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The behavioural consequences of reduced sea water pH in decapod crustaceans

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ABSTRACT

The studies presented in this thesis were designed to investigate the effects of reduced sea water pH on the behaviour of intertidal decapod crustaceans, both within the context of the variations occurring naturally in the pH of rock pool habitats, and in relation to predicted changes to ocean pH resulting from ocean acidification and potential carbon dioxide (CO_2) leaks from carbon capture storage (CCS) sites. Recent studies on marine fish have shown behavioural disruptions as a result of increased CO_2 concentrations in sea water and reduced pH, but the effects on crustaceans are as yet unknown. The first two studies investigated the effects of reduced pH upon the olfactory behaviour of the prawn *Palaemon elegans* and the hermit crab *Pagurus bernhardus*, focussing on their responses to food odours. Short-term (five day) exposures to highly reduced pH (pH_{NBS} = 6.60, 6.80) revealed disruptions to the chemo-sensory behaviour of both species with a reduction in their 'sniffing' response, and the inability of *P. bernhardus* to locate the chemical cue. This was also accompanied by elevated haemolymph chloride ions. in In a further study *P. bernhardus* was subjected to a longer exposure (60 days) and to a range of pH levels (pH_{NBS} = 8.00, 7.90, 7.70, 7.35 and 6.80) in order to detect a threshold for the behavioural disruptions observed, and to determine if there would be any sign of acclimation over a longer period. A clear gradient in the disruptions to the chemo-sensory responses and survival rates of the hermit crabs, and disruption to a physiological marker (elevated haemolymph calcium ions), was found. Possible thresholds for disruption were also identified at levels that match predictions for ocean

acidification and leaks from proposed CO_2 CCS sites. Some of the crabs in the lower pH treatments exhibited a recovery in their responses by day 60, possibly indicating an acclimation effect. The presence of disruption to haemolymph ion concentrations in both the short and longer term hermit crab studies suggest a mechanism for behavioural disruption. In a final study the effects of reduced sea water pH on a more complex behaviour, involving decision making, was investigated. Reduced sea water pH was shown to disrupt the shell assessment and selection behaviour of *P. bernhardus* affecting its decision making processes, although not all crabs were affected in the same way. The work presented here therefore demonstrates that reduced sea water pH could have disruptive effects upon both information gathering, *via* chemo-sensory processes, and decision making in intertidal crustaceans. The mechanism responsible is unlikely to be due to changes in the odour molecule, or physical damage to receptor organs. Rather the observed disruptions could be due (a) to ionic changes, causing metabolic depression or interference with neurotransmitter function, or (b) to disruption to chemoreception *per se*. Such disturbances to key behavioural processes have implications for inter and intraspecific species interactions and population dynamics in the marine environment. Changes in pH are already experienced by intertidal animals for short periods when rock pools are emersed, but future anthropogencially-induced reductions in sea water pH are likely to cause more sustained and widespread disruptions with, as yet, unpredictable consequences. The differential responses observed between individuals in these studies may warrant further investigation as such

differences may provide the basis for selection and adaptation to projected changes in ocean pH.

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AUTHOR'S DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee.

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CHAPTER 1

Introduction

At its most simple, behaviour is defined as any observable activity by an animal in response to internal and external stimuli. Such responses may be caused by physiological and ontological factors, or by adaptive and evolutionary ones, and are the way animals interact with each other and with their environment (Krebs & Davies 1997; Alcock 2005). Behaviours evolve in order to maximise an animal's survival and fitness in response to a constantly changing world, and this plasticity allows certain traits to be maintained by natural selection (Miner et al. 2005). However, behavioural traits are subject to both physiological and environmental constraints that can render them immutable; and environmental changes can occur too rapidly to allow time for evolutionary processes to take place, leading to population extinctions (Krebs & Davies 1997; Schmidt et al. 2010). In this thesis I explore the effects of changes in sea water pH on certain aspects of the behaviour of intertidal decapod crustaceans, both within the context of the environmental variability that already exists in their natural rock pool habitat, and in relation to the reductions that are predicted to take place in ocean pH as a result of anthropoengic activity. I concentrate in particular on the effects on information gathering activities, and responses to chemical stimuli (chemoreception); I also look at how such environmental changes affect decision making processes.

Information gathering - olfaction and chemoreception

Animals continually gather and assess information about their environment *via* their visual, auditory, tactile, gustatory and olfactory senses in order to

make decisions such as where to look for food (Pyke et al. 1977), how to choose a potential mate (Jennions & Petrie 1997), which habitat to settle in (Johnson & Strathmann 1989), whether to hide to avoid predators (Lima & Dill 1990), or whether to enter into a fight (Arnott & Elwood 2009).

Chemoreception is the process by which organisms perceive chemical stimuli in their environment *via* their olfactory and gustatory organs (Bradbury & Vehrencamp 1998). The olfactory behaviour of animals has been widely studied (Cheal 1975; Hoover 2010) and olfaction is an important sensation, as it allows information to be acquired at distance from its source, giving animals time to make decisions about how to respond to such information: whether to avoid the source, in the case of predators or aggressive conspecifics; or seek it out, in the case of food or the proximity of a potential mate, and therefore has significant impacts upon survival and fitness (Bradbury & Vehrencamp 1998). There are two distinct categories of chemical cues (Lima & Dill 1990) One group, named pheromones, are emitted intentionally, as signals, for the purpose of sexual communication between conspecifics, or in agonistic interactions, for example, the female shore crab, *Carcinus maenas*, emits a chemical when it is about to moult indicating its mating receptiveness to male crabs (Hardege et al. 2002); and crayfish release urine during fights conveying information about the signaller's fighting ability (Breitaupt & Eger 2002). Another group of chemicals, are released into the environment, not as intentional signals, but as an unavoidable product of metabolic functions or tissue damage, and are used to identify potential prey, predators, or injured conspecifics that might signal danger (Lima & Dill 1990; Atema 1995; Chivers & Smith 1998). Examples of sensory organs that detect

such chemicals are the noses of vertebrates, and the antennae of arthropods, the surfaces (internal in the case of noses, external in the case of antennae) of which are covered with olfactory receptor cells, although these cells may also be present on other parts of the body. Detection occurs when odour molecules are temporarily bound to specific protein-receptor molecules in the receptor cell. The receptor protein changes, either in shape or chemical composition and depolarises; this depolarisation triggers a nerve impulse carrying information to the brain (Bradbury & Vehrencamp 1998). Animals may also sense and respond to changes in environmental conditions, such as reduced oxygen (hypoxia) or increased carbon dioxide (hypercapnia), and take evasive action to move to a more benign location (Taylor & Spicer 1988; Eriksson & Baden 1997; Taylor & Eggleston 2000; Bernatis et al. 2007); although such sensing is usually a reaction to internal, physiological signals that elicit a response, and not generally mediated by olfaction.

With such a wealth of information available to animals in the environment, they must have some strategy as to which information to prioritise and how much to gather at any one time, as it is an energetically costly activity. So, although information is important, as it forms the basis for decisions that influence animals' lives, the effort expended in gathering it may be traded off against other essential activities or processes (Dill 1987; Schmidt et al. 2010).

Information gathering and decision making in a changing environment

The decisions animals make, based on the information they gather, can have far reaching consequences for both their fitness and survival (Dill 1987; Blumstein & Bouskila 1996; Schmidt et al. 2010). The environment from which animals gather this information may be constant or variable. Some variability constitutes predictable change, such as natural cycles, although these may still suspend or interfere with information gathering activities. For example, changes in light levels can affect foraging, predator-prey interactions and assortative mating in cyprinid fish (Cerri 1983). Environmental variability is common and animals have therefore evolved strategies to cope with such predictable unpredictability (Wells 2007). Information gathering, although energetically costly, can be used to reduce ecological uncertainty; and behavioural plasticity, an evolutionary strategy that enables behaviour to be adjusted in the presence of environmental variability, allows animals to respond to change on the basis of the information that they gather (Dall et al. 2005; Donaldson-Matasci et al. 2008). However, uncertainty can never be entirely eliminated and both stochastic natural events and anthropogenic effects can disrupt the environment and pose problems for animals. Information must be both acquired and processed successfully in order to be useful, and a growing number of studies have shown how changes in the environment, particularly anthropogenically induced ones, can disrupt one or both of these processes (Scott & Sloman 2004; Zala & Penn 2004; Tuomainen & Candolin 2010). Such changes can interfere with the information gathering process itself, by compromising

sensory systems, by altering the stimulus, or by affecting an animal's physiology, thereby disrupting the processing of information and decision making due to neurological disturbance (Dias-Ferreira et al. 2009; Graham et al. 2010; Domenici et al. 2012; Nilsson et al. 2012), and this has been termed 'info-disruption' (Lürling & Scheffer 2007). Environmental changes can also induce metabolic stress, resulting in certain energy-demanding behaviours being allocated a lower priority in favour of maintaining homeostasis (Bernatis et al. 2007). The alternative for an animal when it is exposed to environmental stress is to use what energy it has to take evasive action, where possible, so that the cost of expending that energy is outweighed by the advantage of moving to a more benign location (Taylor & Spicer 1988; Bernatis et al. 2007). Variability and disruption can make information gathering a less successful and reliable activity, and therefore decision-making becomes a more complex process for animals, where the consequences of their choices cannot be known *a priori*, introducing the possibility of maladaptive, as well as adaptive, responses (Dall et al. 2005; Miner et al. 2005; Schmidt et al. 2010). Many decisions, made within the context of environmental uncertainty and disruption, can involve animals in costly trade-offs that may simultaneously afford fitness benefits and incur fitness penalties (Domenici et al. 2007).

Chemoreception in an aquatic environment – the effect of changes in pH

In aquatic environments, where light levels can vary, animals' olfactory senses are particularly important. Aquatic animals are bathed in a cocktail of ambient chemicals, and this has led to the evolution of receptor specificity

and diversity in order to distinguish significant chemicals from background noise (Lima & Dill 1990; Atema 1995). Anthropogenic pollutants (such as heavy metals, pesticides and surfactants), have been shown to affect chemosensory processes (Lürling & Scheffer 2007; Leduc et al. 2004), for example, elevated levels of humic acid, due to Industrial inputs into freshwater streams, result in female swordtail fish losing their preference for conspecific male chemical cues which could promote hybridisation and eventually lead to species extinction (Fisher et al. 2006).

A growing number of studies have demonstrated the adverse effects of changes in pH on the chemo-responsiveness of freshwater organisms. Changes in the hydrogen ion concentration (pH) of water can occur naturally, but are increasingly the result of human activities, and their effects, although often sub-lethal, can be subtle and far-reaching. The reduction in the pH (acidification) of lakes and streams owing to the deposition of sulphur and nitrogen has been a well-studied phenomenon (Schindler 1988), and has been shown to disrupt the olfactory senses of crayfish (Allison et al. 1992), and freshwater fish (Hara 1976; Tembo 2009). Elevated water pH, resulting from agricultural run-off and subsequent eutrophication, has also been shown to adversely affect the perception of predator cues by freshwater snails (Turner & Chislock 2010). In addition to threats to freshwater pH, the future stability of ocean pH is now at risk from both chronic and acute sources, stemming from human carbon emissions. A relatively rapid drop in sea water pH, owing to increased absorption of atmospheric carbon dioxide (CO_2), is a predicted consequence of ocean acidification (OA) where pH levels are projected to decrease by 0.3 points by 2100, and by 0.7 points by 2300 in

coastal waters (Caldeira & Wickett 2003; Raven et al. 2005). Extreme and localised high CO_2/low pH events could also occur as a result of leaks from seabed CO_2 carbon capture storage (CCS) sites, proposed as a mitigating strategy for CO_2 emissions (Hawkins 2004). Leakage scenarios have been modelled that predict a potential drop in ambient sea water pH of up to 0.9 points (Blackford et al. 2009). Periodic upwelling of CO_2-enriched sea water has also been observed as a regular phenomenon in some coastal regions (Feely et al. 2008; Thomsen et al. 2010) with drops in pH of up to 0.7 points, matching those of OA predictions.

The carbonate system, ocean acidification and implications for marine organisms

The pH of a solution is a measure of its acidity/alkalinity and refers to the algorithmic concentration of hydrogen ions (H+) in that solution. Pure water has a pH of 7, acid solutions have a pH of less than 7, and alkaline solutions a pH greater than 7. When CO_2 dissolves in sea water various ionic and non-ionic chemical species are formed. Carbon dioxide becomes hydrated to form carbonic acid (H_2CO_3); carbonic acid then dissociates readily into bicarbonate (HCO_3^-) and carbonate ions (CO_3^{2-}), and hydrogen ions (H^+) are produced. All these reactions are reversible and CO_2-sea water equilibria are dependent upon temperature, pressure and existing ocean alkalinity (Fig. 1.1.).

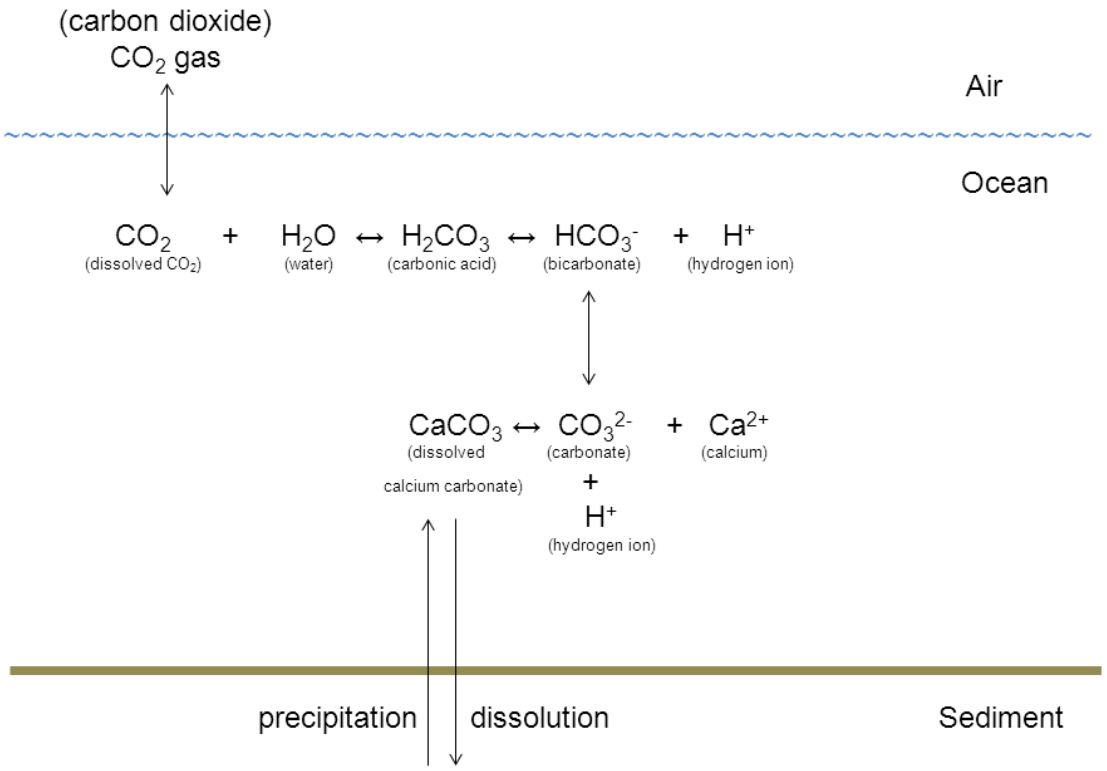

Fig 1.1. The Ocean carbonate system (after Chester 2000) showing the reactions that take place when carbon dioxide dissolves in sea water.

Transitions between all these species tend towards a constant pH because carbonate in sea water has a buffering effect on the positive ions (Chester 2000). But if the ocean has to absorb more CO_2 over a short time period, the concentration of hydrogen ions increases, the buffering capacity of the ocean cannot keep pace, and the pH begins to fall, increasing sea water acidity (Zachos et al. 2005). The current pH of the ocean ranges from 7.9-8.5, averaging about 8.2 (Raven et al. 2005); it has already decreased by 0.1 units since the industrial revolution, and is projected to reduce by up to 0.3 in surface waters by 2100, and by 0.7 by 2300 (Caldeira & Wickett 2003). These may seem small changes but they represent a large increase in the hydrogen

ion concentration as pH is measured on a log scale. Marine organisms often function within narrow pH tolerance limits and are used to relatively stable conditions. Due to its buffering capacity the ocean has possibly not experienced such dramatic changes in pH for more than 20 million years (Feely et al. 2004). The ocean does have the ability to regain equilibrium *via* the dissolution of sinking calcium carbonate debris. Carbonate dissolution increases the alkalinity at depth, and when this water is re-circulated it helps to buffer surface waters (Fig.1.1.). However, oceanic turnover takes 500-1000 years and the oceans' regulatory system cannot match current and projected emission rates (Chester 2000; Raven et al. 2005). This lag in the system, creating a long period of rapidly lowering pH, is what is of concern, coupled with the synergistic effects of increasing sea surface temperatures (Pörtner et al. 2004; Zachos et al. 2005). Another problem arising from the increase in ocean CO_2 is that at current ocean pH, in addition to the reactions already described in Fig. 1.1, CO_2 can also combine with water and consume carbonate ions (CO_3^{2-}), meaning that an increase in CO_2 concentrations will increase this reaction making less CO_3^{2-} available for organisms that build calcium carbonate shells and skeletons (Orr et al. 2005). Many marine organisms may not be able to adapt within the timescales predicted for such change. In some respects the phrase 'ocean acidification' can be seen as misleading, as the pH of sea water is, in fact, slightly alkaline; however, it dramatises the facts in order to convey the potentially rapid and damaging effects upon marine ecosystems.

Reduced sea water pH has already been shown to have a serious effect on marine biota by interfering with their energetic and metabolic

functions, and by making it more difficult for certain groups to build their carbonate shells and skeletons, or forcing them to increase calcification rates to offset the dissolution caused by reduced pH (Pörtner et al. 2004; Orr et al. 2005; Wood et al. 2008). But sea water itself is more ion-rich than freshwater and until recently it had not been clear whether the behavioural effects seen in a freshwater medium would be the same in sea water. However, it has now been shown to affect the chemo-responsive behaviour of the juvenile marine fish *Amphiprion percula* by disrupting its innate ability to correctly identify different odours, and, in some cases, an inability to detect them at all (Munday et al. 2009; Dixson et al. 2010; Munday et al. 2010). Although behaviour has not, so far, been a major focus for ocean acidification studies, particularly with respect to the role of information gathering, chemo-reception and decision making (although see Munday et al. 2009, Dixson et al. 2010; Domenici et al. 2012), behavioural plasticity could be important in allowing a rapid adaptive response to environmental changes, or indeed eliciting a maladaptive response to such changes (Miner et al. 2005).

