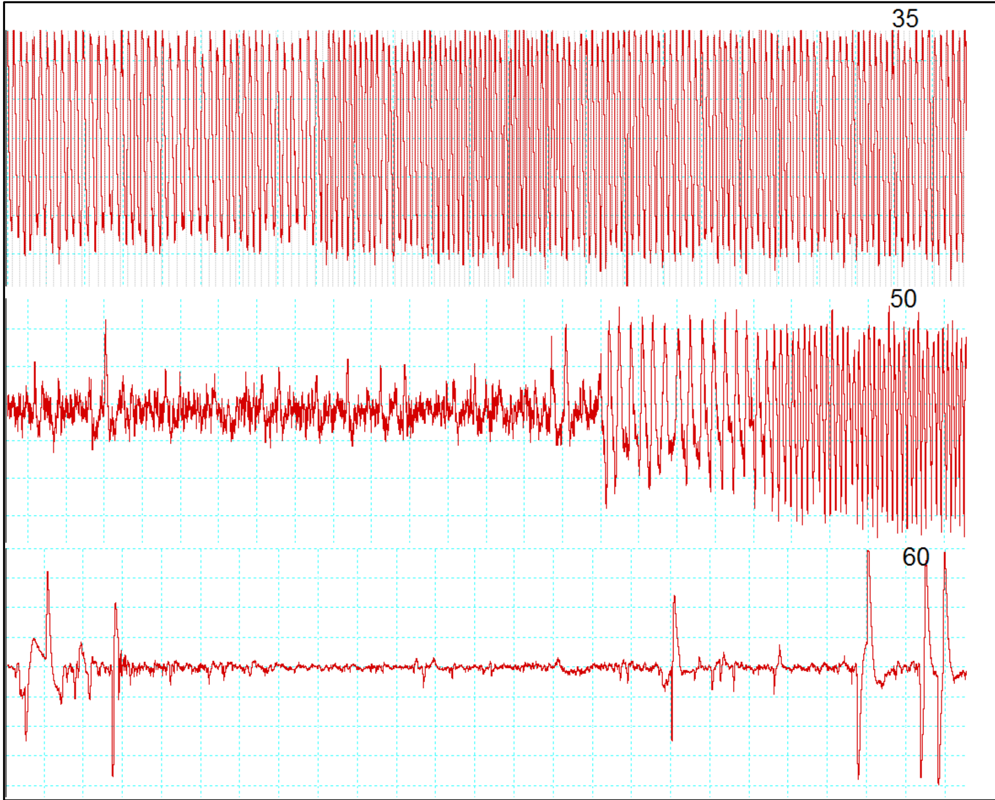


Figure 6.10 Example scaphognathite rate traces from *Necora puber* at different salinities (35, 50 and 60 salinity units). Traces shown are taken over a two minute period.

Table 6.3 Mean heart and scaphognathite beat rates for *Necora puber* per salinity tested (n=5).

Salinity	Mean heart BPM when beating	SE of mean heart BPM when beating	% time heart beating	SE of % time heart beating	bpm heart incl inactivity	SE of bpm heart incl inactivity
35	41.878	5.191	100.000	0.000	41.878	5.191
40	43.258	9.939	100.000	0.000	43.258	9.939
45	32.730	4.952	100.000	0.000	32.730	4.952
50	34.523	6.826	100.000	0.000	34.523	6.826
55	29.893	4.039	90.000	2.887	27.136	4.472
60	33.767	3.583	100.000	0.000	33.767	3.583
Salinity	Mean scaph BPM when beating	SE of mean scaph BPM when beating	% time scaph beating	SE of % time scaph beating	bpm scaph incl inactivity	SE of bpm scaph incl inactivity
35	89.475	11.585	60.966	14.257	55.703	13.780
40	77.845	13.078	52.181	7.608	43.046	10.270
45	116.800	27.487	57.955	16.589	79.213	21.035
50	59.264	14.163	57.414	10.474	39.426	10.041
55	17.739	14.075	22.497	16.392	6.298	6.074
60	12.489	1.806	77.187	22.813	10.052	4.243

The results of a series of statistical tests on the heart and scaphognathite activity of *N. puber* are summarised in Table 6.4 and explained in the text below. The mean bpm over the recording period was analysed as well as the bpm only for the periods the organs were beating (with the % time actively beating included for qualification).

The percentage of time that the heart was active for showed significant differences at salinity 55 (χ^2 = 17.843, p = 0.003, df = 5), specifically, these occur between salinities 40 – 55, 45 – 55, 50 – 55, and 60 – 55 (Table 6.4). Mean values for each salinity showed that at all salinities the hearts of *N. puber* were active for 100% of the time with the exception of salinity 55 where the hearts were active on average for 90% of the time.

There was no significant correlation between the heart bpm (excluding inactivity) and the % time the heart was active, but when the inactive periods were included in the calculation there was a significant correlation between the heart bpm and the % of time it was active for (r_s = 0.468, p = 0.043) (Table 6.4).

The significant difference in the total average scaphognathite beats per minute with regards to salinity indicates that salinity is having an overall affect on the activity of the scaphognathites of

Necora puber. Salinities 35 – 55, 35 – 60, 45 – 50, 45 – 55, 45 – 60 were significantly different in terms of bpm (Table 6.4). This indicates that there is a change in beat activity as salinity increases. Mean bpm decreased from 89.48 bpm at salinity 35 to 12.49 bpm at salinity 60 (Figure 6.10). This level of activity may be insufficient to sustain the life of *N. puber* for a long period of time and may be the cause of high mortality at this salinity level. No significant differences were found when the bpm took into account the periods of inactivity however.

In comparison to the results for *H. gammarus* which showed a linear decrease in heart bpm with increasing salinity (Figure 6.5 Figure 6.6), the heart rate of *N. puber* does not follow the same trend with no significant change in relation to salinity found.

The right scaphognathite of *N. puber* showed significant differences in the % of time it is active for over the salinities tested (Kruskal Wallis; $\chi^2 = 12.889$, df = 5, p = 0.024), with significant differences occurring at the following salinities: 35 – 40, 35 – 55, 40 – 50, 40 – 60, 55 – 60. The mean % activity of the scaphognathites of *N. puber* is very variable over the salinities (Figure 6.12). The significant difference in the right scaphognathite may be due to the left one taking over at salinity 40, however from salinity 45 both scaphognathites show the same trend, with the left showing around 20% more activity. Interestingly the scaphognathites start at salinity 35 with the same level of activity then beat differently until coming together again at salinity 60. However there is overlap in the standard error of the activity at salinities 45, 50 and 55, so there may be not so much of a difference in beat activity as the figure suggests.

There was a decrease in the amount of time that the scaphognathites beat for as salinity increases for both the left and the total average. This decrease may not be enough to sustain the life of *N. puber* when salinity increases beyond their normal range.

There was a significant correlation between the average left scaphognathite bpm and the percentage of time the left scaphognathite was active for and also the average right scaphognathite bpm and the percentage of time the right scaphognathite was active for (Table 6.4). These significant correlations were also all present when the periods of inactivity were taken into account (Table 6.4). Although very close to the 0.05 point (p = 0.054) and despite the left and right individually showing a correlation, there is no significant correlation for the total combined scaphognathite bpm and the % of time the scaphognathites were active for. However this becomes significant when the periods of inactivity are taken into account. These correlations suggest there is

a strong connection between the bpm and the percentage of time the scaphognathites were active for.

Table 6.4 - Statistical analysis of the organ beat data for *Necora puber*. n = 5.