Under high CO_2 conditions, an excess of $[H^+]$ could disrupt chemo-sensory behaviour in four possible ways: firstly, low pH could cause a change in the ionic state of the odour molecules, thereby rendering them unrecognisable (Hara 1976; Leduc et al. 2004); secondly, low pH could reduce chemoreceptive acuity by changing the charge distribution on the odour receptor cells of an animal's sensory organs (Tierney & Atema 1988); thirdly, low pH could cause physical damage to sensory organs, as it has been shown that calcified animals may experience dissolution of their exoskeletons under such conditions (Spicer et al. 2007); and fourthly, rather

than indicating a direct effect on chemoreception *per se*, changes in chemo-responsiveness might simply reflect reduced activity levels, or reduced motivation to respond to chemical cues, occurring as a result of the elevated metabolic load of maintaining acid-base balance or supporting increased calcification rates under conditions of low pH (Pörtner 2004; Wood et al. 2008). It has recently been shown that changes in extracellular ionic concentrations, resulting from ion regulation in high CO_2 conditions, can interfere with the neurotransmitter function in fish, causing sensory and behavioural impairment (Domenici et al. 2012; Nilsson et al. 2012). The same mechanism could cause disruptions to crustacean behaviour, although this has yet to be tested. Acid-base regulation is the process whereby marine organisms equilibrate their internal environment (ion concentrations) with that of the external environment *via* ion exchange and transport across membranes (Truchot 1988). An increase in CO_2 in sea water can result in an internal hypercapnia in marine animals, where the [H+] in their haemolymph rises causing acidification, and such disruption must be compensated through acid-base regulation (Truchot 1983). This is a complex process, but in marine crustaceans it is achieved predominantly *via* ion transport mechanisms where sodium and hydrogen ions (Na^+/H^+) and chloride and bicarbonate ions (Cl^-/HCO_3^-) are exchanged across the gills in order to expel H^+ and accumulate HCO_3^- to reduce the internal acidosis (Henry & Wheatly 1992). This process can be energetically costly, and elicit trade-offs against other behavioural activities and physiological processes (Allison et al. 1992; Kurihara et al. 2008). The dissolution effect of reduced pH has also been shown to actually increase calcification rates in organisms as they attempt to compensate for

such disturbance, but at a cost to other functions (Wood et al. 2008). Calcifying marine invertebrates can actively regulate intracellular calcium ions (Ca^{2+}), along with other ions, in order to create an extracellular micro-environment that is saturated with respect to calcium carbonate, and allows calcification to continue (Pomar & Hallock 2008; Findlay et al. 2009; Nienhuis et al. 2010). Although intertidal animals exhibit adaptive behavioural and physiological mechanisms to cope with the variable environment they inhabit (Taylor & Spicer 1988; Truchot 1988), progressive climate change may create thresholds for dysfunction (Pörtner et al. 2004).

The rock pool environment

The intertidal environment is, by nature, a harsh habitat in which to survive. The animals that live there are well adapted to the variable, and sometimes extreme conditions that exist, but many are already living at the threshold of their tolerance limits (Stillman 2002; Tomanek & Helmuth 2002), so any overall changes to the environment make them vulnerable. Rock pools can act as a modifying factor for organisms in the intertidal by providing a refuge from desiccation during low tide; however, they are also subject to enormous spatial and temporal fluctuations in their physico-chemical characteristics (Truchot 1988). These small, intermittently isolated environments can experience enormous spatial and temporal variability in pH, salinity, temperature, and oxygen concentrations, which are dependent upon weather conditions, the time of day, and the abundance of resident organisms (Huggett & Griffiths 1986). The pH in rock pools can rise and fall rapidly on a

diurnal basis during emersion (eg., 9.5-6.5 pH units, Morris & Taylor 1983, and 10.16-7.29 pH units, Truchot 1988), owing to algal and animal respiration. The average pH in rock pools is therefore likely to be reduced, and perhaps even forced below its natural limits, in the future, owing to the additive effect of ocean acidification. Intertidal organisms, that encounter relatively frequent and extreme variations in pH with tidal cycles, are assumed to be more tolerant to reduced pH than those further offshore (Widdicombe & Spicer 2008), and therefore their behaviour perhaps less susceptible to disruption. However, any loss of chemo-responsive function or disruption to decision-making processes, even over diurnal cycles, could have overall consequences at the level of population health and, if there is a differential effect amongst individuals, fitness consequences.

A survey of rock pools at one of the collection sites, Hannafore Point, Looe, was undertaken to get a vertical and diurnal profile of the pH in high and mid shore rock pools, and give an idea of the variability in pH that my study species are subjected to in their natural environment. The survey showed that pH ranged from a maximum of pH_{NBS} = 9.10 and pH_{NBS} = 8.96 in high and mid shore pools respectively during the day, to a minimum of pH_{NBS} = 7.29 and pH_{NBS} = 7.35 in high and mid shore pools during the night. The pH ranges from this survey, and from previous studies/surveys, informed the pH levels used in the studies in this thesis, along with the future additive effects of ocean acidification, and levels of pH modelled for CO_2 CCS leakage scenarios. Further details of the survey are presented in Appendix 1.

Study species – intertidal decapod crustaceans

Decapod crustaceans have representatives in the intertidal and subtidal regions, and in the deep sea. Decapod crustaceans are an important constituent of the fauna of UK coastal waters and changes in their behaviour and functioning could have consequences at the population, community and ecosystem levels. Many decapods are also commercially valuable and such changes could have economic consequences. The physiology and behaviour of this group, particularly of intertidal and subtidal species, has been extensively studied (Bliss 1982,1983). Their responses to hypercapnia and associated acid-base disruptions (Truchot 1976; Henry & Cameron 1982; Cameron & Iwama 1987; Whiteley et al. 1992; Spicer et al. 2007), and their chemo-sensory behaviour (Brooks 1991; Rittschof 1992; Schmidt & Ache 1996; Steullet et al. 2000; Horner et al. 2004; Zhang & Lin 2006), in particular, have been well characterised in several species. Their olfactory behaviour lends itself well to laboratory experimentation because it is easily visible and recordable. Decapods possess two pairs of antennae that are adapted for chemo- and mechano-reception and possess large numbers of receptor cells. The smaller antennules are used predominantly for chemoreception (Ghiradella et al. 1968a, 1968b) and are flicked through the water to increase water-flow over the cells so that odour molecules can bind repeatedly to receptor sites and has been called ‘sniffing’ (Koehl 2006). This continuous process allows the animal to determine the concentration and direction of the odour plumes (Atema 1995). The structure and function of the olfactory organs of crustaceans, as a group, are highly congruent (Hallberg et al.

1992); therefore any observed effects have the potential to apply to the entire group. Decapods are also calcifying organisms, with their carapace predominantly composed of calcite (Raabe et al. 2006), and are therefore vulnerable to the predicted drop in the availability of calcium carbonate in sea water owing to ocean acidification, as discussed above.

The species I have chosen to use as models from this group are the glass prawn *Palaemon elegans* (Decapoda: Palaemonidae) and the hermit crab *Pagurus bernhardus* (Decapoda: Anomura).These species are from different niches in the intertidal. Both inhabit rock pools, but *P. elegans* is from pools on the upper shore and *P. bernhardus* is commonly found in mid shore pools, they therefore have different tolerances to changes in sea water chemistry.

Palaemon elegans (Rathke) (Fig.1.2.) is a prawn that is common around coastal, shallow UK waters, and is normally found in high-shore intertidal rock pools. Its diet consists of detritus, other small crustaceans, algae, and whatever can be scavenged (Forster 1951a, 1951b; Smaldon et al. 1993). The physiology of *P. elegans* has been shown to be exceptionally tolerant of the variable conditions associated with rock pools, such as daily and seasonal fluctuations in oxygen, pH, salinity and temperature (Truchot & Duhamel-Jouve 1980; Morris & Taylor 1983; Taylor & Spicer 1991). This species has already been used as a model organism in studies on the effects of hypoxia (Morris & Taylor 1985; Taylor & Spicer 1987; Taylor & Spicer 1988) and reduced sea water pH (Dissanayake et al. 2010) on its iono-regulatory capabilities, affording direct comparisons with those studies.

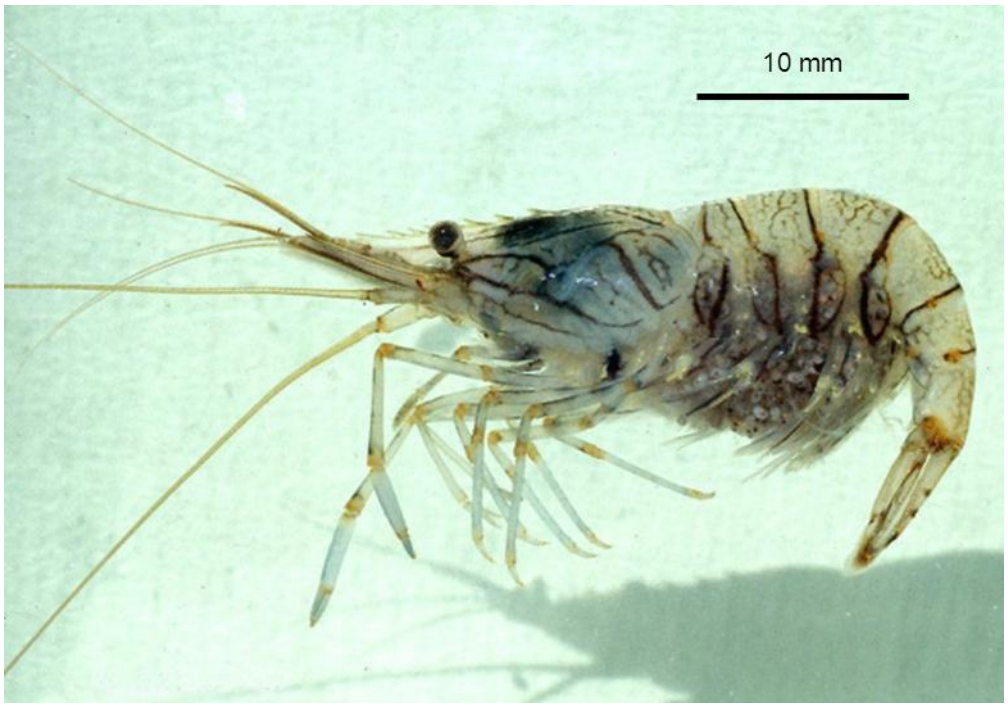

Fig 1.2. *Palaemon elegans*, a female carrying eggs.

Pagurus bernhardus (Linnaeus) (Fig. 1.3.) is common to the coasts of North West Europe and the American Atlantic and, like the majority of hermit crabs, is distinct from other decapods for having an asymmetrical body plan and a soft, curved abdomen that is very lightly calcified, unlike the rest of the exoskeleton, and coiled into an adopted, empty gastropod shell. The gastropod shell offers protection against environmental extremes and predators (Lancaster 1988). *Pagurus bernhardus* can be found subtidally when it is mature, but in its early years it inhabits mid shore, intertidal rock pools. Consequently it can withstand a certain amount of variability in physico-chemical conditions (Shumway 1978), although it is not as robust as *P. elegans*. This species is predominantly a scavenger and detritivore, rather than an active predator, and relies heavily on chemoreception in order to find

food, but also to detect potential mates, predators and prospective shelters at a distance (Lancaster 1988).

The shells that house hermit crabs are a particularly valuable resource to them, and as *P. bernhardus* grows it must constantly upgrade to a larger shell. Shells are acquired either by locating sites where there are empty shells, or by competing with other hermit crabs and engaging in contest behaviour to obtain a better shell (Hazlett 1981). When they find a suitable shell they undertake characteristic shell investigation behaviour to assess the size and quality of the prospective shelter. This involves various sequential activities which often begin with a visual inspection of the shell, then contact may be made with the antennae, followed by manipulation of the exterior and interior of the shell with legs and chelipeds. If the shell is acceptable the crab will pull itself out of the old shell and swing itself rapidly into the new one. Once in the new shell the crab may then decide to remain in it or return to the old shell. All this activity is costly in terms of energy consumption and predation risk, and therefore the costs and benefits of acquiring a new shell must be weighed at each step, and this involves complex decision making based on the information being gathered about the shell itself and the environmental context (Reese 1962; Jackson & Elwood 1989; Cote et al. 1998). Choosing an optimal shell can have important consequences for survival, growth and fitness, as it has been shown that a cramped shell can have deleterious effects on growth and fecundity (Bertness 1981). Shell assessment and investigation behaviour in this species, along with information gathering and chemo-responsive behaviour, is therefore an interesting activity to study in relation to changes in sea water pH. Hermit

crabs have not yet been used as model organisms in reduced pH sea water studies, and yet they may be particularly vulnerable to the effects of dissolution under reduced pH conditions as they not only have a calcified exoskeleton, but have also evolved to rely on the empty, calcified shells of marine gastropods for shelter and protection.

Fig 1.3. *Pagurus bernhardus* investigating an empty shell.

Thesis aims

The aims of this thesis were to investigate the effect of reduced pH on the information gathering activities and chemo-responsive behaviour of intertidal crustaceans, and further to investigate how any disruption to these processes

might affect their subsequent decision making abilities. In this thesis I present a series of experiments that investigate the behavioural effects of reduced sea water pH on the species *P. elegans* and *P. bernhardus*, in the natural context of the variation in rock pool pH, and the wider context of predicted climate change-related shifts in the pH of sea water. Initially I carried out short-term exposures with highly reduced pH levels, in order to identify behavioural effects. I also attempted to address some of the underlying mechanisms for those effects. I then carried out a longer-term exposure with a gradient of pH levels to identify thresholds for dysfunction. Finally I considered the wider ecological implications of pH-induced disruptions in relation to animal decision making. The questions addressed in each chapter are outlined below:

***Chapter 2*:** In this study I took an extremely tolerant species, the upper shore prawn *P. elegans*, and investigated its chemo-sensory and behavioural responses to a food cue under reduced pH conditions. This was to establish whether there were any signs of disruption in a species well adapted to physico-chemical variations in its natural environment, including pH fluctuations. The reduced pH conditions were predicted to affect its antennular flicking rate ('sniffing' response), along with its ability to locate the source of the odour under such conditions. The duration of locomotion was also recorded as a measure of their energetic status.

Chapter 3: In this chapter I investigated whether the disruption to antennular flicking observed in *P. elegans* would be repeated in the hermit crab *P.*

bernhardus, a less tolerant, decapod species from a different niche lower on the shore. The same experimental protocol was used, but the pH was adjusted to reflect the less extreme pH changes lower down the shore. The aim was also to try to investigate possible underlying mechanisms for any disruption by taking a physiological measure of haemolymph ion concentrations, which are an indicator of acid-base disruptions, and also presenting the crabs with both an acidified and non-acidified food cue. *Pagurus bernhardus* was predicted to be more seriously affected by reduced pH than *P. elegans*, being a less tolerant species, showing reduced antennular flicking and an impaired ability to locate, or recognise the food cue. Any disruptions to haemolymph ion concentrations would also indicate whether the reduced pH was affecting the iono-regulatory capacity of the crabs. Duration of locomotion was again recorded as a measure of energetic status.

Chapter 4: The study in this chapter was designed to build upon the results of the study in chapter 3, and the disruptions to *P. bernhardus*' chemosensory behaviour. The aim was to use a range of pH levels (including levels in-line with OA predictions and CO_2 CCS leakage scenarios) in order to identify thresholds for that disruption, and to expose the crabs for a much longer period to test the effects over time. It was predicted that crabs might show a gradient in their response to increasingly lower levels of pH, and that disruptions would be affected by the length of the exposure.

Chapter 5: In this final study I investigated the effects of reduced sea water pH on the resource assessment and decision making activities of *P. bernhardus* in relation to shell choice. The rationale was to observe a more complex behavioural response to reduced pH. A highly reduced pH and a short exposure time were used once again, in order to be able to detect any effects. It was predicted that there would be disruption to *P. bernhardus*' shell investigation and decision-making behaviour, with wider ecological implications for the survival and fitness of this species.

CHAPTER 2

The effect of reduced sea water pH on the chemo-responsive behaviour of the rock pool prawn *Palaemon elegans*

Abstract: The pH of the oceans is decreasing owing to the increased absorption of carbon dioxide from the atmosphere. This relatively rapid change to a previously stable environment is predicted to have serious physiological effects on marine biota. It may also affect their behaviour, and it has recently been demonstrated that the responses of marine animals to olfactory cues is disrupted by the altered water chemistry resulting from ocean acidification. The pH in intertidal rock pools varies naturally, but may be forced below its normal limits with the additive effect of ocean acidification. In this study the responses to food odours of the rock pool prawn *Palaemon elegans* were tested under highly reduced pH conditions. Prawns were kept in untreated artificial sea water (ASW) (pH_{NBS} = 8.20), or acidified ASW (pH_{NBS} = 6.60) for five days; and then tested under those same pH conditions in a choice experiment on day one and day five. There was a significant reduction in antennular flicking (the 'sniffing' response in decapods) under reduced pH conditions compared with the control, both in an initial experiment, without an ablation treatment, and in a second experiment, with antennules ablated. In both cases this effect appeared on day five, so the onset was not immediate. Although disruption was detected, not all chemo-sensory abilities were affected, and the prawns were still able to detect the food odour. This may be owing to the fact that this species has olfactory receptors on other parts of its body, or that it is particularly resilient to pH changes , however, the implications for less tolerant species in the same group may be more serious and warrant further investigation.

INTRODUCTION

As the atmospheric concentration of carbon dioxide (CO_2) is increasing more CO_2 is being absorbed by the oceans, lowering sea water pH (Calderia & Wickett 2003). The dissolution of CO_2 in sea water increases the concentration of hydrogen ions ($[H^+]$), making the ocean more acidic. Ocean pH has already decreased by 0.1 units since the industrial revolution, and is projected to fall by up to 0.3 units in surface waters by 2100, and by 0.7 by 2300 (Caldeira & Wickett 2003). This represents a large increase in the $[H^+]$, as pH is measured on a log scale. Many marine organisms tend to function within narrow pH tolerance limits, particularly those in the deep ocean that are used to relatively stable conditions. Due to its' buffering capacity the ocean has probably not experienced such dramatic changes in pH for more than 20 million years (Feely et al. 2004), and the rate of change leaves organisms little time to adapt, in an evolutionary sense, to such conditions, and evolutionary time lags can lead to extinctions (Raven et al. 2005). Rising sea surface temperatures, associated with global warming, are also expected to have a detrimental synergistic effect (Pörtner et al. 2004). These changes are predicted to have a serious impact on marine biota by interfering with their energetic and metabolic functions, and by making it more difficult for certain groups to build their carbonate shells and skeletons (Pörtner et al. 2004; Orr et al. 2005). It may also affect the behaviour of marine organisms by disrupting their abilities to detect odours. The effect of ocean acidification on the behaviour of marine organisms has, so far, been relatively neglected.

Chemoreception is the way in which animals detect chemical odours in their environment, providing them with information about the location of food and potential mates, the presence of predators, or the status of an opponent in agonistic interactions. In an aquatic environment organisms are bathed in a cocktail of ambient chemicals and it is important for them to be able to discriminate between odours; this has led to the evolution of highly specific chemical receptors (Lima & Dill 1990; Atema 1995). If the ability to detect and identify odours is impaired, then it could have serious consequences for the survival of both individuals and species (Atema 1995; Wisenden 2000). It is hypothesised that the increased [H^+], produced by ocean acidification, could disrupt chemoreception by interfering with the ionic bonding of odour molecules to receptor cells. This hypothesis was developed from freshwater studies on rainbow trout, *Oncorhynchus mykiss* (Tierney & Atema 1988). However, any observed effects on chemo-responsive behaviour may also be caused by either physical damage to the receptor organs (Fabry et al. 2008), or physiological stress that leads responsive behaviour to be allocated a lower priority by animals under these conditions (Allison et al. 1992).

There is already evidence in freshwater species that the chemo-responsiveness of rainbow trout, *O. mykiss* (Hara 1976), and crayfish, *Cambarus bartoni* (Allison et al. 1992), can be disrupted by reduced pH, impairing their ability to detect food odours. Sea water is a more complex medium than freshwater, owing to the greater number of dissolved ions it contains; however, a recent study has also shown olfactory disruption in marine fish, where the larvae of the coral reef fish *Amphiprion percula* were unable to discriminate between different odours, or failed to respond to them

at all, after being reared in reduced pH sea water (Munday et al. 2009). This study investigates the effect of reduced pH on the chemo-responsiveness of the rock pool prawn *Palaemon elegans* to food odours. Decapod crustaceans are an important group in the fauna of UK coastal waters and changes in their behaviour and function could have consequences at the population, community and ecosystem levels. Decapods are also commercially important to the fishing and aquaculture industries. *P. elegans* is common in many UK coastal areas and inhabits mid and high shore rock pools. Like many other decapods crustaceans it has two pairs of antennae that are adapted for chemo- and mechano reception. These antennae possess large numbers of receptor cells. The longer 'antennae' appear to have a predominantly tactile function (Sandeman 1989), and the smaller 'antennules' are used mainly for olfaction, or chemoreception (Atema 1995; Koehl 2006). The aesthetasc tuft region on the outer flagella of the antennules is thought to be responsible for distance chemoreception (Hallberg et al. 1992). The antennules are flicked through the water, also called 'sniffing' (Stacey et al. 2002), to increase water-flow over the cells so that odour molecules can bind repeatedly to receptor sites. This continuous process allows the animal to determine the concentration and direction of the odour plume (Atema 1995).

The aim of this study was to investigate the effect of reduced pH on the olfactory responses and behaviour of *P. elegans*. Two multifactorial experiments were conducted. In the first experiment prawns were held in untreated sea water (pH_{NBS}=8.20) or high CO_2 sea water (pH_{NBS}=6.60) for a period of five days and their chemo-responsiveness to food odours was tested in a choice experiment on days one and five under the same pH

conditions. The second experiment mirrored the first, but with an added treatment level to try and test whether it was specifically chemoreception that was being affected by ablating the part of their antennules, the aesthetasc tuft region, responsible for distance chemoreception. The justification for using the highly reduced pH level of pH_{NBS} = 6.60 is explained in the methods section below. It was predicted that the antennular flicking rate ('sniffing' response) of *P. elegans* would be affected by reduced pH, and that the prawns would also find it harder to locate the food odour, or be less attracted to it, under such conditions.

METHODS

Study organisms

Prawns, *P. elegans*, were collected from high shore rock pools at Mount Batten, Plymouth, Devon, UK (50:21:34N, 04:07:45W) during March 2008 for the first experiment and during August 2008 for the second experiment. To control for size prawns were confined to an average carapace length of 12 mm (±5 mm). In the laboratory the individuals were kept in large holding aquaria with aerated, filtered sea water on a natural light cycle and at a temperature of 15ºC. The holding aquaria contained numerous refuges to limit potentially harmful agonistic interactions. At least two weeks were allowed to elapse between collection and the start of the experiment to acclimate the prawns to the laboratory environment. They were fed with fish during the holding period, and to exclude the effects of satiation, they were starved for three days prior to the experiment. A total of 30 prawns (N = 15) were used in the first experiment and a total of 56 prawns (N = 14) were used in the second experiment.

Treatments

Sea water treatments (experiments one and two): during both experiments prawns were held in one of two sea water pH treatments for a period of five days. The animals were food deprived and kept in aquaria (vol. = 9 l) of either untreated (pH_{NBS} = 8.17 ± 0.03) or reduced pH (pH_{NBS} = 6.67 ± 0.04) artificial

sea water (ASW) (Instant Ocean®) ($S = 35_{PSU}$, $T = 15^{o}C$). Artificial sea water was used because it is assumed to be free of any biological cues. The nominal reduced pH treatment of 6.60 is far lower than predicted levels, the rationale for this is that *P. elegans* inhabits mid and high shore rock pools and is accustomed to significant variations in pH, temperature and salinity. The pH in rock pools has been recorded as low as 6.4 overnight in one study (Morris & Taylor 1983), and at 7.2 in another (Truchot 1988), owing to algal and animal respiration. A preliminary study at local shores showed that pH can fall to as low as $pH_{NBS} = 7.29$ in rock pools overnight (Appendix 1). Consequently *P. elegans* is a resilient species, used to dealing with marked variations in its physico-chemical environment (Taylor & Spicer 1991). Taking into account the extreme conditions in high shore rock pools, along with the predicted fall in sea water pH, it was important to test the prawns at the lower threshold of their tolerance levels.

Prawns in the reduced pH treatments were introduced into aquaria with untreated ASW that was then gradually lowered to the nominated pH, to avoid shocking the animals. This was achieved over a period of 4 hours at a rate of approximately a $pH_{NBS} = 0.37$ reduction per hour, which would mimic the rate of reduction in a rock pool during low tide. To address any aquarium effect there were three aquaria of acidified ASW and three aquaria of untreated ASW. Within the aquaria the prawns were placed in individual cages made from aquatic netting (NCC Supplies Ltd, Crewe, UK) with fixed plastic piping for hides, to prevent agonistic interactions and cannibalism. The ASW pH (SevenEasy™ S20 Mettler Toledo pH Meter, Switzerland) and total CO_2 concentration (Ciba-Corning 965, Carbon Dioxide Analyser, England)

was monitored and recorded daily and one water change was carried out on the third day. Untreated ASW was aerated from the laboratory air supply (pCO_2 = 376.29 ± 1.20 µatm). Reduced pH treatments were created by bubbling through CO_2-enriched air (pCO_2 = 11914.63 ± 41.52 µatm), controlled using a gas analyser (LI-7000, LI-COR, Nebraska USA). The air mixing system was similar to the one described by Findlay et al. (2008) and Dissanayake et al. (2010), and a schematic is shown in Figure 2.1. Behavioural assays were carried out after the first day and on the fifth day to test for an acute and then a short-term response to the treatments.

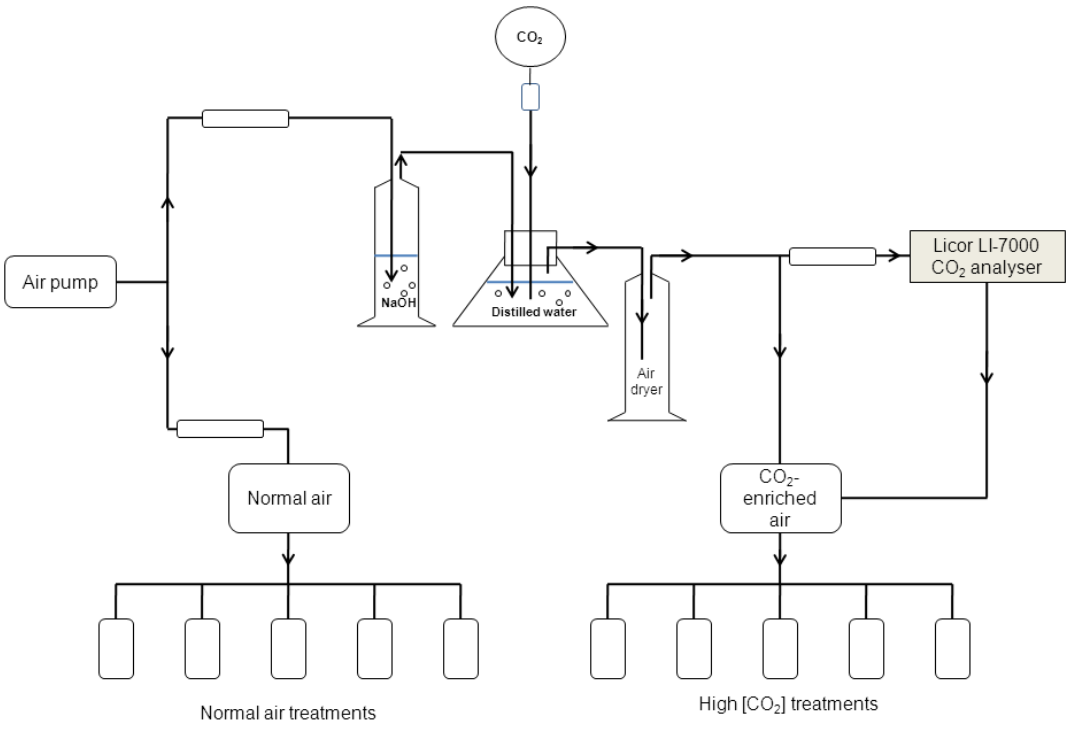

Figure 2.1. A schematic of the air-CO_2 mixing system. Ambient air is pumped through flow meters either directly into normal air treatment aquaria or through the gas mixing system which produces CO_2-enriched air. The air directed to the gas mixing system flows through a

dreschel bottle containing sodium hydroxide (NaOH) to remove all the CO_2. Pure CO_2 is released, through a flow meter, into a mixing vessel, along with the CO_2-free air. The mixed high [CO_2] air flows through a drying bottle. Some of the CO_2-enriched air is then sent through a Licor gas analyser, to monitor and control the [CO_2], and the rest is fed straight into the high-[CO_2] treatments.

Ablations (experiment two): For experiment two the aesthetasc tuft region on the lateral flagella of the antennules, thought to be largely responsible for distance chemoreception, was ablated on half of the prawns and the same length of the medial flagella of the control prawns was removed to control for any ablation effect. Prawns were placed in a small aquarium of sea water at 15ºC and the temperature of the water was reduced in a refrigerator at 5^{o}C for 5 hours to gradually slow down their metabolism. The prawns were removed from the fridge and placed on ice (still in the aquarium of sea water) for a further 30 mins, then kept in Petri dishes on ice for 5 mins, in order to anaesthetise them, before the ablations were carried out. The aesthetasc tuft region was removed with surgical scissors. The ablated prawns were then placed in aquaria within individual cages to recover for 24 hours before being placed in treatment aquaria at the start of the experiment.

Odour preparation

Food odour was produced by crushing 1g of the white fish Coley (*Pollachius virens*) in 50ml of ASW and then filtering it through coarse filter paper (Whatman No. 4). Natural cellulose sponges were used to carry the odour. It

had been established in a preliminary trial that the prawns were attracted to this odour.

Behavioural observations

Behavioural assays were conducted on days one and five in a temperature controlled (15ºC) room, under a natural strip light (80 watts), in behavioural chambers filled with 4 l of either untreated ASW (pH_{NBS} = 8.20) or reduced pH ASW (pH_{NBS} = 6.60). The chambers consisted of a 20 l plastic aquaria which had been painted with black pond paint (Blagdon^{TM}, Interpet Ltd, Dorking, Surrey, England) on three sides and left clear at the front to allow observations to take place. Assays consisted of a choice experiment with the chamber divided into three sections, with sliding Perspex doors separating each section, and cue sponges eventually placed at either end of the chamber (Figure 2.2.). Sponges were soaked in either fish cue or ASW and the odour was allowed to diffuse in still water to mimic a rock pool environment. There is evidence that water currents stimulate antennular flicking, which would be a confounding factor (Snow 1975). The prawns were placed in the centre of the chamber, with the Perspex doors down, and allowed to acclimate for 10 mins. After this period cue sponges were added at random to each end of the chamber to control for any effects of learning, the doors were then lifted and their behaviour observed for a period of 10 mins. Assays were videoed through the front section of the chamber. Videos were later analysed using The Observer Mobile 5.0 Workabout mx handheld computer (Psion Teklogix Inc., Ontario, Canada). The behavioural measures

recorded were: the time spent in the cue section of the behavioural chamber, the time spent in contact with the cue source, the latency to contact the cue source, the rate of antennular flicking, and the duration of locomotion.

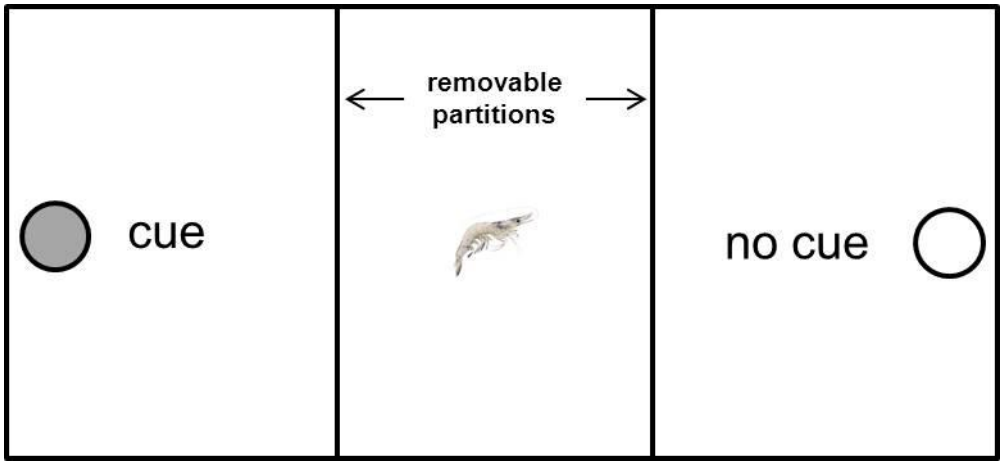

Figure 2.2. Schematic of the behavioural chamber showing the two removable partitions and cue sponges at either end, one soaked in fish cue, the other in ASW.

Statistical methods

Data were Log_{10} transformed, where necessary, and the results were analysed with Statview (5.0, SAS, Cary, NC, USA) using repeated measures ANOVAs. In order to determine the effects of pH and day on the prawns in Experiment one, a series of repeated measures ANOVAs were performed with one between factor (control and reduced pH treatments), and one within factor (response on days one and five). The dependent variables measured were time spent in the cue section of the behavioural chamber, time spent in contact with the food cue sponge, the rate of antennular flicking, activity levels (time spent in motion), and latency to contact with the cue sponge. The second experiment consisted of a multi-factorial design with two between factors (pH treatment and ablation treatment) and one within factor (response on days one and five). A series of repeated measures ANOVAs were carried out in order to determine the effect of pH treatment, ablation treatment and day on the prawns. The dependent variables measured were time spent in the cue section of the behavioural chamber, time spent in contact with the food cue sponge, the rate of antennular flicking, activity levels (time spent in motion), and latency to contact with the cue sponge.

RESULTS

Experiment one

There was a significant effect of treatment ($F_{1,25}$ = 4.601, P = 0.0419) on the time spent in the cue section of the chamber, with prawns in the reduced pH treatment spending more time in the cue section of the chamber than those in the control (Figure 2.3; Table 2.1). There was also a significant effect of day with prawns spending more time in the cue section of the chamber on day five than on day one (Table 2.1). There was a significant effect of sea water treatment on the rate of antennular flicking (Table 2.1) with the prawns flicking their antennules significantly less in the reduced pH sea water than they did in the control (Figure 2.4.). There was also a trend towards an interaction effect between day and sea water treatment on antennular flicking (Table 2.1). There was a trend for prawns to be more active on day five than on day one (Table 2.1).

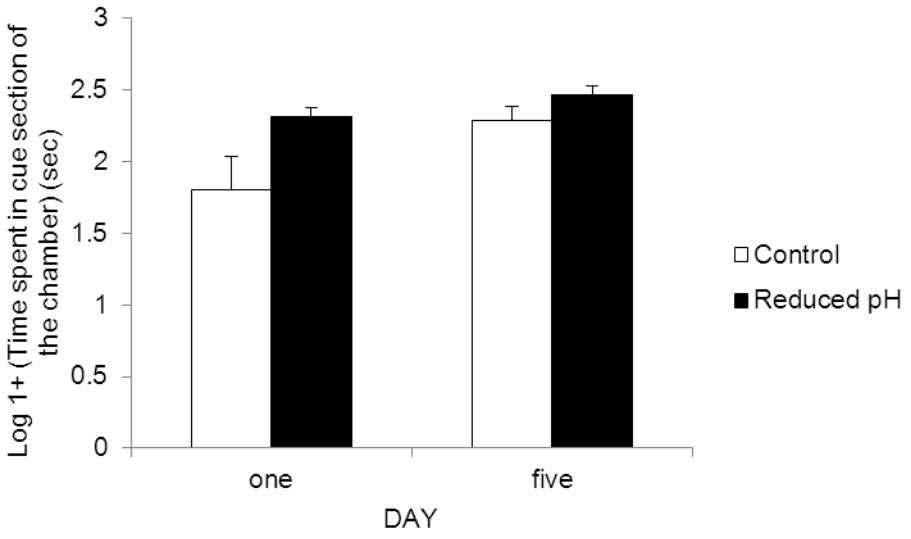

Figure 2.3. Mean time (±SE) spent by prawns from the reduced pH and control treatments in the cue section of the behavioural chamber.

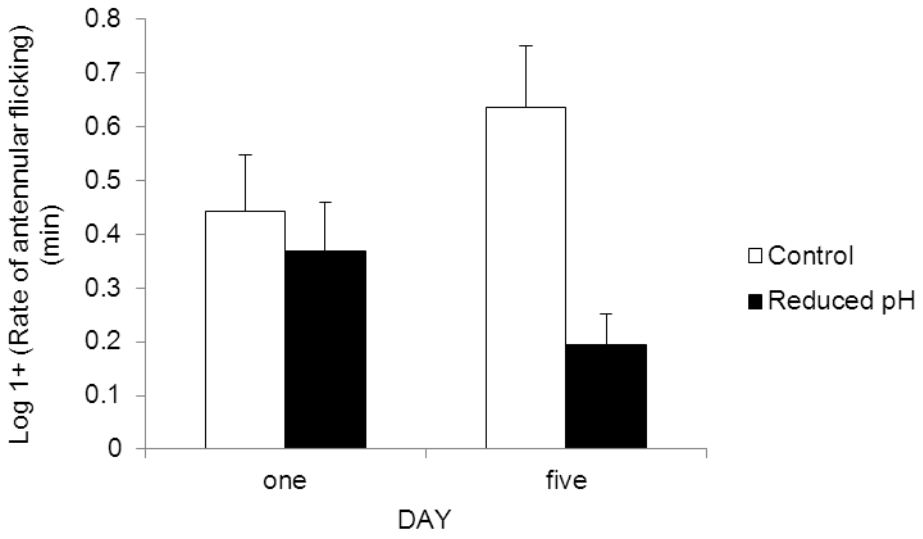

Figure 2.4. Changes in the rate (Mean ± SE) of antennular flicking between reduced pH and control sea water treatments on days one and five.

Table 2.1. ANOVA table for Experiment one showing all treatment interactions. ***** indicates significant result.

	Time in cue section			**Time in contact with cue**			**Latency to contact cue**			**Antennular flicking**			**Activity levels**		
	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Sea water pH	1	4.601	0.0419*	1	1.282	0.2683	1	3.261	0.0830	1	6.763	0.0154*	1	1.804	0.1913
Day	1	8.393	0.0077*	1	0.582	0.4526	1	1.624	0.2143	1	0.011	0.9180	1	3.651	0.0676
pH x Day	1	2.211	0.1495	1	1.128	0.2983	1	0.116	0.7360	1	3.803	0.0624	1	0.235	0.6318
Error	25			25			25			25			25		

Experiment two

There was a significant three way interaction between day, sea water treatment and ablation treatment (Table 2.2) on the time spent in contact with the cue sponge. This was caused by non-ablated prawns in the reduced pH sea water treatment spending more time in contact with the cue on day one than prawns in the other treatments, and ablated prawns in the control treatment spending less time in contact with the cue on day five than prawns in the other treatments. There was also a significant effect of reduced pH on antennular flicking (Table 2.2) with prawns flicking their antennules less in the reduced pH treatment than in the control (Figure 2.5.). There was also a significant effect of day on antennular flicking with prawns flicking their antennules much less on day five than on day one (Table 2.2). Finally, there was a significant effect of day (Table 2.2) on latency to contact the cue sponge, with prawns taking less time to find the cue sponge on day five than on day one.