Null hypothesis	Test used	Results	Answer	Post hoc differences
There is no change in heart BPM with salinity (inactivity excluded)	One way ANOVA	F = 1.568, df = 5. p = 0.237	Accept null hypothesis	n/a
There is no change in heart BPM with salinity (inactivity included)	One way ANOVA	F = 1.975, df = 5. p = 0.150	Accept null hypothesis	n/a
There is no significant difference in the % time the heart was active for dependent on salinity	Kruskal Wallis	χ^2 = 17.843, df = 5, p = 0.003	**Reject null hypothesis**	LSD test*: 35-550 40-55, 45-55, 50-55, and 60-55
There is no correlation between the % time the heart is actively beating for and the average heart bpm (excluding inactivity)	Spearman Rank	r_s = 0.389, p = 0.099, n = 19	Accept null hypothesis	n/a
There is no correlation between the % time the heart is actively beating for and the average heart bpm (including inactivity)	Spearman Rank	r_s = 0.468, p = 0.043, n = 19	**Reject null hypothesis**	n/a
There is no significant difference in the bpm of the left scaphognathite of *Necora puber* in relation to salinity change (excluding inactivity)	One way ANOVA	F = 2.158, df = 5, p = 0.087	Accept null hypothesis	n/a
There is no significant difference in the bpm of the left scaphognathite of *Necora puber* in relation to salinity change (including inactivity)	One way ANOVA	F = 1.861, df = 5, p = 0.132	Accept null hypothesis	n/a
There is no significant difference in the bpm of the right scaphognathite of *Necora puber* in relation to salinity change (excluding inactivity)	Kruskal Wallis	χ^2 = 10.140, df = 5, p = 0.071	Accept null hypothesis	n/a
There is no significant difference in the bpm of the right scaphognathite of *Necora puber* in relation to salinity change (including inactivity)	Kruskal Wallis	χ^2 = 10.876, df = 5, p = 0.054	Close but accept null hypothesis	n/a
There is no significant difference in the bpm of the total combined scaphognathites of *Necora puber* in relation to salinity change (excluding inactivity)	One way ANOVA	F = 2.931, df= 5, p = 0.029	**Reject null hypothesis**	LSD test*: 35-55, 35-60, 45-50, 45-55, 45-60
There is no significant difference in the bpm of the total combined scaphognathites of *Necora puber* in relation to salinity change (including inactivity)	One way ANOVA	F = 2.151, df= 5, p = 0.087	Accept null hypothesis	n/a
There is no significant difference in the percentage of time the left scaphognathite was active for with regards to salinity.	Kruskal Wallis	χ^2 = 3.924, df = 5, p = 0.560	Accept null hypothesis	n/a
There is no significant difference in the percentage of time the right scaphognathite was active for with regards to salinity.	Kruskal Wallis	χ^2 = 12.889, df = 5, p = 0.024	**Reject null hypothesis**	LSD test*: 35-40, 35-55, 40-50, 40-60, 55-60
There is no significant difference in the percentage of time the total combined scaphognathites were active for with regards to salinity.	One way ANOVA	F = 0.713, df = 5, p = 0.619	Accept null hypothesis	n/a

Null hypothesis *(continued)*	Test used	Results	Answer	Post hoc differences
There is no significant correlation between the average left scaphognathite bpm (excluding inactivity) and the percentage of time the left scaphognathite was active for.	Spearman Rank	$r_s = 0.449$, $p = 0.007$, $n = 35$	**Reject null hypothesis**	n/a
There is no significant correlation between the average left scaphognathite bpm (including inactivity) and the percentage of time the left scaphognathite was active for.	Spearman Rank	$r_s = 0.790$, $p < 0.001$, $n = 35$	**Reject null hypothesis**	n/a
There is no significant correlation between the average right scaphognathite bpm (excluding inactivity).and the percentage of time the right scaphognathite was active for	Pearson Correlation	$r = 0.396$, $p = 0.021$, $n = 34$	**Reject null hypothesis**	n/a
There is no significant correlation between the average right scaphognathite bpm (including inactivity).and the percentage of time the right scaphognathite was active for	Pearson Correlation	$r = 0.804$, $p < 0.001$, $n = 34$	**Reject null hypothesis**	n/a
There is no significant correlation between the average total scaphognathite bpm (left and right combined) (excluding inactivity) and the percentage of time the scaphognathites were active for.	Pearson Correlation	$r = 0.328$, $p = 0.054$, $n = 35$	Close but accept null hypothesis	n/a
There is no significant correlation between the average total scaphognathite bpm (left and right combined) (including inactivity) and the percentage of time the scaphognathites were active for.	Pearson Correlation	$r = 0.665$, $p < 0.001$, $n = 35$	**Reject null hypothesis**	n/a

*Despite attempting a number of transformations on the data to normalise the spread, the only *posteriori* test that could pick up any of the differences was the Least Significant Difference (LSD) test

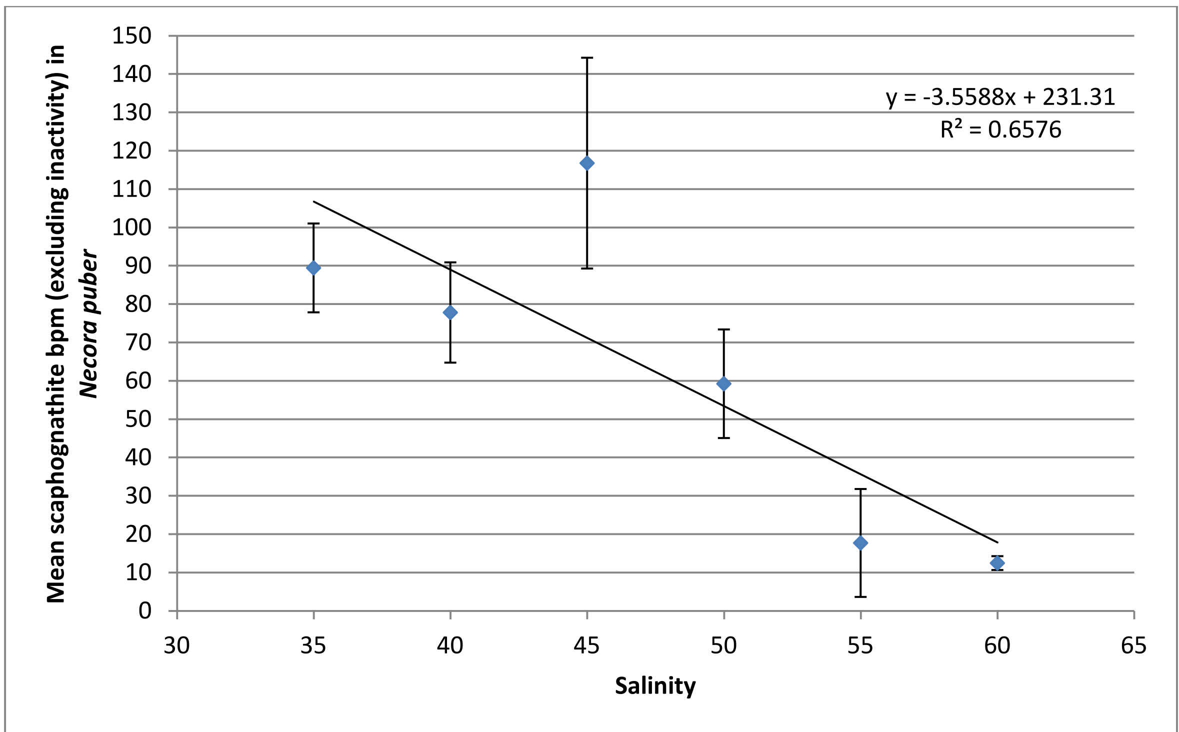


Figure 6.11. Effect of raised salinity on scaphognathite beat rate in *Necora puber* (bpm excludes periods of inactivity). Means ± SE.
Salinities 35-55, 35-60, 45-50, 45-55, 45-60 are significantly different in terms of bpm.

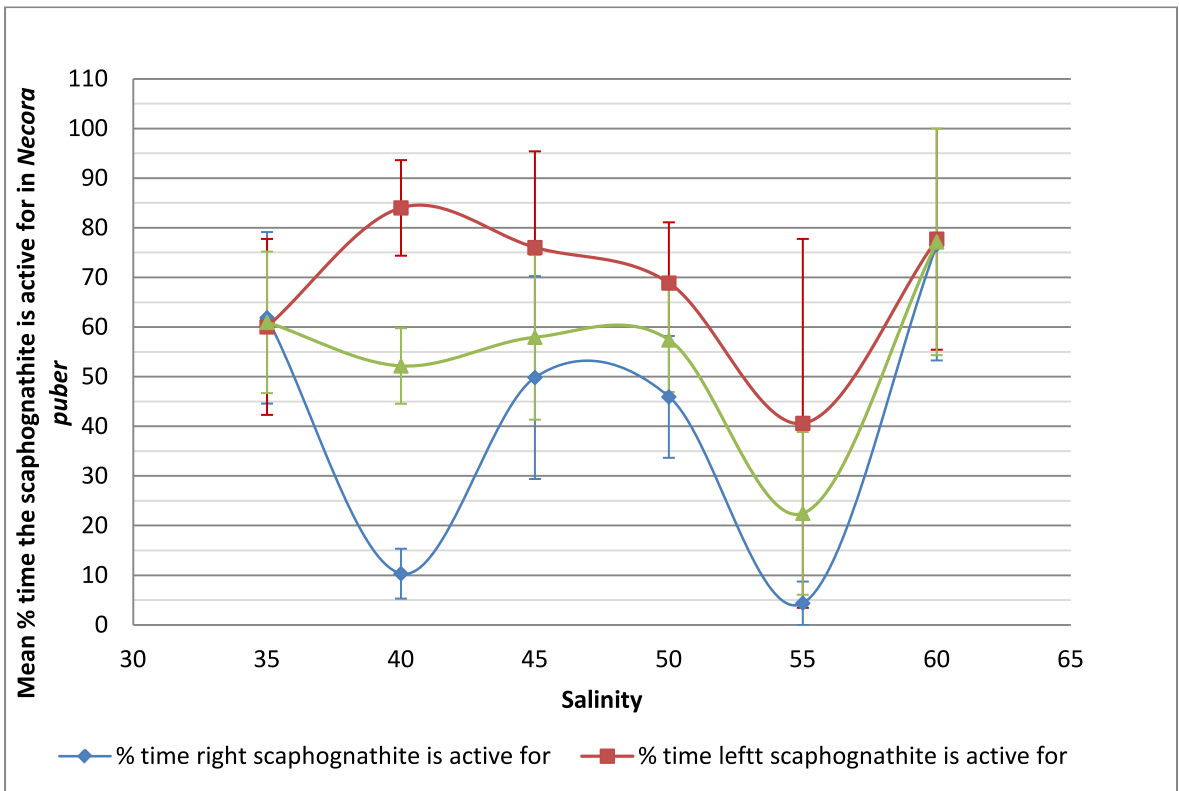


Figure 6.12 Effect of salinity on scaphognathite activity in *Necora puber*. Mean activity ± SE.
Note the considerable overlap in error at salinities 45, 50 and 55.