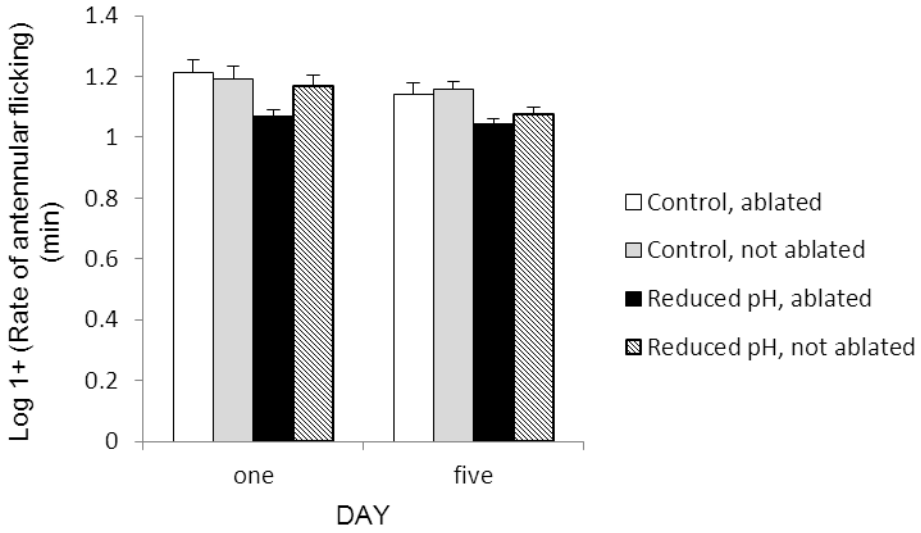

Figure 2.5. Changes in the rate of antennular flicking (Mean ± SE) between reduced pH and control sea water treatments, and ablation treatments on days one and five.

Table 2.2. ANOVA table for Experiment two showing all treatment interactions. * indicates significant result.

	Time in cue section			Time in contact with cue			Latency to contact cue			Antennular flicking			Activity levels		
	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Sea water pH	1	0.006	0.9368	1	0.780	0.3812	1	0.029	0.8656	1	9.494	0.0033*	1	0.688	0.4107
Ablation		0.395	0.5322	1	2.538	0.1172	1	1.211	0.2763	1	1.236	0.2713	1	1.501	0.2260
Day	1	0.716	0.4013	1	0.076	0.7833	1	6.446	0.0142	1	14.356	0.0004*	1	0.164	0.6873
pH x Ablation	1	0.019	0.8902	1	2.125	0.1509	1	0.414	0.5230	1	1.472	0.2304	1	0.167	0.6847
pH x Day	1	0.036	0.8494	1	1.094	0.3003	1	0.003	0.9592	1	0.065	0.8003	1	1.113	0.2964
Ablation x Day	1	0.374	0.5436	1	0.047	0.8293	1	0.024	0.8772	1	0.246	0.6218	1	0.927	0.3401
pH x Ablation x Day	1	1.006	0.3206	1	8.493	0.0052*	1	0.200	0.6486	1	2.913	0.0938	1	0.083	0.7743
Error	52			52			52			52			52		

DISCUSSION

This study shows that reduced pH sea water had a significant impact on the antennular flicking rate ('sniffing' response) of *P. elegans*, implying an effect upon their chemo-responsiveness. This effect appeared after five days of exposure to reduced pH and was consistent across both experiments, and agrees with the findings of Allison et al. (1992) where the rate of antennular flicking of the crayfish *Cambarus bartoni* significantly decreased under reduced pH conditions in freshwater. The rate of antennular flicking has been shown to correlate with the concentration and relevance of an odour (Schmitt & Ache 1979; Atema 1995), so any impairment to this function may compromise the ability to locate food. A recent study by Munday et al. (2009) on clownfish found that their ability to discriminate between odours under reduced pH conditions (pH 7.8) was disrupted, and at even lower pH (7.6) they failed to respond to any of the cues presented to them.

The reduced pH treatment affected other behavioural measures but these results were not consistent between the two experiments. In experiment one the prawns in the reduced pH treatment spent more time in the cue section of the chamber than those in the control, contrary to the expectation that they would not be able to find the source of the food cue. There was also a trend for prawns in the first experiment to spend a higher proportion of their time in motion on day five than on day one. The fact that their activity levels did not differ between treatments, and in one instance increased over time, indicates that they were not incapacitated by the reduced pH; however, their activity levels may not be expected to decrease under such stressful

conditions as Taylor and Spicer (1987; 1988; 1991) found that *P. elegans* will often initiate an escape response when conditions become hypoxic in rock pools, and move to shallower waters, or vacate the water altogether and move onto exposed rocks when it reaches critical levels. This is an adaptive response that allows them to inhabit high shore rock pools where conditions can become extreme overnight.

Prawns in both treatments, and in both experiments, were successful in locating the cue sponge, contrary to expectations that they would show an impaired ability to do so in reduced pH conditions. The size of the behavioural chamber made it unclear at times whether the prawns actually detected the direction of the diffusing fish odour plumes, or whether they came across the cue sponge merely by chance as they were investigating the chamber, or as they were in motion, prompted by an escape response. The reduced pH obviously did not disrupt their attraction to the food cue once they were near the source.

The reduction in antennular flicking under reduced pH conditions observed in both experiments could be due to several factors. The altered sea water chemistry created by increased concentrations of CO_2, and the concomitant increase in hydrogen ions, could interfere with, and change, the state of ionisation of receptors (Tierney & Atema 1988). This would be more likely to be an immediate effect and is difficult to test directly. Munday et al. (2009) attempted to test this indirectly by taking a subset of their fish larvae, that had been reared in untreated sea water, and testing their olfactory responses in reduced pH sea water, to see if there was an immediate effect on their ability to detect odours, which would suggest chemical disruption to

receptors, but the larvae were still able to detect and discriminate between different odours. Munday et al. therefore proposed longer-term tissue damage as a potential cause of olfactory disruption. Bibby et al. (2007) exposed the intertidal snail *Littorina littorea* to a pH of 6.6 for 15 days, and then tested their responses to predator cues. The snails showed increased avoidance behaviour in the presence of predator odour, showing that they could still detect the cue. However, these trials were carried out in untreated sea water, but the implication is that there was no lasting disruption to the function of their receptor organs caused by the exposure to reduced pH.

Physical damage to the receptor organs is another possible cause for a loss of function. Prawns have a calcareous exoskeleton, and the lowered calcification rates and potential dissolution of calcium carbonate exoskeletons resulting from reductions in sea water pH have been demonstrated in other marine animals under such high CO_2 conditions (Riebesell et al. 2000; Orr et al. 2005; Gazeau et al. 2007). Munday et al. (2009) investigated whether there was any physical damage to the olfactory organs of the clownfish larvae by examining the internal morphology of the nasal organ under an electron microscope. They found no differences between treatments and suggest the effect may be owing to elevated CO_2 and reduced pH disrupting the transfer of chemo-sensory signals within the neurosensory system over a longer period.

The lower rate of antennular flicking could also be owing to the effect of metabolic depression on the prawns. It requires energy to perform antennular flicking (Mellon 1997) and is an activity that may be down-regulated under stressful conditions. A study by Dissanayake et al. (2010)

investigated the effects of reduced pH on the osmoregulation and acid-base regulation of *P. elegans* and the closely related species, *P. serratus*. They found that *P. elegans* suffered an acid-base imbalance caused by the reduced pH after 14 days, but was able to compensate for this effect after 30 days *via* iono-regulation, however both the short and long term energetic costs of such regulation are not known, but can be expected to be fairly high. A study on the velvet swimming crab, *Necora puber*, showed that they were able to cope with short-term acid-base regulation under reduced pH, although here too there was likely to be a cost or trade-off involved (Spicer et al. 2007) They also reported an increase in haemolymph magnesium that is likely to have a narcotising effect, slowing metabolism, and thereby affecting an animal's motivational state. Kurihara et al. (2008) carried out long-term experiments (15 and 30 weeks) on the prawn *Palaemon pacificus*, exposing them to sea water containing increased concentrations of CO_2 and found that growth, moulting and fecundity were affected, and they attributed this to the possible costs of maintaining acid-base balance. Interestingly the growth of the second antennae was shown to be significantly inhibited, possibly due to the disruption of the moulting cycle. The brevity of this experiment, however, suggests some more rapid effect on chemo-sensory systems than dissolution.

The ablation treatment in the second experiment of this study was an attempt to elucidate the underlying cause of the lower rates of antennular flicking under reduced pH, to try and establish whether it was primarily an effect on chemoreception rather than an indirect effect mediated by a change in metabolic function. However, the ablations did not have a clear effect on the prawns' ability to detect food odours, or on their rates of antennular

flicking. If they had I would have expected the ablated prawns in the untreated sea water to have flicked their antennules at the same low rate as the ablated and unablated prawns in the reduced pH treatments. It is also possible, however, that the ablations were not wholly effective. Even though the aesthetasc region, which was ablated, has been identified as the area of the antennules largely responsible for distance chemoreception (Hallberg et al. 1992; Stacey et al. 2002), there is evidence that there are chemo-sensory receptor cells on other parts of the antennules of lobsters (Horner et al. 2004; Steullet et al. 2002), and this could also be true of prawns. The lack of any effect of ablations may also indicate that the lower rates of flicking were not owing to damage or disruption to the function of the antennules.

Short-term exposure to reduced sea water pH had a direct effect on the chemo-responsive behaviour of *P. elegans* by affecting its 'sniffing' response. The fact that *P. elegans'* olfactory behaviour was not as severely affected as the coral reef fish in the study by Munday et al. (2009) is likely to be because it is an exceptionally tolerant intertidal species, used to the extreme fluctuations in the pH of high shore rock pools. It is also an efficient iono-regulator, particularly over short timescales (Dissanayake et al. 2010). Further studies with less tolerant intertidal decapod species may reveal greater disruption to chemo-sensory behaviour. Subsequent studies will also continue to investigate the underlying causes of olfactory disruption under reduced pH conditions, alongside any observed behavioural effects. Structural damage to receptor organs could be addressed by examining the gross structure of antennules under an electron microscope. The effects of reduced pH on energetic status could be investigated by taking haemolymph

samples and recording ion concentrations to ascertain the acid-base status of the animals immediately following behavioural trials. The moderate effect on the chemo-sensory behaviour of a tolerant species like *P. elegans* revealed in this study could have far more serious implications for less tolerant decapod species. Any loss of olfactory function, and subsequent changes in behaviour, could have serious consequences for marine crustaceans whose fitness and survival depend upon the detection and recognition of chemical cues in their environment.

CHAPTER 3

Reduced pH sea water disrupts chemo-responsive behaviour in the hermit crab *Pagurus bernhardus*

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Abstract: Chemoreception is a key process by which many aquatic animals perceive their environment, and therefore abiotic disruptions to this process could have serious impacts on the survival and fitness of individuals, and on species interactions. Hermit crabs are subject to cyclical reductions in the pH of the water in the intertidal rock pools that they inhabit. Such reductions may be further exacerbated by on-going ocean acidification and/or leakage of carbon dioxide from geological storage sites and coastal upwelling events. Here I test the chemo-sensory responses of the hermit crab *Pagurus bernhardus* (Linnaeus) to a food odour under reduced pH conditions (pH_{NBS} = 6.80). Acidifying the odour had no effect on its attractiveness indicating no permanent degradation of the cue; however, the pH of the sea water did affect the crabs' responses. Hermit crabs held and tested in reduced pH sea water had lower antennular flicking rates (the 'sniffing' response in decapods); were less successful in locating the odour source, and showed an overall decline in locomotory activity compared to those in untreated sea water. Analysis of their haemolymph revealed a greater concentration of chloride ions ($[Cl^-]$) in the reduced pH treatment group, suggesting iono-regulatory disruption; however, there was no correlation between $[Cl^-]$ and locomotory activity, suggesting a specific effect on chemoreception. This study shows that the chemo-responsiveness of a crustacean may be influenced by both naturally occurring pH fluctuations and future anthropogenically-induced changes in ocean pH.

INTRODUCTION

Successful gathering of information is essential for animals to be able to make decisions that affect their survival and fitness. Most information gathering is mediated *via* visual, tactile, auditory, gustatory and olfactory senses; however, environmental factors can disrupt such activities (Schmidt et al. 2010). In aquatic environments the olfactory senses of animals are often heightened to compensate for reduced visibility (Wisenden 2000). The detection of chemical cues (chemoreception) is essential for the purposes of foraging, maximising reproductive opportunities, and avoiding predation (Atema 1995). Aquatic animals are bathed in a cocktail of ambient chemicals and it is important for them to be able to distinguish between odours (Derby & Sorensen 2008); this has led to the evolution of highly specified chemical receptors (Atema 1995). If the ability to detect and identify odours in this mix is impaired, then it could have serious consequences for the survival and fitness of animals that rely so heavily on the reception and fine discrimination of chemical cues in order to survive in an aquatic environment (Lima & Dill 1990; Wisenden 2000; Hay 2009).

In an aquatic medium chemoreception can be disrupted by a range of anthropogenically-induced changes in water chemistry (Lürling & Scheffer 2007). A growing number of studies have demonstrated the adverse effects of changes in pH on the chemo-responsiveness of freshwater organisms such as fish, crustaceans and molluscs (Hara 1976; Royce-Malmgren & Watson 1987; Allison et al. 1992; Moore 1994; Leduc et al. 2004; Tembo 2009; Turner & Chislock 2010). In a marine environment the pH of intertidal rock

pools fluctuates naturally, along with other physico-chemical characteristics (Truchot 1988), and can rise and fall rapidly on a diurnal basis during emersion (e.g., 9.5 - 6.5 pH units, Morris & Taylor 1983, and 10.16 - 7.29 pH units, Truchot, 1988), owing to algal and animal respiration. As well as undergoing such natural cyclical changes, sea water pH is predicted to decrease on a global scale as a result of anthropogenically induced ocean acidification (Caldeira & Wickett 2003; Raven et al. 2005). This decrease in pH occurs because as sea water absorbs more carbon dioxide (CO_2) a reaction takes place that increases the concentration of hydrogen ions ($[H^+]$). Extreme and localised high-CO_2/low pH events could also occur due to leaks from CO_2 geological storage sites, proposed as a mitigating strategy for CO_2 emissions (Seibel & Walsh 2001; Hawkins 2004); and from the periodic upwelling of CO_2-enriched sea water in some coastal regions (Feely et al. 2008; Thomsen et al. 2010). The average pH in rock pools is therefore likely to be reduced, and perhaps even forced below its natural limits, in the future, owing to the effect of ocean acidification; and leaks from storage sites and coastal upwellings may lead to rapid and severe acidification events. This could have biological consequences for intertidal species many of which, although resilient, are already living at the threshold of their tolerance limits (Stillman 2002; Tomanek & Helmuth 2002). Such reduced sea water pH has already been shown to affect the growth, metabolism and calcification of marine organisms (Pörtner et al. 2004; Orr et al. 2005; Findlay et al. 2010). Recently it has also been shown to disrupt the chemo-responsive behaviour of juvenile marine fish by interfering with their ability to correctly identify different odours, and, in some cases, revealing an inability to detect them at

all (Munday et al. 2009; Dixson et al. 2010; Munday et al. 2010); and a study on the European hermit crab, *Pagurus bernhardus* (Linnaeus), in this thesis, has shown that reduced pH disrupts their resource assessment and decision-making behaviour (Chapter 5: de la Haye et al. 2011).

The underlying mechanisms for reduced chemo-responsiveness under conditions of low sea water pH have yet to be elucidated, but there are four possibilities. Firstly, low pH could cause a change in the ionic state of the odour molecules, thereby rendering them unrecognisable *via* either a reversible or irreversible mechanism (Hara 1976; Brown et al. 2002). Secondly, low pH could reduce chemoreceptive acuity by changing the charge distribution on the odour receptor cells of an animal's sensory organs (Tierney & Atema 1988). Thirdly, low pH could cause physical damage to sensory organs, as it has been shown that calcified animals may experience dissolution of their exoskeletons under such conditions (Spicer et al. 2007). Finally, rather than indicating a direct effect upon chemoreception *per se*, changes in chemo-responsiveness might simply reflect reduced activity levels, or reduced motivation to respond to chemical cues, occurring as a result of the elevated metabolic load of maintaining acid-base balance under conditions of low pH (Pörtner et al. 2004). An increase in CO_2 in sea water can result in an internal hypercapnia in marine animals, where the $[H^+]$ in their haemolymph rises causing acidification, and such disruption must be compensated through acid-base regulation (Truchot 1983). This is a complex process, but in marine crustaceans it is achieved predominantly *via* ion transport mechanisms where sodium and hydrogen ions (Na^+/H^+) and chloride and bicarbonate ions (Cl^-/HCO_3^-) are exchanged across the gills in

order to expel H^+ and accumulate the HCO_3^- that reduces the internal acidosis (Henry & Wheatly 1992). This process can be energetically costly, and elicit trade-offs against other physiological and behavioural activities (Wood et al. 2008). Although intertidal animals exhibit adaptive behavioural and physiological mechanisms to cope with the variable environment they inhabit, including efficient iono-regulatory capabilities (Taylor & Spicer 1988; Truchot 1988), progressive climate change may create thresholds for dysfunction (Pörtner et al. 2004).

The hermit crab *Pagurus bernhardus* (Linnaeus) is a decapod crustacean, common to the coasts of north west Europe and the American Atlantic, that for most of its early adult life inhabits the rocky intertidal, and is commonly found in mid-shore rock pools. Hermit crabs rely heavily on chemoreception in order to detect food, potential mates and predators; and being predominantly opportunistic scavengers, rather than active predators, the ability to detect food sources from a distance is important to them (Lancaster 1988). *Pagurus bernhardus* is a species well suited to studies of reduced pH because it is a calcifying organism that also inhabits the calcified structures (shells) produced by marine gastropods, making it particularly vulnerable to the reduced calcification and dissolution effects of ocean acidification. This species is also an excellent model organism for investigating the potential effects of reduced pH on chemo-sensory behaviour in intertidal crustaceans because, like other decapods, it has two pairs of antennae: the longer antennae are involved mainly in mechano-reception; and the shorter antennules in detecting water-borne chemicals from a distance (Snow 1975). Typically the antennules are flicked rapidly through the

water, allowing chemicals to bind repeatedly to receptor sites (Schmitt & Ache 1979) and this flicking has been called the ‘sniffing’ response in decapods (Koehl 2006). The greatest flicking frequency is observed at the onset of a stimulus (Schmitt & Ache 1979; Reeder & Ache 1980; Devine & Atema 1982; Allison et al. 1992). The antennular flicking of *P. bernhardus* is easily visible and recordable, making it a tractable measure of chemo-responsiveness. The structure and function of the olfactory organs of crustaceans, as a group, are highly congruent (Hallberg et al. 1992); therefore any observed effect in *P. bernhardus* has the potential to affect other marine crustaceans in a similar way.

Here I examine the effects of a five-day exposure to reduced sea water pH on the chemo-responsiveness of the hermit crab *P. bernhardus*. In particular I test its ability to detect and locate a food odour. If reduced pH impairs the olfactory responses of the hermit crabs then I would expect the rate of antennular flicking (the ‘sniffing’ response in decapods) to be affected, and the hermit crabs’ ability to locate the odour source to be disrupted. Furthermore, by recording the effects of reduced sea water pH on locomotory activity and taking a measure of the iono-regulatory capacity of the crabs, I can investigate whether any direct effects on chemo-responsiveness might be owing to an overall increase in metabolic load, affecting foraging motivation.

METHODS

Study organisms

Hermit crabs, *P. bernhardus*, were collected by hand from mid shore rock pools at Hannafore Point, Looe, Cornwall, UK (50:20N, 04:27W), Sept 2009. Crab mass was standardised as much as possible (mass without shell = 1.02 g ± SD 0.22). Only crabs inhabiting *Littorina littorea* shells were collected, as this is the preferred shell species for crabs of this size (Briffa and Elwood, 2007). In the laboratory the individuals were kept in groups of 50-80 in holding aquaria (vol. =80 l) filled with aerated, filtered sea water ($S = 34_{PSU}$), and were provided with numerous refuges to limit potentially harmful agonistic interactions and cannibalism. All aquaria were maintained under conditions of natural light and at a temperature of 15 ± 0.1 ºC. At least one week was allowed to elapse between collection and the start of the experiment, to acclimate the hermit crabs to laboratory conditions. To standardise hunger levels, all crabs were food deprived for two days prior to the experiment. Twenty-four hours before the experiment the hermit crabs were carefully excised from their original shells using a bench vice, and their sex determined. Only males with a full complement of undamaged limbs, and free from obvious parasites, were used to control for any intersexual differences in behaviour. Each crab was presented with an empty, optimal shell (determined from a previous shell-selection experiment by Briffa & Elwood 2007), and allowed to recover for 24 h before being placed in treatment aquaria.

Experimental design

The study used a two-way design with two factors: sea water pH and odour pH. Crabs were kept in either untreated sea water (pH_{NBS} = 8.20), or reduced pH sea water (pH_{NBS} = 6.80) for five days, after which both groups underwent behavioural trials, under those same pH conditions, where they were presented with either an untreated or reduced pH food odour, and their behaviour observed. A 5-day exposure at a pH of 6.80 could mimic a CO_2 storage leak (Blackford et al. 2009), or indeed the most extreme scenario where water pH in rock pools can decline to such levels during spring tides, albeit in the latter case the depression is constant over periods of hours rather than days. A preliminary study of pools at the collection site showed that pH can fall to as low as pH_{NBS} = 7.29 in rock pools overnight. Either way, the behavioural consequences of exposure to low pH in this study are not totally dissimilar to what could be experienced under normal field conditions. The use of a manipulation that is not variable and relatively chronic, but ecologically informed, is necessitated by the high degree of variance encountered in behavioural data. By taking this experimental approach, I am able to identify behavioural effects that may be present, but would be difficult to discern following a brief or cyclical exposure. A total of 60 hermit crabs (N = 15 in each treatment group) were used in the experiment.

Treatments

Sea water treatments

During the experiment hermit crabs were kept in individual aquaria (vol = 1l) with either untreated (pH_{NBS} = 8.20) or reduced pH (pH_{NBS} = 6.80) artificial sea water (ASW) (Instant Ocean®) (S = 34_{PSU}, T = 15°C). Artificial sea water was used because it is assumed to be free of any biological cues. Untreated ASW was aspirated with untreated air, supplied under pressure (pCO_2 = 373.08 ± 4.37 µatm). In the reduced pH treatments CO_2-enriched air (pCO_2 = 12061.24 ± 141.38 µatm) was aspirated through the ASW using an air mixing system identical to that described by Findlay et al. (2008). In both cases actual [CO_2] was measured using a gas analyser (LI-7000, LI-COR Nebraska USA). Hermit crabs in the reduced pH treatments were introduced into aquaria with untreated ASW that was then gradually lowered to the nominated pH, to avoid shocking the animals. The ASW pH_{NBS} (SevenEasy™ S20 Mettler Toledo pH Meter, Switzerland) and total CO_2 concentration (Ciba-Corning 965, Carbon Dioxide Analyser, England) was recorded daily (see Results,Table 3.1.), and water changes were carried out every other day.

Odour treatments

Food odour was produced by crushing the white fish Coley (*Pollachius virens*) in either control or reduced pH ASW (concentration standardised by using 1 g fish : 50 ml ASW), and then filtering the resultant fluid through coarse filter paper (Whatman No. 4), the pH was then maintained by aspirating it with the appropriate CO_2 concentration. Aquaria air stones were

soaked for 20 mins prior to being placed in the behavioural chamber. Air stones were used to carry the odour because they are semi-porous and the crabs were not able to destroy them with their chelipeds. It had been established in a preliminary trial that the hermit crabs were attracted to the odour of this white fish.

Behavioural observations

After five days in each treatment, behavioural observations were conducted on the hermit crabs in a 15ºC temperature-controlled (CT) room, in a behavioural chamber filled with 4 l of either untreated ASW (pH_{NBS} = 8.20) or reduced pH ASW (pH_{NBS} = 6.80). The chamber consisted of a 20 l plastic aquarium, painted with stone-coloured pond paint (Blagdon™, Interpet Ltd, Dorking, Surrey, England) on three sides, and left transparent at the front to allow observations to take place. The chamber was then placed inside a lit observational chamber with a two-way mirror, and the lights of the CT room switched off, so that the observer did not distract the crab. Assays consisted of a choice experiment with the chamber divided into three sections, with sliding Perspex doors separating each section, and small, shallow Petri dishes fixed at each end of the chamber to hold the cue stones (see Chapter 2, Figure 2.2.). Air stones were soaked in either untreated or acidified fish cue for 20 mins. The hermit crabs were placed in the centre of the aquarium, with the Perspex doors down, and allowed to acclimate for 10 min. After this period cue stones were added, using a randomised system, to each end of the chamber. The odour was allowed to diffuse in still water to mimic a rock

pool environment. There is evidence that water currents stimulate antennular flicking, which would be a complicating factor (Snow 1975). The doors were immediately lifted and the behaviour of the hermit crabs was observed for 10 min, and recorded using The Observer Mobile 5.0 Workabout mx handheld computer (Psion Teklogix Inc., Ontario, Canada). The behavioural measures recorded were: the time spent in each section of the behavioural chamber, the time spent in contact with the cue source, the latency to contact the cue source, the number of antennular flicks, and the duration of locomotion.

Haemolymph analysis

Haemolymph samples were removed from a sub-sample of the hermit crabs from each treatment (14 from pH_{NBS} = 6.80, and 11 from pH_{NBS} = 8.20). Immediately after their behavioural trials, crabs were carefully removed from their shells using a bench vice, and haemolymph samples (≤ 20 µl) were extracted from each crab using a micro syringe (BD Micro-Fine1 ml, Becton, Dickinson and Company, Franklin Lakes, NJ, U.S.A.), the needle of which (29 G) was inserted into the infrabranchial sinus, *via* the arthrodial membrane at the base of the third pereiopod. Samples were analysed for the cations sodium (Na^+), magnesium (Mg^{2+}), potassium (K^+), and calcium (Ca^{2+}) using an ICP Optical Emission Spectrometer (Varian 725-ES, Mulgrave, Australia); and the anion chloride (Cl^-) using a chloride meter (Jenway PCLM3, Essex, UK). For the haemolymph ions a strong ion difference (SID) analysis was carried out. The SID in any solution is defined as the sum of all the strong cation concentrations, minus the sum of all the strong anion concentrations,

and can be used to characterise acid-base disturbances (Stewart 1981). This method has been used to try and explain the observed changes in haemolymph pH during acid-base regulation in marine animals (Cameron & Iwama 1987; Luquet & Ansaldo 1997; Whiteley et al. 2001).

Additional experiment - baseline flicking

An additional study was undertaken to establish a baseline flicking rate under normal sea water pH conditions in the presence and absence of a chemical cue. Hermit crabs, *P. bernhardus*, were also collected from Hannafore Point in April 2011, and maintained as before. Hermit crabs were food deprived for 48 h prior to the experiment and 24 h before the experiment crabs were excised from their shells, sexed and given a replacement shell, as described earlier. A repeated measures design was adopted using 20 crabs that randomly underwent behavioural trials, both with a cue and without a cue. Crabs were placed in a 1L aquarium of ASW ($S = 34_{PSU}$, $T = 15^{o}C$, $pH_{NBS} = 8.20$). The aquarium was then placed in a lit observational chamber with a two-way mirror, and the lights of the temperature controlled ($15^{o}C$) room switched off, so that the observer did not distract the crab. Crabs were left for 10 min to settle. Following that settling period, 1 ml of either ASW or ASW mixed with a fish cue (see odour treatments for preparation) was injected gently into the water using a syringe (BD 10 ml, Becton, Dickinson and Company, Franklin Lakes, NJ, U.S.A.), 2 mins were allowed to elapse for the crabs to calm down and for the introduced water to disperse, and then antennular flicking and duration of locomotion were recorded using the

Observer Mobile 5.0 Workabout mx handheld computer (Psion Teklogix Inc., Ontario, Canada).

Ethical note

No crabs were injured during the process of removing them from shells by use of the bench vice. The removal of 10-20 µl of haemolymph samples from crabs of this size is not fatal, and following this procedure crabs were supplied with new shells then returned to the shore after re-acclimation, and a recovery period of at least two weeks. I minimised the number of individuals used by keeping sample sizes smaller for the haemolymph analysis.

Statistical methods

Data were Log_{10} transformed, where necessary, and for the main experiment were analysed with StatView 5.0 (SAS, Cary, NC, U.S.A.) using a three-way one within (position in the chamber), two between (cue pH and sea water pH), repeated measures ANOVA, in order to investigate the time spent in each section of the behavioural chamber (the cue end, the middle, or the end with no cue). Two-way ANOVAs were performed for the time spent in contact with the cue source, the latency to contact the cue source, the number of antennular flicks, and the duration of locomotion. For the haemolymph ions one-way ANOVAs were used to compare SID and individual ion concentrations between untreated and reduced pH sea water groups. A Pearson product-moment correlation was used on a subset of the data to see

if there was any relationship between the time spent in motion and haemolymph ion concentrations in the reduced pH sea water group. In order to compare the magnitude of the effects of reduced sea water pH treatment on different behavioural measures, I calculated their effect size estimates, partial eta^2 (η^2_p) (Briffa et al. 2008), where appropriate. Data for the additional base-line flicking experiment were analysed using paired t-tests (SPSS, 18.0 Chicago, IL, USA).

RESULTS

Main experiment

There was no significant effect of cue pH ($F_{1,56} = 1.0$, $P = 0.322$), or sea water pH ($F_{1,56} = 1.0$, $P = 0.322$) on the time spent by the hermit crabs in each section of the behavioural chamber. There was also no significant interaction effect between cue pH and sea water pH ($F_{2,112} = 1.0$, $P = 0.322$), section and cue pH ($F_{2,112} = 0.433$, $P = 0.649$), or section, cue pH and sea water pH ($F_{2,112} = 0.610$, $P = 0.545$). There was, however, a significant effect of section ($F_{2,112} = 14.083$, $P < 0.0001$) and a two-way interaction effect between section and sea water pH ($F_{2,112} = 14.374$, $P < 0.0001$) indicating that the time spent in different sections of the chamber varies between the sea water pH treatments, with hermit crabs in the control sea water spending longer in the cue section of the chamber (Figure 3.1.).

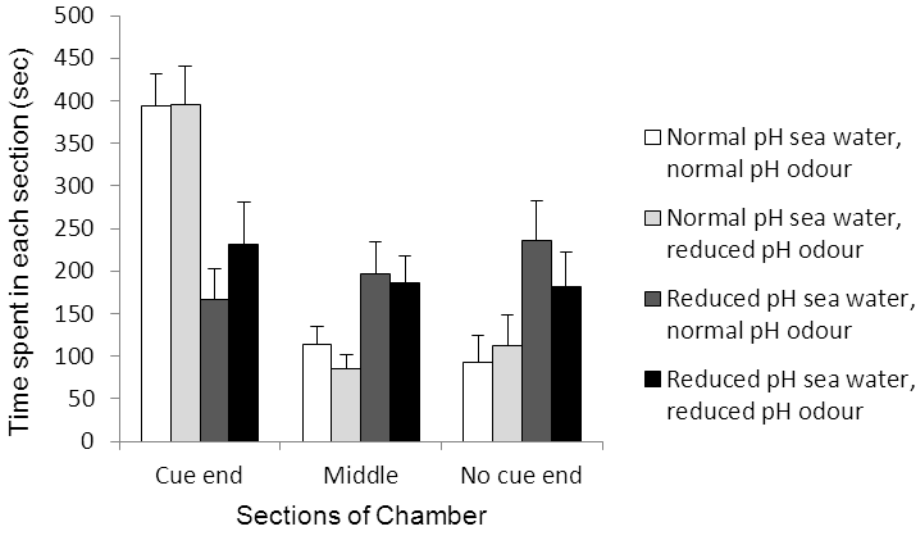

Figure 3.1. Mean time (± SE) spent by the hermit crabs in each section of the behavioural chamber in either untreated sea water or reduced pH sea water, and in the presence of either a control pH odour or a reduced pH odour.

There was no significant effect of cue pH ($F_{1,56} = 0.271$, $P = 0.6047$) on the time spent in contact with the cue, and no significant interaction effect between cue pH and sea water pH ($F_{1,56} = 0.067$, $P = 0.796$). There was, however, a significant effect of sea water pH on the time spent in contact with the cue, with the hermit crabs in the reduced pH sea water groups spending significantly less time in contact with the cue than those in the control sea water groups ($F_{1,56} = 22.757$, $\eta^2_p = 0.289$, $P < 0.0001$) (Figure 3.2.).

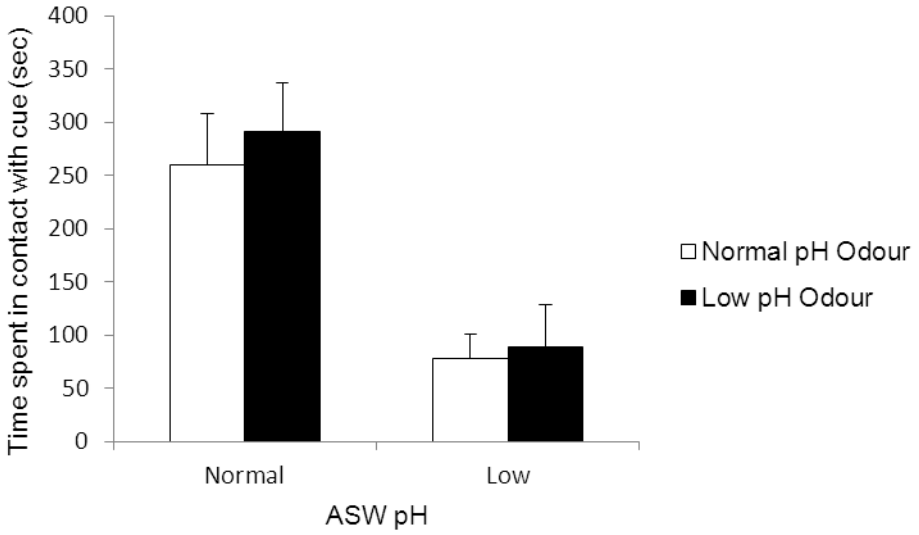

Figure 3.2. Mean time spent in contact with the food cue (± SE) by the hermit crabs in either untreated sea water or reduced pH sea water, and in the presence of either a control pH odour or a reduced pH odour.

There was no significant effect of cue pH on the latency to contact the cue ($F_{1,56} = 0.344$, $P = 0.560$), and no significant interaction effect between cue pH and sea water pH ($F_{1,56} = 0.216$, $P = 0.644$). There was, however, a significant effect of sea water pH on the latency to contact the cue, with the hermit crabs in the reduced pH sea water groups taking significantly longer to contact the cue than those in the control sea water groups ($F_{1,56} = 4.685$, $\eta^2_p = 0.077$, $P = 0.0347$) (Figure 3.3.).

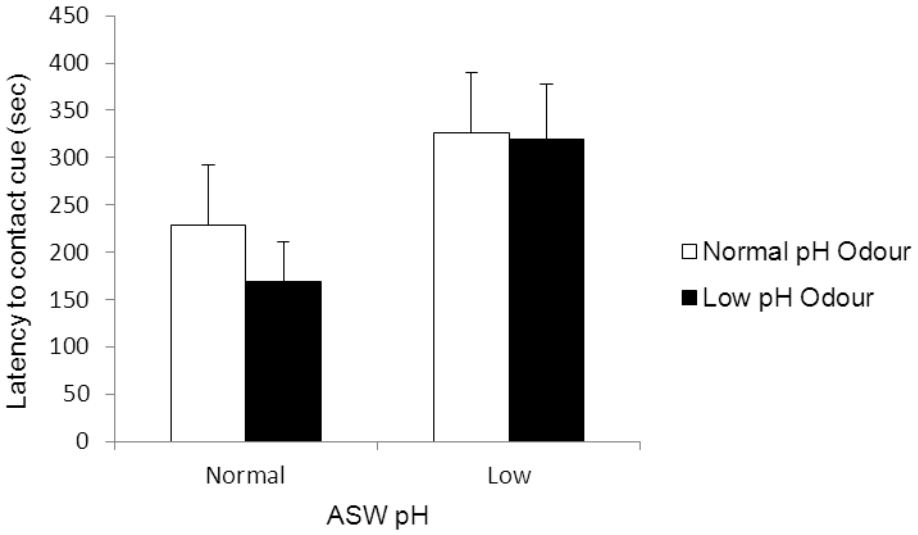

Figure 3.3. Mean time taken to contact the food cue (± SE) by the hermit crabs in either untreated sea water or reduced pH sea water, and in the presence of either a control pH odour or a reduced pH odour.

There was no effect of cue pH on antennular flicking ($F_{1,56} = 0.281$, $P = 0.598$) and no interaction effect between cue pH and sea water pH on antennular flicking ($F_{1,56} = 2.061$, $P = 0.157$). There was, however, a significant effect of sea water pH on the rate of antennular flicking, with lower rates of flicking from hermit crabs in the reduced pH sea water groups compared to the control sea water groups ($F_{1,56} = 106.642$, $\eta^2_p = 0.656$, $P < 0.0001$) (Figure 3.4.).

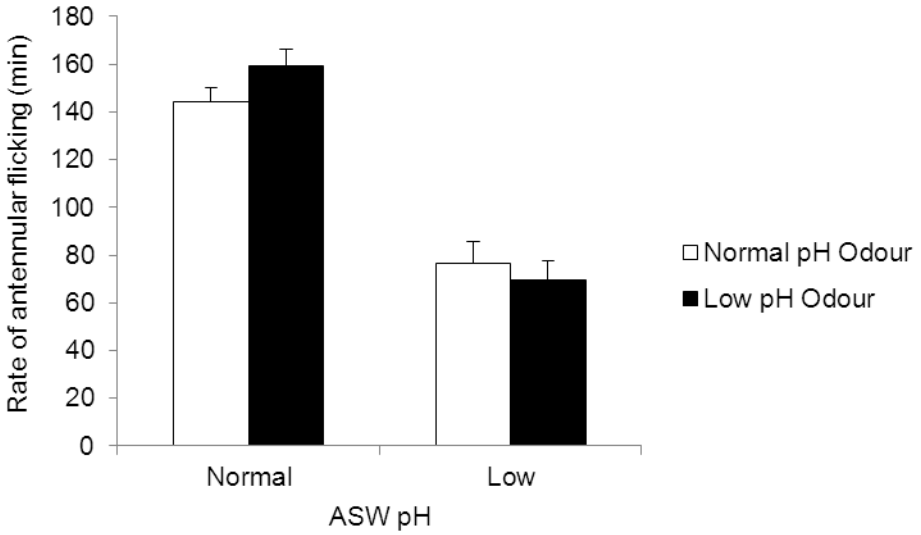

Figure 3.4. Mean rate of antennular flicking per minute (± SE) performed by the hermit crabs in either untreated sea water or reduced pH sea water, and in the presence of either a control pH odour or a reduced pH odour.