6.4.3 Comparison between cardioventilatory activity after 0h and 24h hypersaline exposure in *Homarus gammarus*.

The previous results have been based on a combined mean of the beats from the 3 recording sessions following introduction to hypersalinity and the 3 recording sessions after 24 hours had passed. To assess whether there was any degree of acclimation to the hypersalinity used in the trial, the same tests have been performed but separately on the 0h and 24h data. Both heart data and scaphognathite data were analysed but when regarding scaphognathites, only the values for total combined scaphognathite beats have been analysed. The individual scaphognathite responses have not been looked at due to the high variability indicated by the previous statistical analyses. The analyses have shown that there is no difference between the beat rates and organ activity between 0 hours and 24 hours. The only exception is for heart bpm where there appears to be some degree of acclimation to the hypersalinities when tested after 24 hours, where the bpm at salinities 50 and 55 fail to become significantly different from 35.

The difference in *N. puber* between 0 hours and 24 hours was not analysed due to large mortalities at the higher test salinities resulting in a sample size too small to divide and still produce reliable results.

6.4.4 Summary of results

For *Homarus gammarus*:

- Salinity had a significant effect on the mean heart bpm of *H. gammarus* with the impact becoming different from the normal rate once salinity reaches 50 psu.
- The activity of the heart was consistent across all test salinities and was not affected by hypersalinity (within the test range 35-60 psu).
- Overall there was a positive correlation between the mean heart bpm and the activity of the heart, hence a higher beat frequency correlated with a higher % of time active.
- The total bpm of the scaphognathites showed a significant decrease with increasing salinity at salinity 45. Individually the right scaphognathite showed a difference once salinity reaches 60 psu.
- The scaphognathites showed a positive correlation between the percentage of time active for and the mean bpm both when left and right scaphognathites were considered individually and when combined.
- There was no relationship evident between the mean heart bpm and the mean scaphognathite bpm suggesting that cardio and ventilatory activities in *H. gammarus* are not related.
- There appears to be some degree of acclimation in terms of heart bpm after 24 hours in high salinity media. The beat rate at salinities 50 and 55 changes from being statistically lower than the bpm at salinity 35, to not different from the bpm at salinity 35 after 24 hours has elapsed.

For *Necora puber*:

- Increasing salinity has no significant effect on the mean heart rate of *N. puber.*
- Salinity does have a significant effect on the percentage of time the heart of *N. puber* beats, with the heart only beating for less than 100% of the time at salinity 55.
- Overall there was a positive correlation between the mean heart bpm and the percentage of time the heart was active for, hence a higher beat frequency correlated with a higher % of time active.
- Once salinity reaches 55 psu there was a significant decrease in the total scaphognathite bpm when compared to the normal 35 psu bpm. This only occurred when apnoeic periods are excluded.
- Only the right scaphognathite of *N. puber* showed a significant difference in the percentage of time it is active for, this occurred at salinity 40.
- Salinity affects the scaphognathites, the scaphognathites showed a positive correlation between the percentage of time active for and the mean scaphognathite bpm both when considered individually and combined. Rates decreased with increasing salinity.

6.5 Discussion

The cardioventilatory activities of both the lobster *Homarus gammarus* and the velvet crab *Necora puber* were found to be salinity-dependent within the range of salinity 35 to 60. This represents potential qualitative and quantitative ways with which to evaluate the impact of salinity challenges on species that present few, or no, overt (visible to the eye) behavioural responses to such environmental events.

Although the obvious behavioural responses of *H. gammarus* and *N. puber* to hypersaline challenges are very similar (Chapter 3), their cardioventilatory behavioural responses to the same challenges show several differences. The mean heart rate of *H. gammarus* showed an almost linear, direct relationship with test salinity ($r^2 = 0.91$) but that of *N. puber* showed an initial increase at salinity 40 and then decreased with the subsequent test salinities. However there was no statistically significant difference in the beat rate of *N. puber* over any of the salinities tested. The variable heart beat rate of *N. puber* is highlighted by the high standard errors associated with the mean values which may have effectively disguised any salinity-dependence that may have been present. In fact, intraspecific (and interspecific) variability that is associated with movements of the animals has been considered to seriously reduce the value of absolute rate data (as maximum, minimum or mean values) in comparative studies (Walters and Uglow 1981) and high variability may itself be an indicator of stress as animals do not necessarily respond in a uniform way to environmental changes.

Transfer from 100% to 15% sea water causes an increase in the heart beat rate of the euryhaline *Carcinus maenas* (Hume and Berlind 1976), suggesting that the degree of euryhalinity/stenohalinity of crustaceans could affect their response to salinity change. Both *H. gammarus* (Gilles 1973) and *N. puber* (Ingle 1980; Rainbow and Black 2001; Rainbow and Black 2005) are considered to be stenohaline, though *N. puber* can make some physiological responses to ambient salinity change (Rainbow and Black 2001). Although there is limited information available on the effects of hypersalinity on crustaceans, the effects of hyposalinity are relatively well studied. In a review of cardioventilatory responses of other crustaceans in Hume and Berlind (1976), it has been noted that the euryhaline amphipod *Gammarus duebeni* and the euryhaline shrimp *Crangon crangon* also show a increased heart bpm in response to reduced salinity. The stenohaline crab *Libinia emarginata*, however, shows a decrease in heart rate when transferred from 100% to 80% sea

water. The results for *H. gammarus* and *N. puber* are consistent with these findings in terms of departure from the normal beat rate as salinity departs from ambient, therefore indicating salinity change is a stressor to crustaceans in the hypersaline (as well as the hyposaline as shown by the authors above) direction.

Initially, both species tested showed a direct increase of mean scaphognathite beat rate with salinity and both reached a maximum mean value in the middle of the test salinity range after which ventilatory activity was negatively related with test salinity. Such a pattern of salinity-induced scaphognathite change suggests a critical salinity (psu_{cr}) exists beyond which maximum beat rates cannot be sustained and that this varies interspecifically with values of 50 and 45 respectively for *H. gammarus* and *N. puber*. The retardation of the mean scaphognathite beat rate was due principally to the increased occurrence and duration of inactive periods that were not compensated for by any increases in beat rate. Cessation of scaphognathite behaviour is seen in other studies of the responses of crustaceans to a variety of environmental stressor challenges (Uglow 1973; Cumberlidge 1986; Paterson and Thorne 1995).

Cardiac or ventilatory output is the result of a combination of stroke or beat rate, stroke volume and stroke frequency. Due to technical limitations the present data set does not include information on possible changes to the stroke efficiency of the heart or scaphognathites and so precludes information on outputs. However, a direct relationship between salinity and cardiac stroke volume has been shown to occur in *Callinectes sapidus* but, in dilute media, this was more than compensated for with an increased cardiac output caused by an increase in beat rate (McGaw and Reiber 1998). What seems clear is the positive relationship between cardioventilatory activities and metabolic rate with heart rate being shown to be a reliable indicator of oxygen consumption in *Carcinus maenas* (Rovero et al. 2000) and a general lowering of heart rate with quiescence in a variety of marine decapod species (Walters and Uglow 1981). Figure 6.12 suggested that in *N. puber,* the right scaphognathite was consistently less active than the right. Once cause of this could be the way the crab was oriented within the experimental tank. If the right side of the crab was consistently closer the wall of the tank, it is possible that this scaphognathite may be used less. However light conditions were carefully controlled so as not to induce any phototaxic response that could prompt such orientation and during the trial there appeared to be no consistent favouring of any particular location in the tank (*pers obs*).