There was no significant effect of cue pH on the activity levels of the hermit crabs ($F_{1,56} = 0.174$, $P = 0.678$), and no significant interaction effect between cue pH and sea water pH ($F_{1,56} = 0.156$, $P = 0.695$). There was, however, a significant effect of sea water pH on the activity of the hermit crabs, with the amount of time spent in motion significantly lower in the reduced pH sea water groups compared to the control sea water groups ($F_{1,56} = 10.855$, $\eta^2_p = 0.162$, $P = 0.0017$) (Figure 3.5.).

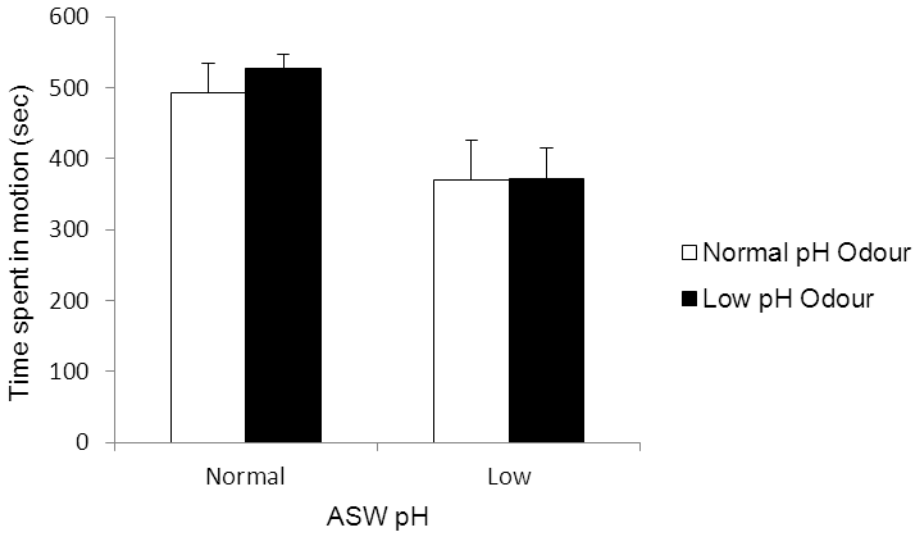

Figure 3.5. Mean time spent in motion (± SE) by the hermit crabs in either untreated sea water or reduced pH sea water, and in the presence of either a control pH odour or a reduced pH odour.

There were no significant difference between the treatments for the SID ($F_{1,23} = 1.132$, $P = 0.298$), or for the individual ions Na^+($F_{1,23} = 2.686$, $P = 0.115$), Mg^{2+}($F_{1,23} = 0.061$, $P = 0.808$), Ca^{2+}($F_{1,23} = 0.166$, $P = 0.688$), or K^+($F_{1,23} = 3.422$, $P = 0.077$). There was, however, a significant difference between the treatments for Cl^- ($F_{1,23} = 4.423$, $P = 0.047$) with the crabs in the reduced pH sea water showing increased concentrations of Cl^-. There were no correlations between the time spent in motion and antennular flicking rate, and the [Cl^-] in the reduced pH sea water group ($r_s = 0.145$, $N = 14$, $P = 0.620$; $r_s = 0.235$, $N = 14$, $P = 0.419$).

Additional experiment

In the presence of the cue hermit crabs flicked their antennules at a significantly greater rate ($t_{(19)}$ = -11.254, $P < 0.001$) than when the cue was absent, and their activity levels were also significantly greater ($t_{(19)}$ = -11.677, $P < 0.001$) than when the cue was absent.

Table 3.1. Mean ± SD for experimental aquaria ASW physical parameters measured throughout the 5 day exposure period (90 measurements from each treatment group). The parameters were calculated with CO2sys (Pierrot et al. 2006) using the pH and pCO_2 values with dissociation constants from Mehrbach et al. (1973) refit by Dickson and Millero (1987) and KSO_4 using Dickson (1990).

Variable	**Experimental Aquaria**	
Nominal pH	pH 8.20	pH 6.80
Measured pH		
NBS	8.21 ± 0.05	6.81 ± 0.05
Total	8.09 ± 0.05	6.70 ± 0.05
TCO_2 (mmol l^{-1})	2.19 ± 0.24	3.05 ± 0.30
Salinity	34.04 ± 0.06	34.02 ± 0.04
Temperature (ºC)	15.10 ± 0.09	15.08 ± 0.08
pCO_2 (µatm)	373.08 ± 4.37	12061.24 ± 141.38
Alkalinity (µEq kg^{-1})	2445.42 ± 286.46	2611.96 ± 307.00
Calcite saturation	4.43 ± 0.88	0.23 ± 0.06
Aragonite saturation	2.844 ± 0.57	0.15 ± 0.04
HCO_3^- (mmol l^{-1})	1995.25 ± 206.13	2588.90 ± 302.42
CO_3^{2-} (mmol l^{-1})	184.84 ± 36.84	9.64 ± 2.30

DISCUSSION

This study is the first to clearly demonstrate that reduced sea water pH adversely affects the chemo-sensory behaviour of an intertidal crustacean. Both groups of hermit crabs held in the reduced pH sea water flicked their antennules (the 'sniffing' response in decapods) at a significantly reduced rate than those in the untreated sea water; they took longer to locate the odour source, and spent less time in contact with it, than those in untreated sea water. There did not appear to be any permanent degradation of the cue under reduced pH conditions, as crabs in the control sea water were still able to detect a cue that had been acidified. Crabs in the reduced pH group showed less locomotory activity, a sign either of a lack of stimulation, or of a metabolic depression. An elevated concentration of chloride ions (Cl^-) in crabs from the reduced pH group, suggests some disruption to iono-regulatory ability and/or acid-base balance. Thus physiological stress, and perhaps its effects on the motivation to forage, might be partly responsible for the reduced chemo-responsiveness under low pH conditions. However, the effect size of low pH on the specific chemo-responsive behaviour, antennular flicking, was greater than that for activity levels. Moreover, there was no correlation between [Cl^-] and activity levels, suggesting that there must also be a direct effect of low pH on the chemoreception process itself.

Hermit crabs kept and tested in reduced pH sea water spent less time in the vicinity of (Figure 3.1.), and in contact with (Figure 3.2.), the cue stone; took longer to locate the odour source (Figure 3.3.); and had lower antennular flicking rates (the 'sniffing' response in decapods) (Figure 3.4.), compared to

those in untreated sea water. These results are similar to a study on the freshwater crayfish, *Cambarus bartoni*, which flicked its antennules at a reduced rate and failed to locate a food odour under low pH conditions (Allison et al. 1992). This absence of a detectable response in the hermit crabs and the crayfish could have been due either to an inability to detect the odour through disruption or damage to chemoreceptors, or to low motivation brought on by the energetic costs of iono-regulatory and/or acid-base disturbance. It is possible that reduced pH could cause physical damage to sensory organs, as it has been shown that calcified animals may experience dissolution of their exoskeletons under such conditions (Spicer et al. 2007; Hall-Spencer et al. 2008). However, the antennules of hermit crabs in a previous study were viewed under an electron microscope and did not reveal any visible damage after a 5-day exposure to reduced pH sea water (see Appendix 2). Similarly, Munday et al. (2009) did not find any evidence of visible damage to the olfactory organs of their fish larvae using electron microscopy. A hypothesis that it is the receptor sites themselves that are affected by the reduced pH was proposed by Tierney and Atema (1988) who suggested that the increased [H^+] might alter the charge distribution on the odour receptor cells of an animal's sensory organs. This is difficult to test directly, however, the greatest antennular flicking frequency occurs at the onset of chemical stimulation (Schmitt & Ache 1979), and this was borne out in my additional study on base-line flicking where crabs in normal pH sea water flicked their antennules at a significantly faster rate in the presence of a chemical cue than when it was absent. Therefore the decreased flicking observed under reduced pH conditions, coupled with less time spent near the

source of the cue, or in contact with it, could imply an impaired ability to detect the chemical stimulus.

Crabs in the control sea water presented with an acidified odour were still able to detect it. This indicates that there was no permanent pH-induced degradation of the cue, altering its conformation and rendering it either undetectable or unrecognisable to the crabs. However, as the pH of the odour could not be monitored once it had been placed in the behavioural chamber, it is possible that any effects of CO_2 upon the odour were reversible and that in the control sea water the cue was re-buffered to a normal pH level. Brown et al. (2002) showed that loss of response in salmonids to a conspecific alarm cue was owing to a non-reversible degradation of that cue, possibly as a result of a covalent change to the alarm pheromone molecule. However, the chemical structure of cues differs and they may not all be affected by pH changes in the same way. Further studies would have to be carried out to determine whether there is any temporary reversible effect on this particular food odour cue.

The pH-related reduction in locomotory activity could indicate metabolic depression owing to the energy required to regulate acid-base balance, or support enhanced metabolic activity and calcification, in high-CO_2 conditions (Pörtner et al. 2004; Wood et al. 2008). Physical stress is likely to affect an animal's motivational state, and energetically demanding activities, such as foraging, could be suspended in such circumstances. Reduced locomotion has been demonstrated in response to physiological stress in a range of crustaceans (Taylor & Spicer 1988; Eriksson & Baden 1997; Taylor & Eggleston 2000; McAllen & Taylor 2001), but may also indicate a lack of

olfactory stimulation (Reeder & Ache 1980). The additional base-line flicking study revealed that crabs in normal pH sea water showed significantly less locomotory activity in the absence of a chemical cue, implying that a lack of stimulation can also be the cause of reduced activity, and agreeing with a study by Reeder and Ache (1980) on lobsters. If both a metabolic depression and a lack of stimulation can elicit a reduction in movement, conclusions about the proximate cause of such behaviour should be made with caution.

In an attempt to shed some light on whether the observed reduction in the locomotory activity of the crabs in reduced pH sea water indicated a lack of stimulation, or a metabolic depression, an ion analysis of the haemolymph from a subset of the crabs was carried out. There was no significant difference in SID between the reduced pH and untreated sea water groups. However, analyses of the individual ions detected a difference between the groups in the concentrations of Cl^- present, with greater concentrations of Cl^- in the haemolymph of those hermit crabs in the reduced pH sea water groups. Other studies on hypercapnia in decapod crustaceans found a decrease in [Cl-] owing to the uptake of HCO_3^- to compensate a hypercapnic acidosis (Truchot 1979; Cameron & Iwama 1987). However, the increase in [Cl-] in the hermit crabs is similar to Dissanayake et al. (2010), where two species of palaemonid prawns, *Palaemon serratus* and *Palaemon elegans*, showed differential haemolymph ion concentrations after 14 days exposure to reduced pH sea water. *Palaemon serratus*, a lower shore species like *P. bernhardus*, had elevated concentrations of Na^+, Ca^{2+} and Cl^-, while *P. elegans*, a high shore species, had reduced concentrations of the same ions; indicating different mechanisms for ionic regulation. However, because of

their freshwater origins, the Palaemonidae, as a group, show a pronounced hypo-regulatory pattern, and maintain their ions at a level considerably less than that present in full-strength sea water (Parry 1954), therefore any comparison between *P. serratus* and *P. bernhardus* should be made with caution. The elevated Cl^- concentration in the haemolymph of *P. bernhardus* in the reduced pH treatment could be interpreted as some form of ion exchange activity taking place, either in order to compensate for disruption of acid-base balance, or because of disruption in Cl^- exchange itself. However, without additional data on haemolymph pH and $[HCO_3^-]$ this remains unclear. The fact that the hermit crabs were observed spending less time in motion, could support the hypothesis of metabolic depression, and represent a trade-off between the energetically costly activities of acid-base regulation and foraging. However this effect was statistically weak when compared to the reduced function of the antennules and the impaired ability to locate the food odour; and there was no relationship between the duration of locomotion, or the rate of antennular flicking, and the concentration of haemolymph Cl^-. The fact that the behavioural effects were far more pronounced than the physiological ones, suggests that the change observed in their behaviour is unlikely to be solely owing to acid-base disruption, and low motivation, but also to a disruption to the chemo-receptive process itself. However, it is also possible that, as has recently been seen in reef fish (Nilsson et al. 2012), changes in the Cl^- levels due to ionic regulation might interfere with nerve transmission, thus altering perception or muscular control. The direction of Cl^- change observed in the hermit crabs is different to that observed in fish, therefore the precise mechanism remains unknown at present.

The inhabitants of rock pools may experience disruptions to their normal behaviour for extended periods during tidal cycles owing to a reduction in sea water pH, and such reductions are likely to be exacerbated in the future by ocean acidification, potential CO_2 sequestration leaks, and CO_2 upwelling events. This study clearly shows that reduced sea water pH disrupts chemo-sensory behaviour in an intertidal crustacean, and is consistent with recent studies on marine fish (Munday et al. 2009; Dixson et al. 2010). It also shows that the odour was not irreversibly degraded by reduced pH, and that the observed disruption to the chemo-sensory behaviour of *P. bernhardus* is likely to stem from disruption to either the reception of the odour, or to the crabs' physiological status. The comparative strength of the behavioural responses, and the lack of any association between haemolymph [Cl^-] and activity levels, indicates that a change in overall physiological condition cannot explain all of the reduction in chemo-responsiveness, suggesting that there must also be a direct effect of low pH on chemo-receptive ability. A pH-induced disruption to chemo-responsiveness in crustaceans, and potentially other groups, is indicated, and such dysfunction could generate widespread confusion in an environment where animals must regularly, and rapidly, process large amounts of olfactory information. There is also likely to be differential effects between species, depending on their tolerance to pH changes, and this has the potential to re-order intertidal community dynamics and structure.

CHAPTER 4

Medium-term exposure to CO_2-acidified sea water reveals a gradient in behavioural responses: evidence of a threshold for disruption?

Abstract: A reduction in sea water pH is one of the predicted effects of ocean acidification, and could be a consequence of leaks from proposed carbon dioxide (CO_2) sequestration sites and upwelling events; it is also a regular feature of conditions in intertidal rock pools. CO_2-acidified sea water has been shown to disrupt the behaviour and olfactory responses of marine animals. However, it is not clear what the threshold for dysfunction is, or whether behavioural responses are a good indicator of overall condition. Here I investigate the chemo-sensory responses of the hermit crab *Pagurus bernhardus* over time to a number of different pH levels. Crabs were kept in 5 different sea water pH treatments (nominal pH_{NBS} = 6.80, 7.35, 7.70, 7.90 and 8.00) for a period of 60 days. Their responses to a food odour were tested periodically (on days 7, 14, 30 and 60) under those same pH conditions. Haemolymph samples were taken at the end of the experiment, to test for evidence of extracellular acid-base disturbance. Crabs showed a gradient in response, with antennular flicking rates ('sniffing' response) decreasing as pH decreased, with significant step decreases in response at pH_{NBS} = 7.70 and 6.80. Responses over time differed with an increase in the flicking response on day 60. Crabs in the pH_{NBS} = 6.80 treatment had significantly lower activity levels than those in the other treatments. Of the haemolymph ion concentrations measured (calcium (Ca^{2+}), chloride (Cl^-), magnesium (Mg^{2+}), potassium (K^+) and sodium (Na^+)), crabs in the low pH treatments had progressively greater concentrations of Ca^{2+}, indicating the possibility of some iono-regulatory disturbance. This increase in Ca^{2+} concentrations was negatively correlated with antennular flicking rate on day 60. Survival also decreased as the pH level decreased. This study reveals a gradient in

disruption to a behavioural response with thresholds for dysfunction; with a correlation between a behavioural response and a physiological measure, and a similar pattern in response to reduced sea water pH in both behavioural and physiological measures, and in survival rates. There is also evidence of possible acclimation to low pH conditions by surviving individuals on day 60. These results link behavioural and physiological measures and suggest that behaviour may be a sensitive indicator of overall condition. They also reveal some interesting differences in the responses of individuals within the same treatment groups which, alongside the evidence of possible acclimation, warrant further investigation. This study demonstrates that behavioural responses may be a fruitful area of research in relation to environmental disturbances if changes in behaviour can be sensitive indicators of overall condition.

INTRODUCTION

Chemoreception is the mechanism by which animals detect chemical odours in their environment, providing them with information about the location of food, potential mates, or other resources, and alerting them to the presence of predators. In aquatic environments, where low light levels often limit visibility, well-developed olfactory senses can be crucial to survival (Zimmer-Faust 1987, Lima & Dill 1990, Jacobsen & Stabell 2004). Marine animals exist in a medium that is rich in chemicals emitted by a variety of organisms, and it is important for them to be able to discriminate between odours; this has led to the evolution of highly specific chemical receptors (Atema 1995). If the ability to detect and identify odours is impaired in any way, then it could have serious consequences for survival (Wisenden 2000).

Reduced sea water pH, one of the predicted consequences of ocean acidification (Caldeira & Wickett 2003; Raven et al. 2005) and carbon dioxide (CO_2) carbon capture storage (CCS) leakages (Hawkins 2004; Blackford et al. 2009) have already been shown in numerous studies to have serious effects on marine biota by interfering with their energetic and metabolic functions (Pörtner et al. 2004; Widdicombe & Spicer 2008), and by affecting calcification rates in species that build carbonate shells and skeletons (Orr et al. 2005; Wood et al. 2008; Findlay et al. 2010, 2011; Neinhuis 2010). Recently it has also been shown to affect the behaviour of marine fish by disrupting their ability to detect vital settlement and predator cues (Munday et al. 2009, Dixson et al. 2009), and by affecting the chemo-responsiveness (Chapter 3: de la Haye et al. 2012) and resource assessment and decision

making activities (Chapter 5: de la Haye et al. 2011) of hermit crabs, *Pagurus bernhardus*. Although the behavioural consequences of low pH have not, so far, been a major focus for ocean acidification research, the study of behavioural responses to environmental disruption is important as behaviour can link physiological function with ecological processes (Scott & Sloman 2004). Behavioural plasticity can also allow animals to respond rapidly to environmental disruption where physical acclimation might take longer to achieve, or be too costly (Miner et al. 2005; Sih et al. 2010; Tuomainen & Candolin 2010).

As yet, few studies have directly investigated the effects of low pH on the behavioural activities performed by intertidal animals (although see Bibby et al. 2007 and Chapter 3 for exceptions). In comparison with sub-littoral organisms, which are adapted to relatively stable environments, intertidal organisms must cope with frequent and extreme variations in pH within tidal cycles. Rock pools and under stone environments are therefore intertidal environments that experience enormous spatial and temporal variability in their physico-chemical characteristics (Agnew & Taylor 1986; Truchot 1988). In addition to pH, salinity, temperature, and oxygen concentrations are all dependent upon weather conditions, the time of day, and the presence of algae and animals (Huggett & Griffiths 1986). The pH in rock pools can show wide diurnal fluctuations during emersion (eg., 7.2 - 9.5 pH units, Daniel and Boyden 1975, 6.5 - 9.5 pH units, Morris and Taylor 1983, and 7.29 - 10.16 pH units, Truchot 1988), owing to algal and animal respiration. It may be assumed, therefore, that rock pool species might be more tolerant to reduced pH, and other variables related to CO_2-induced climate change, than sub

littoral or deep-sea organisms (Widdicombe & Spicer 2008), and their behaviour could be less susceptible to disruption. Rock pools might, under certain conditions, offer a refuge from any decline in ocean pH when oxygen tension is boosted by algal photosynthesis; but at other times, they could experience even more extreme reductions in pH, if average sea water pH is already depressed as a result of ocean acidification. Ocean pH is projected to fall by up to 0.3 units in surface waters by 2100, and by 0.7 by 2300 (Caldeira & Wickett 2003), and a high CO_2 CCS leak is predicted to lower ambient sea water pH by up to 0.9 units (Blackford et al. 2009). More prolonged periods of exposure to such extremes could have biological consequences, as many intertidal species are already living at the threshold of their tolerance limits (Stillman 2002, Tomanek & Helmuth 2002). Consequently, any observed effects on an intertidal species would mean that the implications for closely related sublittoral species might be far more serious.