When ventilation ceases, the effect will be to retain a reservoir of water in the branchial chambers (Curtis et al. 2007). The absence of ventilation means that the salinity of these reservoirs will remain relatively stable and independent of any deterioration of external conditions. At the same time, the accompanying general lowering of metabolism will result in a decreased efflux of ammonia and carbon dioxide into, and a decreased influx of oxygen out of these reservoirs. The sites of salinity detection in *Homarus americanus* (the American lobster) are believed to be located within the branchial chambers (Dufort et al. 2001). When branchial chamber salinity was reduced but external salinity maintained constant, Dufort et al (2001) found a rapid change in heart rate in *H. americanus*. During salinity change *Cancer gracilis* were able to maintain the salinity of water within the branchial chambers at a level that was about 30% higher than that of the surrounding medium (Curtis et al. 2007). When stressed the ventilation of *C. maenas* becomes independent of the oxygen content of the water and the crabs become oxygen conformers (Jouve-Duhamel and Truchot 1985). This behaviour of isolating the branchial chambers may constitute a short term adaptation that would have a distinct survival value to the species concerned as it would allow them to prolong their tolerance when under adverse conditions.

Presumably, the duration of this tactic will also be metabolic-rate dependent and thus temperature dependent. Consequently, hypersalinity tolerance data are more informative if accompanying temperature data are included with them. Worden et al (2006) found that the contraction amplitude of isolated *H. gammarus* hearts decreased by more than 60% over the temperature range 2°C to 22°C and was accompanied by a decreased stroke volume but an increase in beat frequency. This suggests that the animals are able to manipulate their cardiac beat behaviour to maintain cardiac output under increasingly stressful temperature conditions.

Crustacean cardioventilatory beat frequency has been shown to display considerable interspecific and intraspecific variability e.g. Walters & Uglow (1977a), Cumberlidge & Uglow (1981). *C. maenas* shows some decline in scaphognathite beat rate with decreasing salinity, but this is again variable between different individuals (Hume and Berlind 1976). A decreased scaphognathite beat rate may be a mechanism for limiting the exchange of materials across the gills and this could be an important adaptation in hypersaline media where the external concentration of ions is far greater than what the tissues of the animals are used to experiencing.

At any one set of environmental conditions, individual animals may show a wide range of beat behaviours over their normal activity range. This range may be wider in species that may spend large periods inactive (e.g. when buried in substratum) but with bursts of activity, compared with animals that spend most of their time active.

When taking into account periods of organ inactivity, there was a significant correlation between the mean bpm and the percentage of time the heart was active ($p < 0.05$ in all cases) for both *H. gammarus* and *N. puber*. There were also significant correlations between the bpm of the scaphognathites (both left, right and combined total mean) and the percentage of time the scaphognathites were active. As the percentage activity and/or bpm decreased with increasing salinity, these correlations indicate that hypersalinity is having an effect on the crustaceans tested, even though they may not be showing any outward signs of distress. When the cardioregulatory nerves of *H. americanus* were severed, the lobsters showed no cardiac response to salinity reductions (Dufort et al. 2001), indicating that there is a cardiac response in lobsters to changing salinity.

Arrhythmic scaphognathite beats have been discovered in the species in this study and occur in only the stressed state in *H. gammarus* and in both the normal and stressed state in *N. puber*. The heart of *N. puber* shows constant activity (continuous beating with no periods of inactivity) regardless of the salinity, but *H. gammarus* shows arrhythmic periods as salinity increases. However, though the organs may be active for 100% of the time, the actual bpm may be lowered by salinity. In contrast, in *C. maenas* the resting heart beat rate is naturally arrhythmic and ranges from 20 – 60 bpm with the stressed rate ranging between 80 – 120 bpm (Cumberlidge and Uglow 1977a; Rovero et al. 2000), although the crab may not display any observable behaviours at this time *e.g.* movement of legs or antennae (Cumberlidge and Uglow 1977a). However arrhythmia is only a common occurrence when the crab is resting (Cumberlidge and Uglow 1977a).

The cardioventilatory responses seen here are typical of those shown by crustaceans undergoing hypoxic stress (e.g. lowered ventilation rate, reduced heart rate etc) as they attempt to conserve oxygen. When taking into account these cardioventilatory responses in addition to the raised haemolymph lactate levels and the acidification of the haemolymph discovered in chapter 5, it appears that a similar hypersalinity-induced hypoxic effect is likely happening here. In response to increased environmental salinity the animals attempt to reduce the flux of ions into the body by

reducing the bpm of the cardioventilatory organs, as a by-product reducing the opportunity for an O_2—CO_2 exchange across the gills. *Cancer pagurus* has been shown to become bradycardic (reduction in heart rate) when exposed to hypoxic conditions (Bradford and Taylor 1982). In *C. maenas*, scaphognathite beat frequency has a direct linear relationship with absolute ventilation volume (Cumberlidge and Uglow 1977b). If it is assumed that the amplitude of scaphognathite beats is relatively constant in these crustaceans [as shown for *C. pagurus* (Pilkington and Simmers 1973) and *C. maenas* (Young 1975)], then the force required to move water in and out of the branchial chambers must be variable, requiring variable amounts of energy. If beat frequency drops far enough, ventilation may become inadequate to cope with the oxygen demands of the animals when under hypersaline stress, even when in an O_2 saturated environment.

Decreases in heart rate and/or increased cardiac dysfunction are thought to be related to hypoxia in a number of crustacean species *e.g.* the decapod *Callinectes danae* (Rantin et al. 1996) and the Thalassinid shrimp *Trypaea australiensis* (Paterson and Thorne 1995). Many studies have been undertaken on the response of decapod crustaceans to hyposalinity and the overall result is a notable rise in heart rate (tachycardia) (Hume and Berlind 1976; Cumberlidge and Uglow 1977a; Spaargaren 1982; McGaw and McMahon 1996; McGaw and Reiber 1998; Dufort et al. 2001). It is thought that these changes may be indicative of an increased energy requirement for active ion uptake (Taylor 1977; Jury et al. 1994a) that may be brought about by increased activity levels. In the current study, when under hypersaline challenge, activity levels are decreased and due to higher concentrations of ions exteriorly, less energy should be needed for active uptake. This may explain why both heart and scaphognathite beat frequencies are reduced once salinity increases from the normal of 35. However is also possible that the animals are quiescent due to putting more energy into keeping high concentrations of ions from entering into the body's tissues and this response is aided by seriously retarded cardioventilatory organ activity *e.g.* Curtis (2007), Depledge (1984a).

These readings do not take into account the beat strength and therefore whilst the beats may be regular, the strength of the beat may be very weak. Therefore even if beats are occurring they may not be bringing sufficient water (and hence oxygen) into the gill chamber. Also the decrease in beat activity, and any concurrent decrease in amplitude, may indicate the crabs are trying to avoid taking the hypersaline water into their bodies.

The results given here also do not account for any mortalities that occurred as the experiment progressed, hence the data used in all statistical calculations came from surviving crabs and lobsters. These therefore may have a good level of organ activity but obviously dead specimens had no heart or scaphognathite activity whatsoever. *N. puber* in particular experienced a decline in physical state when under hypersaline challenge consistent with what has been observed in previous chapters. So whilst it may appear that the crabs can cope well with the higher salinities, this refers to only the fittest individuals. Mortalities appeared at the salinities discovered in chapters 4 and 5 and were not included in any calculations of organ beat rate or activity so as to not bias results.

6.6 Conclusions

Null hypotheses

1. hypersalinity does not cause any change to heart beat behaviour in the crustacean species tested; **REJECTED**. Hypersalinity caused a significant decrease in heart rate in *Homarus gammarus* but not in *Necora puber*.
2. hypersalinity does not cause any change to scaphognathite beat behaviour in the crustacean species tested; **REJECTED**. Hypersalinity caused a significant decrease in scaphognathite rate in both *H. gammarus* and *N. puber*.
3. the crustaceans tested cannot distinguish between normal and hypersalinity; **REJECTED**. The significant decrease in cardioventilatory behaviour shows that both species can distinguish when salinity deviates from normal.

It is assumed that the movement away from hypersalinity discovered in Chapter 3 at salinity 50 for both *Homarus gammarus* and *Necora puber* is the animals' main escape response from unfavourable conditions. The concealed responses discovered here mainly happen at salinities higher than those that prompt preference behaviour, being closer to those that prompt the salinity induced changes in blood chemistry seen in Chapter 5 (*e.g.* 55 in *N. puber* and from salinity 45 upwards in *H. gammarus*) and it may be that change in beat behaviour effects the change in blood chemistry and vice versa. The changes in cardioventilatory behaviour when trapped in unavoidable hypersaline conditions involve a reduction in the ventilation of the branchial chamber via a reduction in scaphognathite beat rate in both *H. gammarus* and *N. puber*. A critical salinity exists beyond which maximum scaphognathite beat rates cannot be sustained and that this varies inter-specifically with values of 50 and 45 respectively for *H. gammarus* and *N. puber* which is consistent with the onset of the negative halotaxis. The heart rate of *H. gammarus* is also significantly reduced with increasing salinity. When unable to avoid hypersaline conditions, the overall effect of this is likely to be to maintain a reservoir of favourable salinity inside the body, however this is expected to be only a short term solution and should the species tested be forced to endure hypersaline conditions above those that prompt the behavioural tolerances of Chapter 3, their long term survivability is likely to suffer. The important result is that salinity caused a deviation away from normal organ activity in both *H. gammarus* and *N. puber* and so it can be concluded that there was indeed an effect which could potentially lead to the lowered fitness of exposed animals.

Chapter 7

General Discussion and Conclusions

7.1 General Discussion

The decapod crustacean species tested had a detection threshold to ambient hypersalinity beyond which avoidance behaviour was induced. There was also an interspecific variability of this hypersalinity-induced behavioural threshold which ranged from 40 psu for *Carcinus maenas* to 55 psu for *Pagurus bernhardus*. Intermediate values of 45 psu were shown by *Homarus gammarus* and 50 psu for *Cancer pagurus* and *Necora puber*. The presence of a shelter in a hypersaline medium did not influence avoidance of hypersalinity by *H. gammarus* or *C. pagurus* suggesting that the detrimental effects of hypersalinity override the risks associated with lack of cover and likelihood of predation. *P. bernhardus* showed the highest test salinity to elicit a preference response and this high value may be related partly to this species retreating into its gastropod shell and thereby minimising direct contact with the outside environment, possibly by retaining a reservoir of seawater in the shell. *P. bernhardus* did not recover as soon as the salinity became favourable again such as better-adapted species, instead they had to spend a long time in normal salinity conditions before becoming active again, suggesting the body tissues were still suffering osmotic stress. The findings of Davenport (1985) agree with this.

Study here has also suggested that the encroachment of hypersaline waters over existing fishing grounds above the threshold values for these species may displace a population from such areas with consequent impacts on the local fisheries. In the wild, *H. gammarus* appear to undertake a limited range of movements alongshore or in/offshore. In a mark and recapture study by Smith *et al* (2001) over periods of up to 862 days 95% of recaptured lobsters had moved < 3.8 km from first release site, helping to explain how stable fisheries exist in several areas around the UK. The literature suggests that under normal conditions stable lobster fisheries exist, and the current study indicating that under hypersaline conditions there is a threshold salinity that prompts a preference for normal (35 psu) salinity, it seems likely that populations around a hypersaline diffuser would vacate the area completely if the salinity is too high, resulting in a loss of the fishery.

The possible effects of hypersalinity on *H. gammarus, C. pagurus* and *N. puber* indicate hypersalinity-induced quantitative changes to some of the haemolymph constituents. There was clear interspecific variability, and some evidence of intraspecific variability, of the threshold salinity at which the responses were statistically significant. *H. gammarus* was the least tolerant species with a salinity threshold of 45 for intermoult animals but as low as 40 for post-moult specimens. *C. pagurus* was the most tolerant species and showed a strong regulatory ability with salinities of 55 and above needed to induce significant quantitative changes to the haemolymph composition. *N. puber* showed the most marked changes with significant increases in all blood parameters tested for at 50 psu including haemolymph glucose and lactate levels. Variability in response may itself be an indicator of stress as animals do not always respond in a predictable and consistent way to environmental changes. Therefore the general order of ability to cope with hypersalinity is *C. pagurus* > *N. puber* > *H. gammarus*.

The hyperglycaemic response and the significant increase in haemolymph lactate levels are typical responses of crustaceans under hypoxia (Bridges and Brand 1980; Fincham and Rainbow 1988; Zou et al. 1996; Sneddon et al. 1998; McMahon 2001). Here it is taken as evidence that an internal hypoxia occurs at salinities above the threshold value, even though the external hypersaline medium remained normoxic. It was conjectured here that this apparently anomalous situation occurred because of a shutdown of cardio-ventilatory activities as a response of the animals to reduce any changes to their osmotic equilibrium at the high salinities which would be likely to induce an exosmosis (the passage of a fluid through a semipermeable membrane toward a solution of lower concentration). This hypothesis was accepted after a series of studies on the effects of hypersalinity on the cardioventilatory activities on *N. puber* and *H. gammarus* which showed a significant reduction in cardioventilation.

The principal quantitative response of hypersalinity on cardioventilatory behaviour were bradycardia and decreased ventilation in *H. gammarus*, at salinities of 50 and 45 respectively, whereas *N. puber* showed a significant decrease in mean scaphognathite beat rate at salinity of 55 but the mean heart rate was not altered significantly in any of the test media used. Decreased cardioventilatory behaviour can, as described in chapter 6, lead to insufficient oxygenation of the body and so an internal hypoxia in otherwise fully oxygenated media.

The findings here show a group of behavioural and physical responses of a hypersaline challenge to a number of crustacean species which are fished commercially in an area of the North Sea where brine discharges occur. It is reasonable to assume that the ability to detect and respond defensively to adverse environmental conditions has considerable survival value implications for any species. In these studies, despite interspecific differences in detail, it is clear that the primary response of each species is a preferential movement away from the source of the hypersaline challenge. These responses were elicited at salinities slightly lower than the non-visible regulatory responses of haemolymph composition adjustments and altered cardio-ventilatory activities. Presumably, a simple, rapid, primary response, such as escape, is an effective one in many situations and will avert the need for more long-term defence against an environmental stressors. Only if the stressor persists and/or intensifies will further resistance measures such as the physiological adjustments found in the present studies be called upon. If the salinity challenge does persist, the animals may either adapt to the changed conditions and survive indefinitely or they will temporarily survive in a situation that will be ultimately lethal. Either way, the responses to hypersalinity shown here have demonstrable survival value.

The types of response observed were much the same for each species but interspecific and some indications of intraspecific differences did occur and it is of interest to examine these more closely in terms of their relationship with the preferred habitat of these species. *Carcinus maenas,* which has a littoral/shallow sublittoral distribution, had the lowest threshold level for behavioural avoidance of hypersalinity which, at a salinity of 40, was only 5 salinity units above the value of normal seawater. Such sensitivity to a changed intensity of an environmental variable would be an advantage to a species which, as an adult, occurs in the intertidal, a habitat recognised for its environmental instability. *C. maenas* is known to be a hyper/hypo-osmoregulating species (Siebers et al. 1982; Siebers et al. 1985). Although as shown here it is able to detect and respond to hypersalinity, it does if needed, have a wide range of tolerance and can cope well within the salinity range of 17 – 41 psu (Thomas et al. 1981; Ameyaw-Akumfi and Naylor 1987). The other species studied are rarely found intertidally and probably experience far fewer, and far less sudden changes in the intensity of environmental variables such as those experienced daily by littoral species. This may be why they have a higher behavioural tolerance for hypersalinity. As they have little need to detect changes that are unlikely to occur in their environment, this ability is not required for their day to day existence in the way that is needed by an intertidal species such as *C.*

maenas which can get trapped in tidal pools and similar and hence be exposed to dilution effects through rainwater and surface runoff and concentration effects through evaporation.

All of the species tested became inactive at the higher salinity levels. Such induced quiescence may be attributed to a number of factors including the effects of external magnesium levels. Although all species tested showed some regulation of haemolymph magnesium there was still a direct relationship found between the external and internal magnesium levels. *H. gammarus* and *C. pagurus* showed strong regulation and maintained their haemolymph magnesium levels at *ca*. 10-15% of their hypersaline external medium but *N. puber* showed very weak regulation of magnesium with haemolymph levels remaining at ≈95% of the external values tested. These results compare with those of Walters & Uglow (1981) who showed that, in normal seawater, *H. gammarus*, *C. maenas* and *Nephrops norvegicus* regulated their internal magnesium levels at 20-30% of the external medium values.