The underlying mechanisms for reduced chemo-responsiveness under conditions of reduced sea water pH have yet to be elucidated, but there are four possibilities. Firstly, low pH could cause a change in the ionic state of the odour molecules, thereby rendering them unrecognisable *via* either a reversible or irreversible mechanism (Hara 1976; Brown et al. 2002). Secondly, low pH could reduce chemoreceptive acuity by changing the charge distribution on the odour receptor cells of an animal's sensory organs (Tierney & Atema, 1988). Thirdly, low pH could cause physical damage to sensory organs, as it has been shown that calcified animals may experience dissolution of their exoskeletons under such conditions (Spicer et al. 2007). Finally, rather than indicating a direct effect on chemoreception *per se*,

changes in chemo-responsiveness might simply reflect reduced activity levels, or reduced motivation to respond to chemical cues, occurring as a result of metabolic depression brought on by the energy required to regulate acid-base balance, or support enhanced metabolic activity and calcification in high-CO_2 conditions (Pörtner et al. 2004; Wood et al. 2008). An increase in CO_2 in sea water can result in an internal hypercapnia in marine animals, where the $[H^+]$ in their haemolymph rises causing acidification, and such disruption must be compensated through acid-base regulation (Truchot 1983). This is a complex process, but in marine crustaceans it is achieved predominantly *via* ion transport mechanisms where sodium and hydrogen ions (Na^+/H^+) and chloride and bicarbonate ions (Cl^-/HCO_3^-) are exchanged across the gills in order to expel H^+ and accumulate the HCO_3^- that reduces the internal acidosis (Henry & Wheatly 1992). This process can be energetically costly, and elicit trade-offs against other physiological and behavioural activities (Wood et al. 2008). Although intertidal animals exhibit adaptive behavioural and physiological mechanisms to cope with the variable environment they inhabit, including efficient iono-regulatory capabilities (Taylor & Spicer 1988; Truchot 1988), progressive climate change may create thresholds for dysfunction (Pörtner et al. 2004). Impairment is likely to have a differential effect upon both different species and upon individuals from the same species, leading to a change in ecosystem dynamics affecting both intra- and interspecific interactions (Fabry et al. 2008).

The hermit crab *Pagurus bernhardus* (Linnaeus) is a decapod crustacean, common to the coasts of northwest Europe and the American Atlantic, that for most of its early adult life inhabits the rocky intertidal, and is

commonly found in mid-shore rock pools. Hermit crabs rely primarily on chemoreception in order to detect food, potential mates and predators; and being predominantly opportunistic scavengers, rather than active predators, the ability to detect food sources from a distance is important to them (Lancaster 1988). *Pagurus bernhardus* is a species well suited to studies of reduced pH because it is a calcifying organism that also inhabits the calcified structures (shells) produced by marine gastropods, making it particularly vulnerable to the dissolution effects of ocean acidification. This species is also an excellent model organism for investigating the potential effects of reduced pH on chemo-sensory behaviour in intertidal crustaceans because its behavioural repertoires are already well understood (Reese 1962; Jackson & Elwood 1989; Briffa & Elwood 2000). In addition, like other decapods, it has two pairs of antennae: the longer antennae are involved mainly in mechano-reception; and the shorter antennules in detecting water-borne chemicals from a distance (Snow 1975). Typically the antennules are flicked rapidly through the water, allowing chemicals to bind repeatedly to receptor sites (Schmitt & Ache 1979) and this flicking has been called the 'sniffing' response in decapods (Koehl 2006). The greatest flicking frequency is observed at the onset of a stimulus (Schmitt & Ache 1979; Reeder & Ache1980; Devine & Atema 1982; Allison et al. 1992). The antennular flicking of *P. bernhardus* is easily visible and recordable, making it a tractable measure of chemo-responsiveness. The structure and function of the olfactory organs of crustaceans, as a group, are highly congruent (Hallberg et al. 1992); therefore any observed effect in *P. bernhardus* has the potential to affect other marine crustaceans in a similar way.

In Chapters 2 and 3 I demonstrated that chemo-responsive behaviour is disrupted by low pH in the short term. Pörtner et al. (2004), however, identified the need for long-term studies that identify critical threshold levels for adverse effects of CO_2 in tolerant, as well as sensitive species. It is important to establish when, and at what pH level, effects start to appear, if the causes of them can be mitigated. Tolerant species can also act as indicators for sensitive species. Consequently the aim of this study was to expose *P. bernhardus*, an intertidal species whose chemosensory behaviour has already been shown to be adversely affected by highly reduced sea water pH (pH_{NBS} = 6.80) over a short-term exposure of five days (Chapters 3 and 5), and expose it to a range of different pH levels (including levels in line with future OA predictions and CO_2 CCS leakage scenarios) over a longer time period, in order to identify thresholds for disruption in chemo-sensory behaviour. If reduced pH impairs the responsiveness to olfactory cues, as shown in previous studies, then I would expect the rate of antennular flicking ('sniffing' response) to be affected along a gradient, with thresholds for reduced flicking rates, and for the hermit crabs to find it increasingly difficult to locate food odours, or exhibit less interest in them, either over time, or with decreasing pH, or both. Furthermore, by measuring the crabs' haemolymph ion concentrations (calcium (Ca^{2+}), chloride (Cl^-), magnesium (Mg^{2+}), potassium (K^+), and sodium (Na^+)) I can investigate an aspect of their iono-regulatory capacity at the different pH levels, and by recording their survival rates over the 60 days I am able to check for associations between both these measures and the behavioural variables, and look for similar patterns in response.

METHODS

Study organisms

Hermit crabs, *P. bernhardus*, were collected by hand from mid shore rock pools at Hannafore Point, Looe, Cornwall, UK (50:20N, 04:27W) in April 2010. Crab mass was standardised as far as possible (mass = 1.02 g ± SD 0.22). Only crabs inhabiting *Littorina littorea* shells were collected, as this is the preferred shell species for crabs of this size (Briffa & Elwood 2007). In the laboratory individuals were kept in groups of 30-40 in holding aquaria 40 l) supplied with aerated, filtered sea water, and were provided with numerous refuges to limit potentially harmful agonistic interactions and cannibalism. All aquaria were maintained under conditions of natural light and in a temperature controlled room at 12 ºC. At least one week was allowed to elapse between collection and the start of the experiment, to acclimatise the hermit crabs to laboratory conditions. To standardise hunger levels, all crabs were food deprived for two days prior to the experiment. Twenty-four hours before the experiment the hermit crabs were carefully excised from their original shells using a bench vice, and their sex determined. Only males with a full complement of undamaged limbs, and free from obvious parasites were used, to control for any intersexual differences in behaviour. Each crab was presented with an empty, optimal shell (determined from a previous shell-selection experiment by Briffa and Elwood (2007)), and allowed to recover for 24 h before the start of the experiment. At the start of the experiment crabs were placed in temporary holding chambers where the pH of the sea water

was lowered gradually to the appropriate level over a 4 h period (a rate of approximately a pH_{NBS} = 0.30 reduction per hour), to mimic the rate of change in rock pools and avoid shocking the animals before they were placed in treatment aquaria.

Experimental design

I used a repeated measures design with two factors: sea water pH and time. Crabs were kept in five different pH treatments pH_{NBS} = 8.00, 7.90, 7.70, 7.35 and 6.80 for 60 days. Behavioural trials, under those same pH conditions, were carried out on days 7, 14, 30 and 60, where they were presented with a food odour, and their behaviour observed. There were 12 crabs in each treatment group, giving a total of 60 hermit crabs. As two aquaria had to be used per pH treatment, aquaria was included as a random factor in the design to test for any effects within treatments.

Sea water treatments

The experiment was carried out in the benthic mesocosm of the Plymouth Marine Laboratory, a controlled temperature laboratory containing a total of sixteen 1 m^3 aquaria. These aquaria are situated in 4 parallel rows of 4 aquaria. Ten aquaria were used in this study and each aquarium was filled with approximately 800 L of 1 µm-filtered sea water. Aquaria were randomly assigned to one of five CO_2 treatment levels, with 2 replicate aquaria per treatment. The desired pCO_2 treatment levels were “ambient” (approximately

390 µatm), 785 µatm, 1300 µatm, 3000 µatm and 11,000 µatm. Corresponding pH values were calculated in CO2SYS (Pierrot et al. 2006), using measured temperature, salinity and alkalinity values for each aquarium; dissociation constants from Mehrbach et al. (1973) refit by Dickson and Millero (1987) and KSO_4 constant of Dickson (1990) were used. The pH values were then used to set the carbonate chemistry of the system. Two of these values (pH_{NBS} 7.90 and 7.70) represent realistic pH values for near-surface temperate waters by the year 2100, under the IS92a and A1F1 "business as usual" scenarios from the IPCC (Meehl et al. 2007), pH_{NBS} 7.35 represents a year 2300 scenario based on IPCC A1F1 "business as usual" (Caldiera & Wickett 2003), or a mild Carbon Capture Storage (CCS) leakage scenario, while pH_{NBS} 6.80 represents a high CO_2 CCS leakage scenario (Blackford et al. 2009). These pH values were then used to actively control the bubbling of CP grade 99.95 % CO_2 gas (BOC) into each aquarium. A pH controller (Aqua Digital pH-201, accuracy ± 0.1%) monitored the pH_{NBS} and controlled CO_2 gas flow *via* a solenoid valve feedback system connected to the gas cylinder. Natural air was also bubbled through each aquarium from an external source (pCO_2 ~390 µatm) to maintain the same starting point of CO_2 across all treatments. Each aquarium was fitted with two Eheim Aquaball pumps in addition to the internal pump, to promote mixing of the entire water body. Treatments were set up and allowed to stabilise for two weeks prior to the crabs being introduced. On 12th April 2010, six hermit crabs *Pagurus bernhardus*, (N = 12 per pCO_2 treatment) were placed into each aquarium. The acidification exposure lasted for a period of 60 days.

The exposure was carried out as follows: hermit crabs were placed individually (to prevent agonistic interactions) into cages specially constructed from pre-seasoned aquatic mesh (NCC Supplies Ltd, Crewe, UK) and square plastic guttering (112 x 60 mm, Marley, Maidstone, Kent, UK), stitched together with nylon fishing line (Trilene XT, Berkley Outdoor, Iowa, USA). The plastic guttering was inserted to create a rigid cage (cylinder shape, length = 120 mm, height = 60 mm). Cages were then distributed equally between the 10 experimental aquaria, and kept submerged for the duration of the exposure period (excluding 2 hours each week for feeding when the hermit crabs were removed from the treatment aquaria and transferred into smaller aquaria with the appropriate pH sea water). Thereafter each cage was opened and the individual crab removed for behavioural trials (as described below). Behavioural observations were carried out on days 7, 14, 30 and 60. Throughout the exposure period, individual crabs were hand-fed with white fish (*Pollachius virens*) at the outset and then every 7 days thereafter. A mesh bag containing activated charcoal was placed in each mesocosm, to prevent accumulation of ammonia in the water. The salinity and temperature of treatment aquaria were measured every other day for the duration of the experiment using a combination temperature and salinity probe (WTW LF187). pH was measured independently of the acidification system using a pH meter (SevenEasy^{TM} S20 Mettler Toledo pH Meter, Switzerland). Samples for alkalinity analysis (250 ml) were taken twice weekly from each tank and collected in brown borosilicate glass bottles, poisoned with $HgCl_2$ according to Dickson et al. (2007) then analysed using pentiometric titration (Apollo SciTech Alkalinity Titrator Model AS-ALK2 and

Batch 100 certified reference materials from Andrew Dickson). Measured sea water parameters for the experiment are given at the end of the Results section, Table 4.2.

Odour preparation

Food odour was produced by crushing the white fish (*P. virens*) in sea water (concentration = 1 g fish : 50 ml sea water) and then filtering it through coarse filter paper (Whatman No. 4). Aquaria air stones were used to carry the odour because they are semi-porous and the crabs were unable to destroy them with their chelipeds. It had been established in a preliminary trial that the hermit crabs were attracted to the odour of this white fish.

Behavioural observations

Behavioural observations were conducted on days 7, 14, 30 and 60 in a partitioned area of the mesocosm room surrounded by blackout cloth (CT 12^0C), in a behavioural chamber filled with 4 l of the appropriate pH sea water. The chamber consisted of a 20 l plastic aquarium that was painted with stone-coloured pond paint (Blagdon^{TM}, Interpet Ltd, Dorking, Surrey, England) on three sides, and left clear at the front to allow observations to take place. The behavioural chamber was then placed inside a lit observational chamber, so that the crab was not distracted by the observer. Assays consisted of a choice experiment with the behavioural chamber divided into three equal sections (cue, middle, no cue), with sliding Perspex

doors separating each section, and cue stones eventually placed at either end of the chamber (as in Chapters 2 and 3). Air stones were soaked in either fish cue (a food odour) or sea water, and the odour was allowed to diffuse in still water to mimic a rock pool environment. There is evidence that water currents stimulate antennular flicking, which would be a confounding factor (Snow 1975). The hermit crabs were placed in the centre of the aquarium, with the Perspex doors down, and allowed to acclimatise for 10 min. After this period cue stones were added, at random, to each end of the tank, the doors were then lifted and their behaviour observed for a period of 10 min, and recorded using The Observer Mobile 5.0 Workabout mx handheld computer (Psion Teklogix Inc., Ontario, Canada). The behavioural measures recorded were: the time spent in the cue section of the behavioural chamber, the time spent in contact with the cue source, the latency to contact the cue source, the number of antennular flicks, and the duration of locomotion. Following trials hermit crabs were kept in the appropriate pH sea water, fed and then returned to their treatment aquaria.

Haemolymph analysis

Haemolymph samples were removed from a sub-sample of the hermit crabs from each treatment at the end of the study, and immediately after their 60 day behavioural trials, except for those crabs in treatment pH_{NBS} = 6.80 where mortality was too high. The crabs were carefully removed from their shells, and haemolymph samples (up to 20 µl) were extracted from each crab using a microsyringe (BD Micro-Fine1 ml, Becton, Dickinson and Company,

Franklin Lakes, NJ, U.S.A.), the needle of which (29 G) was inserted into the infrabranchial sinus, *via* the arthrodial membrane at the base of the third pereiopod. Samples were analysed for the cations sodium (Na^+), magnesium (Mg^{2+}), potassium (K^+), and calcium (Ca^{2+}) using an ICP Optical Emission Spectrometer (Varian 725-ES, Mulgrave, Australia); and for the anion chloride (Cl^-) using a chloride meter (Jenway PCLM3, Essex, UK). For the haemolymph ions a strong ion difference (SID) analysis was carried out. The SID in any solution is defined as the sum of all the strong cation concentrations, minus the sum of all the strong anion concentrations, and can be used to characterise acid-base disturbances (Stewart 1981). This method has been used to try and explain the observed changes in haemolymph pH during acid-base regulation in marine animals (Cameron & Iwama 1987; Luquet & Ansaldo 1997; Whiteley et al. 2001).

Shell analysis

Six undamaged, unoccupied *L. littorea* shells, the shell of choice for intertidal *P. bernhardus*, were placed in each pH treatment at the beginning of the experiment, and at the end of the 60 day study they were removed from the mesocosms, washed and dried, and then tested for strength using an INSTRON 3345 Testing Machine (INSTRON, High Wycombe, UK). The force required to produce an initial crack in the shell was recorded.

Ethical note

None of the crabs were injured during the process of removing them from shells using the bench vice. The removal of haemolymph samples from crabs of this size is not fatal. At the end of the study crabs that survived were supplied with new shells then returned to the collection site after re-acclimatisation, and a recovery period of two weeks. I minimised the number of individuals used by keeping sample sizes smaller for the haemolymph analysis. However, a number of crabs died during the study in the low pH treatments (9 in the pH_{NBS} = 6.80, 3 in pH_{NBS} = 7.35 and 1 in pH_{NBS} = 7.70).

Statistical methods

Analysis of effects after 7 Day's exposure to pH treatments: To compare the results of this experiment with those from previous experiments with 5 day exposures, the data were initially analysed for the 7 day observations only, followed by the analyses of the results for the 60 day experiment in its entirety. The effect of aquaria was tested for each variable using linear mixed models (LMMs) with aquaria as a random factor, but, as it was shown to have no significant effect, it was removed from subsequent analyses. The effect of pH treatment on flicking was analysed using a one-way ANOVA followed by a Tukey test to identify significant pairwise differences between treatments. The other dependent variables (time spent in the cue section of the behavioural chamber, time spent in contact with the cue, latency to contact the cue, and time spent in motion) were not normally distributed so Kruskal-Wallis tests

were used, followed by a Mann-Whitney *U*-test to check for significant pairwise differences between treatments (SPSS 19.0, IBM, Chicago, IL, USA).

Analysis of full time series (60 days): For the behavioural measures (antennular flicking, time spent in the cue section of the behavioural chamber, time spent in contact with the cue, latency to contact the cue, and time spent in motion) LMMs (with hermit crab as the subject and day as a repeated measure) were used to conduct two-way analyses looking at the effects of both time and pH treatment. The effect of aquaria was again tested for each variable for the entire 60 day dataset with aquaria as a random factor. Once again it was shown to have no significant effect, and was removed from subsequent analyses. Linear mixed models were followed by Bonferroni tests to check for pairwise differences between treatments for the main effects, and since *post-hoc* tests are limited to main effects in repeated measures designs, a one-way ANOVA was carried out to investigate the flicking response of crabs on day 60, in order to make a comparison with the day 7 analysis.

Survivorship over time and between pH treatments was analysed using Life Tables and Gehan's Generalized Wilcoxon Test (Gehan 1965). For the haemolymph ions a strong ion difference (SID) analysis (Stewart 1981) was carried out followed by one-way ANOVAs to compare SID and individual ion concentrations between treatment groups, with Tukey tests to check for pairwise differences between treatments. Spearman's Rank Correlations were used on a subset of the data to see if there were significant correlations between the antennular flicking frequency and time spent in motion, and

haemolymph calcium concentrations on day 60. The force required to crush shells was analysed using a one-way ANOVA. Any data that were not normally distributed were Log_{10} transformed prior to analysis. All analyses were carried out using SPSS 19.0 (IBM, Chicago, IL, USA).

RESULTS

Analysis of effects after 7 Day's exposure to pH treatments

After 7 days antennular flicking rate had declined with decreasing pH (ANOVA: $F_{4,59} = 23.373$, $P < 0.001$) (Figure 4.1.). *Post hoc* tests (Tukey tests, see Appendix 3: Table A3.1.) indicate that there was no difference between $pH_{NBS} = 8.00$ and $pH_{NBS} = 7.90$, but that compared to treatment $pH_{NBS} = 8.00$, flicking rates in treatments $pH_{NBS} = 7.70$, $pH_{NBS} = 7.35$ and $pH_{NBS} = 6.80$ were significantly and increasingly lower. (See Appendix 3 Table A3.1. for a full set of pair wise comparisons.)