Magnesium is known to act as a neurotransmitter and Mg^{2+} is known to inhibit neuromuscular activity in crustaceans (Lang et al. 1979; Walters and Uglow 1981). Excess Mg^{2+} has been shown to have a narcotising effect on a number of marine invertebrates (Pantin 1931) and magnesium chloride and magnesium sulphate are often used in laboratory experiments for narcotising and anaesthetising aquatic invertebrates (Moore 1989; Spooner et al. 1991; Culloty and Mulcahy 1992; Wilson 2005). Generally, species that maintain low blood Mg^{2+} values were more active than those maintaining high blood Mg^{2+} levels (Robertson 1949) which accords with the narcotic properties of Mg. Increases in Mg as salinity increases coincide with the inability to move in the species of this study. In the intermoult *H. gammarus* acute exposure trial there was a significant correlation between Mg concentration in the haemolymph and activity ($p = 0.001$, $n = 32$, $r = 0.556$) with increasing Mg levels correlating with decreasing activity levels. There is a significant linear regression between these two variables: ($p = 0.001$, $F = 13.436$, $y = 0.314x + 8.310$). No significant correlation or liner relationship was found in late postmoult adult lobsters, though this is likely to be due to the small sample size and the fact that none survived past a salinity of 40. A negative linear relationship between the relative haemolymph magnesium values and the relative heart activity data has been shown for a number of species (Walters and Uglow 1981) and hence supports the findings of this thesis with regards to the lobsters. However no significant correlation or linear relationship between Mg and activity was found for acutely exposed *C. pagurus. C. pagurus* was

shown here to be able to regulate haemolymph Mg such that there was no change with increasing environmental salinity, whereas *H. gammarus* was not able to regulate in this way and therefore the ability to regulate the haemolymph Mg level may explain why the lobsters showed a relationship between raised Mg and lowered activity, whereas the crabs did not. The overall general quiescence observed in all test crustaceans at the highest salinities indicates that there is some factor that is causing this behaviour. Mg may be one explanation for this, another may be that it is to avoid much transfer of high solute levels from the external media to the body or conserving energy in the face of increased energy costs associated with removing excess internal Mg against a high external Mg gradient.

As Mg inhibits neuromuscular transmission and also acts as a narcotising agent, it suggests that the high levels in hypersaline media may play a role in the significant quiescence of all three species tested under the extremes of hypersalinity and may also play a role in the decrease of cardioventilatory activity by influencing the reduction in beat rate. The heart of crustaceans is neurogenic (where heart beat originates from a small cardiac ganglion located on or in the heart) rather than myogenic (where the contraction is controlled by the heart muscle cells rather than an external source such as a cardiac ganglion nerve cluster). Therefore if magnesium acts to inhibit neuromuscular transmission it is likely that the activity of the heart would therefore be inhibited too. This hypothesis is supported by the findings of Walters and Uglow (1981) who showed a negative linear relationship between the relative haemolymph magnesium values (Mg^{2+}) and the relative heart activity. In *Homarus americanus* neuromuscular transmission was severely depressed by 40 mM Mg^{2+} but this was not the case in the spider crab *Hyas areneus.* It would appear that the spider crab is physiologically adapted to function at relatively high blood Mg^{2+} concentrations where the lobster is not (Lang et al. 1979).

Haemocyanin (HCY) is the respiratory pigment in the haemolymph of crustaceans, in the same way that haemoglobin is the respiratory pigment in mammals. Whereas the metal in haemoglobin is iron, in haemocyanin the molecule instead contains copper. There is a direct relationship between HCY level in the blood and the activity of lobsters. Those with little or no HCY in the blood can survive for several months, but are not capable of appreciable activity and it is doubtful whether they can complete their normal life cycle (Spoek 1974). There is also a potential relationship between copper, HCY and hypersalinity. Bamber and Depledge (1997) found a direct relationship between copper levels and heart rate in *Carcinus maenas.* They noted that when Cu was

increased, the heart rate of *C. maenas* experienced a matching increase with the elevated rates indicating respiratory stress in both resting crabs and crabs subjected to physical stress, suggesting an impairment to one or more of the processes associated with normal respiratory functioning. Copper has been shown to induce a tissue hypoxia in crabs over several days (Nonotte et al. 1993). Tissue hypoxia is a condition which has the potential to influence respiratory and cardiac physiology.

Copper is the metal in HCY and it appears from an additional analysis of present study data here (using data presented in Chapter 5), that that environmental Cu has little effect on the HCY level in the study species. Under chronic hypersaline exposure intermoult lobsters showed no significant correlation or linear regression between Cu and HCY level. The same was true for late-postmoult lobsters under acute hypersaline exposure and velvet crabs under chronic hypersaline exposure. Acutely exposed intermoult lobsters however showed a significant positive correlation between HCY and Cu ($p = 0.005$, $r = 0.481$, $n = 33$) and a weak yet significant positive linear regression ($p = 0.005$, $F = 9.323$, $y = 181.892x +1 2.701$, $r^2 = 0.231$). There was also a positive correlation between copper and HCY in acutely exposed *C. pagurus* ($p = 0.002$, $r = 0.447$, $n = 45$), and a positive linear regression which was weak but still significant ($p = 0.002$, $f = 10.759$, $y = 2.369x + 1.023$, $r^2 = 0.20$). The same was true for acutely exposed velvet crabs with a positive correlation between Cu and HCY ($p = 0.003$, $r = 0.747$, $n = 13$), and a positive linear regression ($p = 0.003$, $f = 13.899$, $y = 0.774x + 5.700$, $r^2 = 0.558$). Therefore, when they experienced an acute hypersaline exposure, the test species revealed a weak relationship between the ambient copper and their circulating haemocyanin levels. Under chronic exposure long enough for the animals to become acclimated they displayed no relationship between these two variables. Allowing time for animals to acclimate to hypersaline conditions suggests a reduced chance of sublethal impacts occurring which may otherwise be detrimental to the health and survival of the species concerned.

In *C. pagurus*, the HCY has a high affinity for oxygen (p_{50} = 5-10 torr) and shows a large positive Bohr shift, however under normal conditions this affinity is largely irrelevant to the crab with over 91% of the oxygen supplied to the tissues being carried in solution rather than by the haemocyanin (Bradford and Taylor 1982). Bottoms (1977) worked on *C. pagurus* in a Scottish sea loch and found the crabs to be largely inactive with no measurable haemolymph copper indicating that basal metabolic rate oxygen requirements are very low. Therefore low HCY may play a role in low activity levels in decapod crustaceans. During prolonged food deprivation, the brown shrimp *Crangon*

crangon, catabolised its HCY and the liberated copper was removed from the blood and stored in vesicles in the hepatopancreas (Djangmah 1970). It was found that these vesicles had the capacity to store the complete HCY copper content should all the pigment be catabolised. In *Crangon crangon*, 60-93% of the haemolymph protein comes from haemocyanin (Djangmah 1970), and Uglow (1969) found that this could attain 100% in *Carcinus maenas*. Consequently, the significant changes in protein seen in the experiments here may be due to the breakdown in HCY under hypersaline pressure.

Both *H. gammarus* and *N. puber* showed changed scaphognathite beat behaviour in hypersaline media. This resulted in a reduced beat rate and qualitatively, in a change from constant regular beating to long periods of inactivity interspersed with short periods of beating. At the highest salinities tested, the mean beat rate dropped as low as 20 beats per minute in a number of cases (from 90 bpm) and this raises questions regarding the possible functional significance of altered beat behaviour. Regular beating ensures constant flushing of the respiratory surfaces to promote gas and ion fluxes but short bursts of beating punctuating lengthy periods of non-beating are unlikely to overcome the inertia of the water, although they may disrupt the development of a static, hypoxic layer forming at the gill surface. It is probable that this would ensure some measure of flux of respiratory gases but it is unlikely to be sufficient to prevent an internal hypoxia developing.

Lobster species such as *H. gammarus* can compensate for hypoxia by improving gill oxygen transfer (Butler et al. 1978). It has been suggested that under hypoxic conditions oxygen supply can be maintained by pumping blood exclusively to vital organs and by decreasing heart rate and activity (Reiber and McMahon 1998). This reduction of activity was also found in the lobster *Palinurus interruptus* when nearing hypoxia (Ocampo et al. 2003). In fact *P. interruptus* appeared dead with no observable movements which is the same response seen in the three species tested here. The possibility of an internal hypoxia caused by a shutdown of cardioventilatory activity has already been discussed and complete quiescence in extreme hypersaline conditions (salinities 50 and above) was common for all species. Ocampo et al (2003) suggested that this reduction in activity may be a compensatory mechanism to supply the energy required to survive stressful conditions such as hypoxia. This response is likely to apply to other environmental challenges such as hypersalinity and therefore could be used to maintain homeostasis at least in the short term.

Another possible cause of the inactivity is a build up of acid metabolites in the haemolymph. Lactic acid is produced in the body as a by-product of carbohydrate metabolism. Blood lactate arises primarily from muscle cells and reflects both production and metabolism. Lactic acidosis is caused by deprivation of oxygen and can result in weakness, fatigue, stupor and coma (Biotech Undated). The effects of the significant lactate acid build up (over a twofold increase in haemolymph level once salinity reached 50 and 55 in *H. gammarus* and *N. puber* respectively) seen in this study could explain why the crustaceans went into an apparent stupor/coma at high salinities. Hypoxia shows in the blood as raised levels of lactate and treating the hypoxia will cause a reduction in lactate levels. In the study crustaceans the increase in lactate was probably caused by an internal hypoxia (as lactate is produce by anaerobic respiration and the lack of high activity means exercise was not the cause of anaerobic respiration), therefore lactate is likely to be a factor in, but not the overall cause of the catatonic state induced in the test species in hypersaline environments.

The breakdown of regular and sufficient cardioventilatory activity seems to be the key factor in contributing to the lowered fitness and survival potential of the test species during hypersaline challenges. This change in beat behaviour effectively stops respiratory gas and ammonia fluxes and prompts a possible internal hypoxia which would induce a switch from aerobic to anaerobic metabolism (as shown by elevated pH and lactate in the haemolymph). The links between hypersalinity, physiological and behavioural changes in the test species are shown in the conceptual model given in Figure 7.1. This model shows the way behavioural and physiological responses to hypersalinity in the test species are linked and cannot be considered as separate processes, along with the potential outcomes possible if a crustacean encounters a hypersaline environment. A number of crustacean species such as *N. norvegicus* (Hagerman et al. 1990; Schmitt 1995), *Eriochier sinensis* (Zou et al. 1996) and *P. interruptus* (Ocampo et al. 2003) show responses to hypoxic conditions that match those found in this study such as elevated blood lactate and hyperglycaemia. This is again indicative of an internal hypoxia induced by the hypersalinity in otherwise normoxic conditions. Schmitt (1995) showed that *N. norvegicus* could alter the blood oxygen supply to help tolerate progressive hypoxia through the changes in lactate concentration.

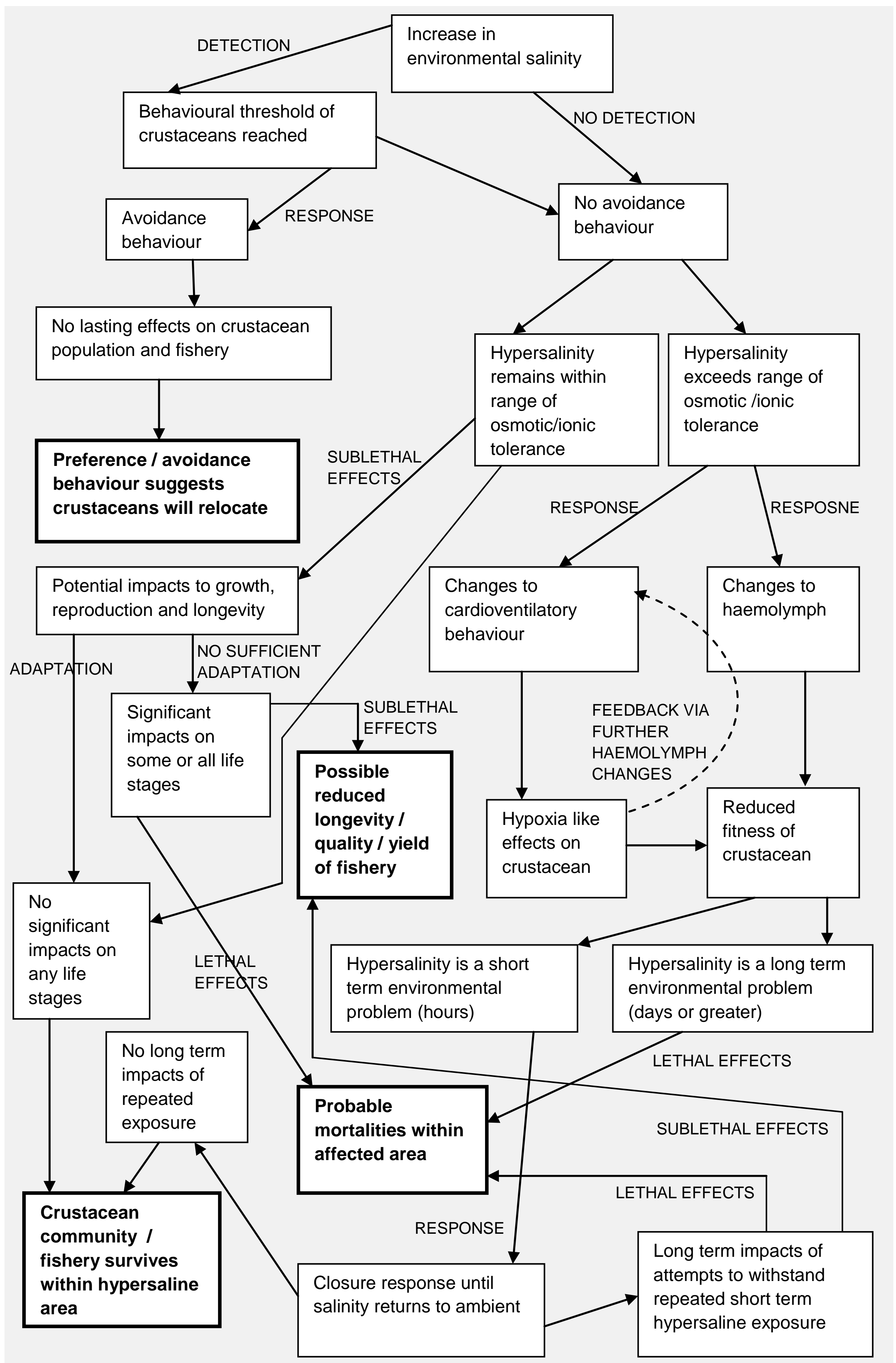


Figure 7.1 The potential effects of raised salinity on adult decapod crustaceans within the area affected by hypersalinity. Showing links between the potential effects on cardioventilation, behaviour and haemolymph properties.

Osmoregulation and salinity tolerance in aquatic crustaceans are highly correlated (Charmantier et al. 1988; Charmantier and Charmantier-Daures 1991). The findings of this study indicate that in general, under hypersaline conditions, *H. gammarus* is a limited hypo-osmoregulator, *C. pagurus* is a relatively strong hypo-osmoregulator and *N. puber* is an osmoconformer with regards to the ionic constituents of sea water.

When under hyposaline challenge, *C. pagurus* acts as an osmoconformer as when exposed to salinities only 5 to 10% less than ambient (salinity 35) their body gains water and they die within a few hours (Péqueux 1995). The present findings suggest therefore that the crab is more tolerant of relatively high hypersalinity than slight hyposalinity, as it had the highest 96 hour LC_{50} value of all study species at salinity 55.5 which is 58 % above normal. Hypo-osmoregulation appears to be a widespread method for coping with hypersalinity; the semiterrestrial crab *Chasmagnathus granulatus* is a strong hyperosmoregulator in diluted seawater as well as hypoosmoregulating in hypersaline conditions (Charmantier et al. 2002).

Ionic regulation is defined as the maintenance in a body fluid of concentrations of ions differing from those in the passive equilibrium (Robertson 1949) and there is a strong correlation between a high osmoregulatory ability and salinity tolerance in aquatic crustaceans (Charmantier et al. 1988; Charmantier and Charmantier-Daures 1991). These studies were carried out at a temperature of 8 °C ± 1 °C, but in the North Sea where the local brine discharge occurs, temperatures can range from around 6 °C to 14 °C (winter-summer) (Cutts et al. 2004). It has been shown that environmental conditions, such as salinity change, that may be tolerable for a number of marine species at low temperatures may not be tolerable at higher temperatures (*e.g.* in the crabs, *Cancer irroratus* and *Cancer borealis* (Charmantier and Charmantier-Daures 1991) and cottid fish, *Oligocottus maculosus, Clinocottus globiceps* and *Leptocottus armatus* (Morris 1960). Mortalities of juvenile of *H. gammarus* reared at 15 °C (approx summer temperature in the North Sea), occur only in salinities <17 and >46 with isosmotic regulation in high salinities and slightly hyperosmotic regulation in low salinities (Charmantier et al. 1984). Therefore it appears probable that the physiological effects of the hypersaline discharge will be more marked during the summer months when the surrounding seas are warmer than in the winter.

Whilst it has been demonstrated here that when faced with hypersalinity the study species will preferentially move to a more favourable salinity environment, an abrupt, severe stressor of

salinities above 50 - 55 results in the onset of quiescent behaviour. This behaviour also occurs if there is no behavioural 'escape route.' Quiescence in unfavourable conditions and hence attempts to regulate the internal body chemistry either by employing iono- and osmoregulation techniques such as ion pumping, or by just shutting down all cardioventilatory activity to stop the flux of ions across the gills, are probably effective as a short-term measure rather than a means of prolonged survival. Jury et al (1994a) studied the energetic cost in *H. americanus* for its limited osmoregulation in low salinity conditions and whether it would be more energy efficient to increase active transport of ions or to employ avoidance behaviour. They found that at salinity 10, females required more energy to osmoregulate and that for both sexes the physiological stress imposed by hyposalinity played a part in determining their distribution and movements in estuarine habitats. Therefore it was preferable for the lobsters to avoid the unfavourable hyposaline conditions rather than staying and attempting to cope. The crab *Cancer magister* also shows reductions in the number of animals feeding, the amount of food consumed and the time spent feeding in salinities where it actively osmoregulates (Curtis et al. 2010).

Scope for growth and energetic repercussions of coping with the effects of stressors have been used as indicators of sublethal impacts to toxicants in the marine environment (Moriarty 1993; Forbes and Forbes 1994; Salazar and Salazar 1996). If the crustaceans of this study become inactive due to putting energy into regulation, then this energy will not be available for other biological processes or functions. Perhaps the most well known and extensive study of scope for growth is for the bivalve mollusc *Mytilus edulis* (Widdows and Johnson 1988; Widdows et al. 1995) where reduced scope for growth occurred in the presence of toxicants such as copper, diesel oil and organic contaminants. In terms of crustaceans, salinity stress has been shown to reduce scope for growth in a number of species; *Gammarus oceanius* (high salinity stress) (Normant and Lamprecht 2006), *Callinectes sapidus* (low salinity stress) (Guerin and Stickle 1992), *Callinectes similis* (low salinity stress) (Guerin and Stickle 1997), *Cherax quadricarinatus* (high salinity stress) (Meade et al. 2002), *Farfantepenaeus californiensis* (high salinity stress) (Villarreal et al. 2003) and *Cancer magister* (low salinity stress) (Curtis et al. 2010).

In terms of the management of hypersaline discharges into marine areas, the results of this study have indicated that allowing time for animals to acclimate to hypersaline conditions means a reduced chance of sublethal impacts occurring which may otherwise be detrimental to the health

and survival of the species concerned. Hypersaline discharges, if not constant in their operation should be started and stopped gradually so as to allow animals time to acclimate to increased salinity in their environment rather than an abrupt onset which could induce the acute effects seen here. However the timescale over which this should happen needs further investigation in order to make a suitable compromise between the needs of the crustaceans and the operational capacity of the hypersaline discharge. In general, when unable to move away from areas of hypersalinity (which is the primary response) or if the onset of hypersalinity is so abrupt as to induce quiescent behaviour, the crustaceans will experience marked reduction in their cardioventilatory behaviour which may not be sufficient to sustain the organisms indefinitely. These changes are represented by increased haemolymph pH and lactate levels which indicate switch to anaerobic respiration due to hypoxia. Impacts of the stress caused by hypersalinity and the inadequate respiration are shown by increased haemolymph ammonia and hyperglycaemia, and inadequate iono-regulation by increased levels of ionic components of seawater in the haemolymph (*e.g.* Na and Mg). Overall hypersalinity, when acute, or above the behavioural tolerances for each species, produces first an avoidance response, then if inescapable, significant physiological changes that lower the fitness of exposed animals which if prolonged or beyond the range of homeostasis, are ultimately lethal (Figure 7.1).

7.2 Final summary and conclusions

This study has assessed some of the behavioural and physiological effects of hypersalinity on commercially important crustacean species of the North Sea. The primary response to hypersaline challenge is a movement away from areas of hypersalinity once each species' threshold is reached. If the crustaceans cannot move (*e.g.* rapidity of salinity change is sufficiently high as to prompt quiescence) then physiological changes begin to happen (Figure 7.1). There is a breakdown in cardioventilatory behaviour which most likely leads to an internal hypoxia and anaerobic respiration as evidenced by increased haemolymph pH and lactate levels. The combined effects of hypoxia and the external hypersaline environment results in changes in properties of the haemolymph and together the altered cardioventilatory activity and haemolymph properties cause a reduction in fitness and survival potential. In terms of management of fisheries it may be advisable, not only to limit discharges to the lowest tolerance of all the commercially fished species in the area, but to also limit discharges during the months of moulting. This could be achieved by slowing down the rate of pumping to allow the brine to diffuse more easily, or to not excavate the caverns at all during the typical moult months. This practice, though beneficial for the crustaceans, may not be feasible for the industry as it would increase cavern excavation time and operating time. A cost-benefit exercise (or similar) would need to be carried out in order to assess the financial viability for the industry of operating hypersaline discharges in such a way.

Despite the potential for complete recovery of select specimens from hypersaline exposure it is emphasised that their inability to move even as a consequence of direct physical stimulus at the extremes tested raises the question of their long term survival and reproductive potential in a hypersaline environment. Hypersalinity is an increasing environmental issue and the results of this work have demonstrated that, in addition to the wealth of existing knowledge on hyposaline limitations in marine organisms, crustaceans are also limited on the hypersaline side. All of the species studied exhibited hypersaline upper preference limits and without exception were severely affected upon reaching their specific upper threshold, in addition limited survival was observed at salinities ≥ 50, with at least 50% morality under these conditions. There is much attention focused on the effects of visual contaminants to the marine environment such as the 2010 Deepwater Horizon oil spill, however the data presented herein provides compelling evidence for the need for increased monitoring of the hidden effects of contaminants on ecosystems proximal to man made sites of hypersalinity such as those generated by the gas and desalination industries.

7.3 Further study and improvements

The present studies have furthered our knowledge of the effects of hypersaline discharges on the behaviour and physiology of some commercially-important decapod crustacean species and have shown that hypersalinity significantly affects the studied species on both the behavioural and physiological level. Clearly, the findings raise other questions the answers to which were beyond the scope of the present work. It is suggested that further studies would be useful not only in terms of their addition to our knowledge of crustacean ecophysiology, but also in terms of the their relevance to the management and protection of crustacean fisheries on which the livelihoods of many are dependent directly and indirectly, as well in terms of management of brine discharges themselves. Suggested areas of further study include:

- an assessment of impact of hypersaline discharges on all the moult stages of *Homarus gammarus, Cancer pagurus* and *Necora puber* to test the possibility of moult stage-dependent variability and also moult progression. Hints of such variability occurred in the present studies with the observation that late-postmoult lobsters had markedly lower hypersalinity tolerance than intermoult ones. It is therefore hypothesised that newly moulted individuals may have an even lower tolerance for high environmental salinity due to the lack of a physical barrier (the carapace). If individuals can survive in a hypersaline environment, but cannot successfully complete or survive the moult the survival of the local populations and the continuation of their attendant fishery is questionable.

- an assessment of the impact of hypersaline discharges on the eggs and larval and juvenile stages of the species studied should be made for the same reasons as given in the suggestion above. It is unknown whether ovigerous females can carry out normal ventilation of the developing eggs attached to their abdominal limbs, and if hypersalinity means that eggs do not fertilise or hatch and/or that larval stages have delayed or halted metamorphosis, the fishery will not survive in the long term.

- an assessment of the effects of hypersalinity on the prey items of the test species. The long-term health and survival of a population of any species is dependent on an adequate food supply.

- investigation into why a number of *C. pagurus* chose a location in the multi-choice salinity preference test and then stayed there for 24 hours, regardless of the prevailing salinity. This behaviour may have implications for populations and commercial fishing operations in areas affected by brine plumes.

- the trials undertaken here were carried out at one temperature only. An improvement to this would be to repeat each trial at both colder and warmer temperatures. This would be beneficial in helping to provide a more comprehensive overview of the responses of these crustaceans to hypersalinity during different seasons.

- although time and space meant it was impossible for this study, to enhance further results it would be preferable to increase the number of specimens used in each trial. This is especially true in the case of *C. pagurus* where poor availability meant this species was unable to be used in some of the experiments.

- investigating alternative methods of nitrogen metabolism in crustaceans challenged by hypersalinity. The stability of haemolymph ammonia in some of the animals tested suggests that an alternative biochemical pathway may be being used as a detoxification strategy. This hypothesis was realised too late for this study but warrants further investigation. Analyses should be made of other principal nitrogenous metabolites, particularly urea and uric acid/urate in the haemolymph and other possible storage sites (e.g. muscles and bladder) after treatments which duplicate those described here.

- determining conclusively whether the breakdown of cardioventilatory behaviour is causing an internal hypoxia of sufficient magnitude to cause the mortalities observed. Such studies would require the monitoring gill chamber water oxygen levels and simultaneous *in situ* haemolymph oxygen levels. The magnitude of the internal hypoxia could then be assessed in terms of whether or not it was the cause of the mortalities that occur at such times.

- in terms of fisheries management it would also be advantageous to map the extent of the affected area in hectares and hence the normal catch of each species per hectare would be useful information for the discharging body and the fishermen as it would facilitate a sensible value for compensation by the discharging body should such a need arise.

The suggestions made above would be useful for industry managers in helping to site hypersaline diffusers and providing further data that would inform suitable discharge concentrations and operational strategies that would benefit both the industry and the benthic crustaceans in the affected area.

The studies herein are laboratory based and hence very controlled. Without results from field trials (where environmental conditions are naturally variable) to support these conclusions, caution should be appropriately extended by anyone wishing to extrapolate the results of these laboratory trials to impacts in the sea, especially as due to both anthropogenic and natural diffusion, the affected area is likely to be relatively small in the context of the local environment.

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