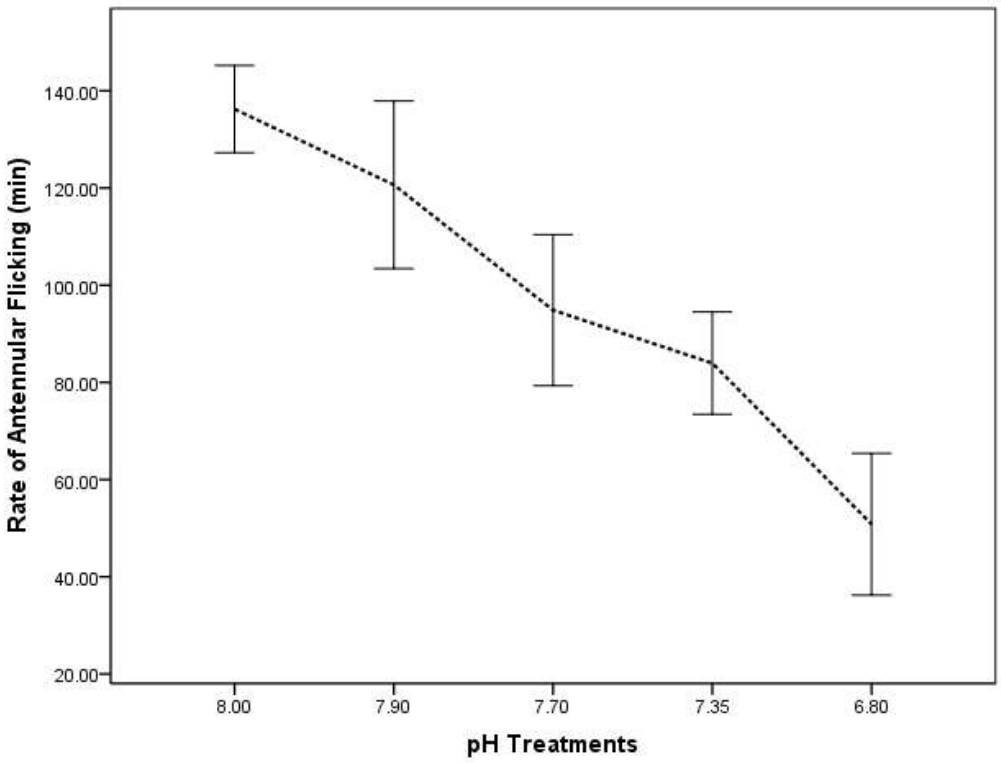

Figure 4.1. Mean rate of antennular flicking per minute (± SE) performed by the hermit crabs across the five different sea water pH treatments after seven days of exposure.

There was a significant effect of sea water pH (Kruskal-Wallis: χ^2 = 11.828, *df* = 4, *N* = 60, *P* = 0.019) on the activity levels of the hermit crabs (Figure 4.2.). Results for *post hoc* tests (Mann-Whitney *U*-tests) are summarised in Appendix 3 Table A3.2., and revealed that hermit crabs in treatment pH_{NBS} = 6.80 moved significantly less than crabs in all the other treatments, except for treatment pH_{NBS} = 7.90. Crabs in treatment pH_{NBS} = 7.90 moved significantly less than crabs in treatment pH_{NBS} = 7.70, but showed no significant differences in movement to crabs in the other treatments.

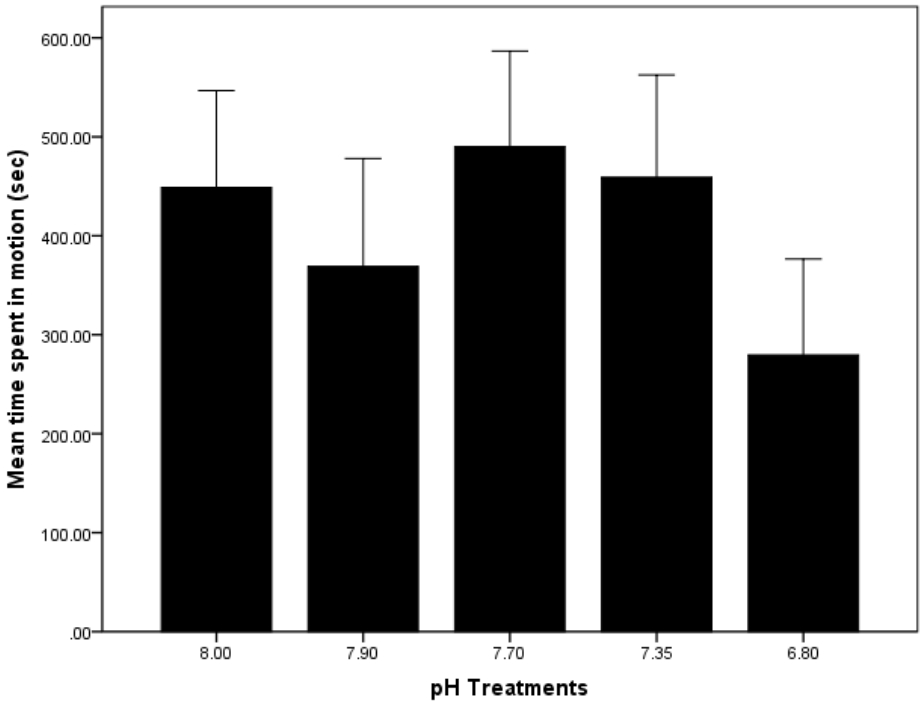

Figure 4.2. Mean time spent in motion (± SE) by the hermit crabs in the five different sea water pH treatments after seven days of exposure.

There was no significant effect of sea water pH on the time spent in the cue section of the aquarium (Kruskal-Wallis: $\chi^2 = 6.408$, $df = 4$, $N = 60$, $P = 0.171$), the latency to contact the cue (Kruskal-Wallis: $\chi^2 = 6.367$, $df = 4$, $N = 60$, $P = 0.173$), or on the time spent in contact with the cue (Kruskal-Wallis: $\chi^2 = 7.121$, $df = 4$, $N = 60$, $P = 0.130$).

Analyses for entire 60 day experiment

Behavioural analyses

Antennular flicking decreased with decreasing pH (LMM: $F_{4,68.049} = 26.454$, $P < 0.001$) (Figure 4.3.). Results for *post hoc* tests are summarised in Appendix 3 Table A3.3., but revealed that there was no difference in the flicking rates of hermit crabs in treatments $pH_{NBS} = 8.00$ and $pH_{NBS} = 7.90$, but flicking rates in both these treatments were higher than all other pH treatments. There was also no difference in the flicking rates of crabs in treatments $pH_{NBS} = 7.70$ and $pH_{NBS} = 7.35$, but both were significantly lower than treatments $pH_{NBS} = 8.00$ and $pH_{NBS} = 7.90$, and significantly higher than in treatment $pH_{NBS} = 6.80$.The flicking rate of crabs in treatment $pH_{NBS} = 6.80$ was significantly lower than in all other treatments.

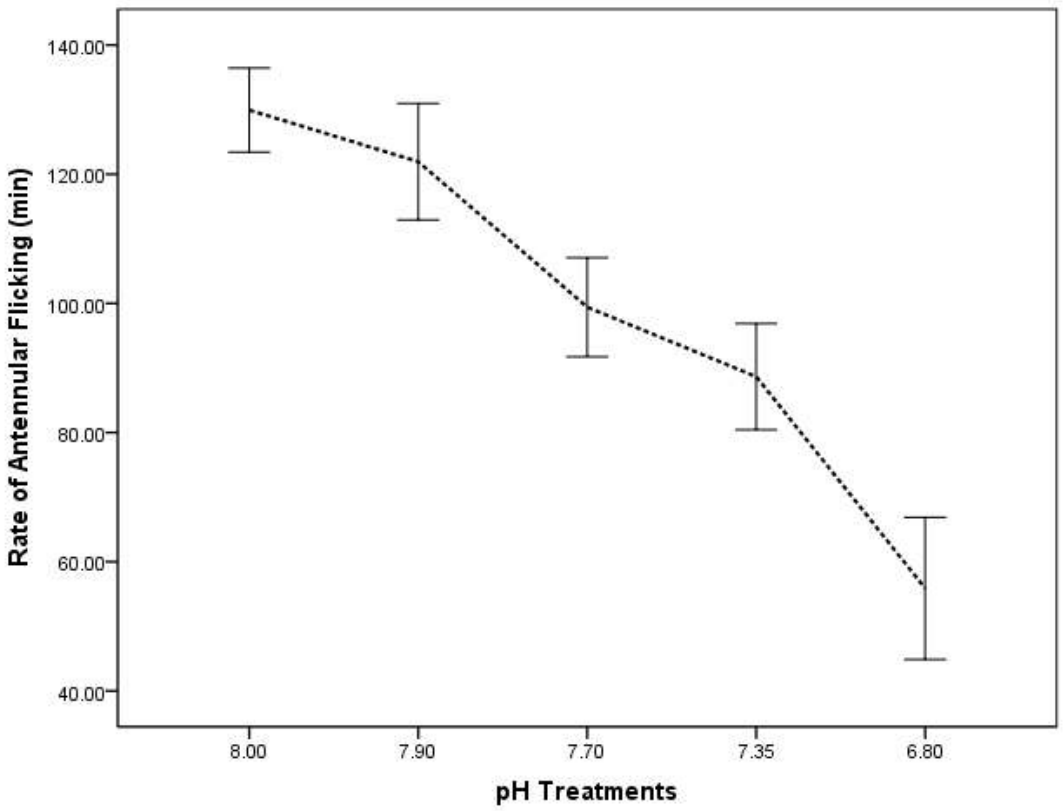

Figure 4.3. Mean rate of antennular flicking per minute (± SE) performed by the hermit crabs across the five different sea water pH treatments during the 60 day study.

Flicking rate also differed significantly across days (LMM: $F_{3,141.675}$ = 7.770, $P < 0.001$) and results for *post hoc* tests are summarised in Appendix 3 Table A3.4., and showed that crabs flicked their antennules significantly more on day 60 than on all other days (Figure 4.4.)

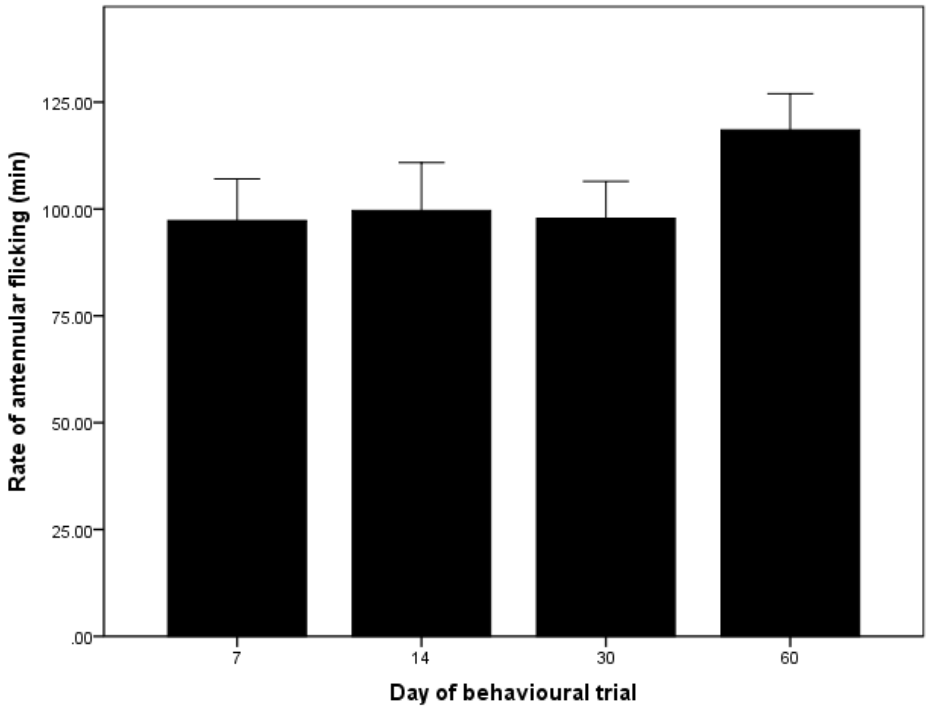

Figure 4.4. Mean rate of antennular flicking per minute (± SE) performed by the hermit crabs across four repeated behavioural trials at intervals of 7, 14, 30 and 60 days during the 60 day study

There was also a significant interaction effect between treatment and day (LMM: $F_{12,137.955} = 2.537$, $P = 0.005$) (Figure 4.5.). In order to investigate this interaction effect a one-way ANOVA was carried out for day 60 so that it could be compared to the analysis for day 7. It showed that there was no significant difference in the flicking rate of the crabs between treatments on day 60 (ANOVA: $F_{4,46} = 0.496$, $P = 0.738$).

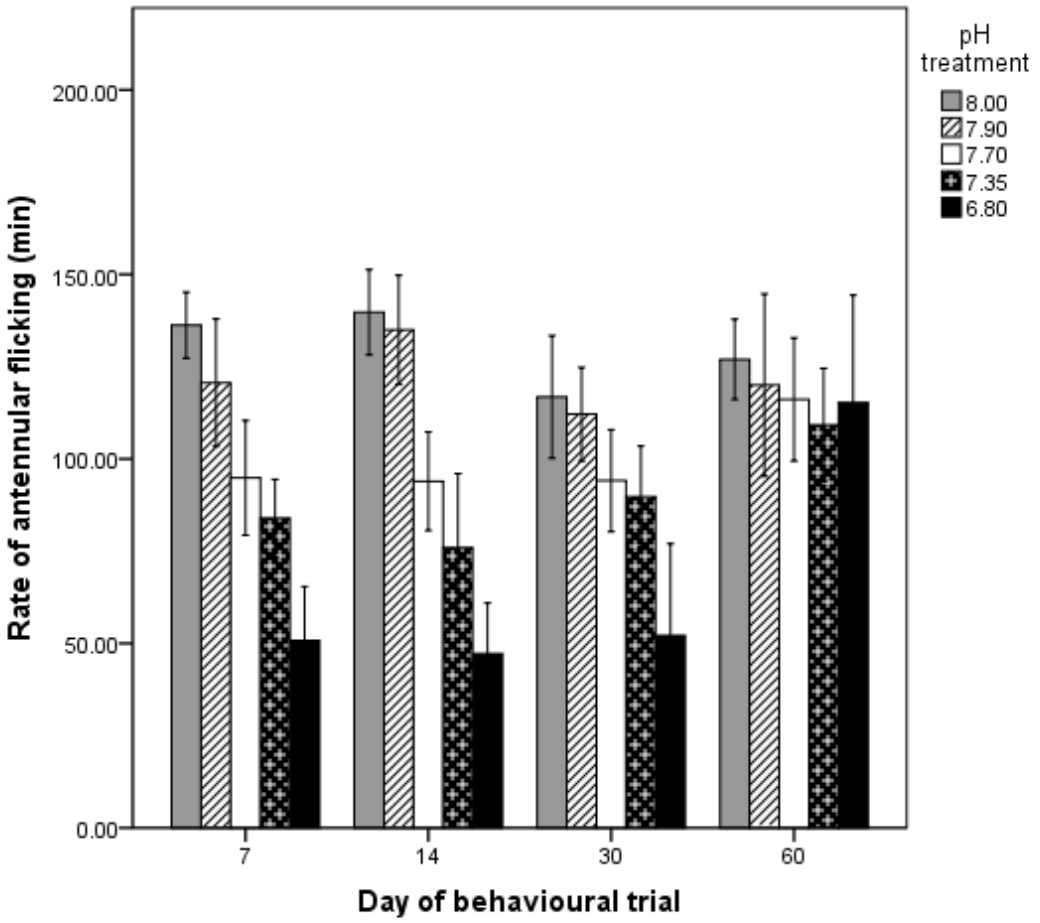

Figure 4.5. Mean rate of antennular flicking per minute (± SE) performed by the hermit crabs in the five different sea water pH treatments across four repeated behavioural trials at intervals of 7, 14, 30 and 60 days during the 60 day study.

There was no significant effect of day (LMM: $F_{3,141.177}$ = 1.136, P = 0.337), and no interaction effect between day and sea water pH (LMM: $F_{12,136.904}$ = 0.1.590, P = 0.101) on the activity levels of the hermit crabs. However, there was a significant effect of sea water pH (LMM: $F_{4,68.157}$ = 3.164, P = 0.019) on the activity of the hermit crabs (Figure 4.6.). *Post hoc* pairwise comparisons showed that activity was significantly lower in the

pH_{NBS}=6.80 treatment than in any of the other treatments (Appendix 3: Table A3.5.).

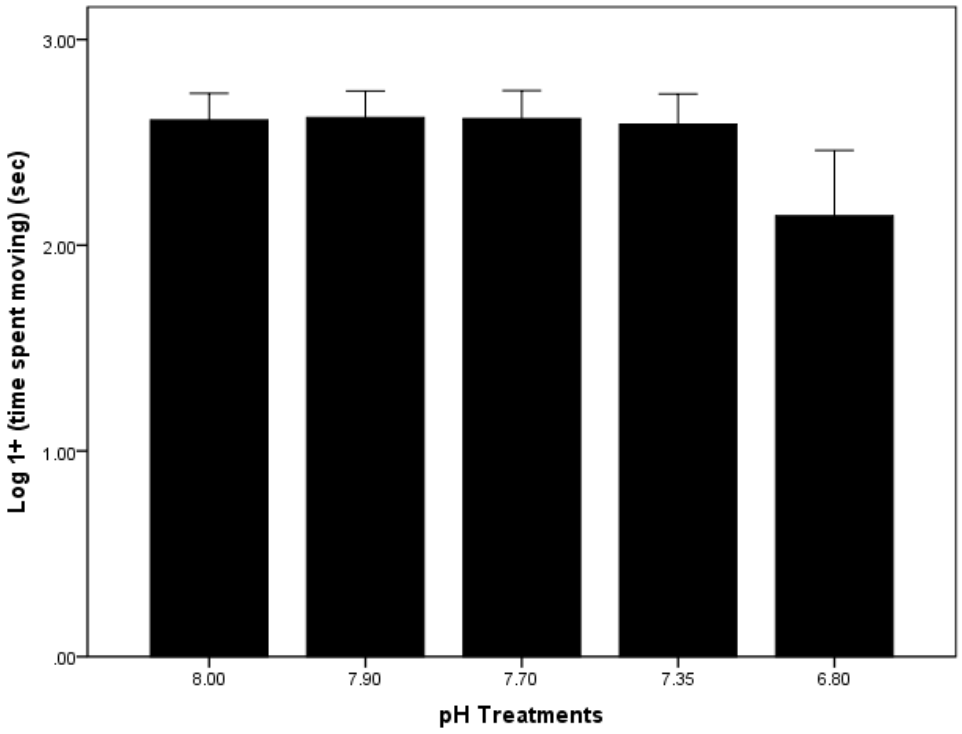

Figure 4.6. Mean log 1+ of time spent in motion (± SE) by the hermit crabs in the five different sea water pH treatments across four repeated behavioural trials at intervals of 7, 14, 30 and 60 days during the 60 day study.

There was no significant effect of sea water pH (LMM: $F_{4,72.138} = 0.847$, $P = 0.500$), or day (LMM: $F_{3,147.383} = 0.798$, $P = 0.497$) on the time spent by crabs in the cue section of the behavioural chamber. There was also no significant interaction effect between sea water pH and day (LMM: $F_{12,139.175} = 1.511$, $P = 0.127$). There was no significant effect of sea water pH (LMM: $F_{4,81.319} = 1.306$, $P = 0.275$), or day (LMM: $F_{3,161.434} = 0.627$, $P = 0.599$) on the time spent in contact with the cue. There was also no significant interaction

effect between sea water pH and day (LMM: $F_{12,147.619}$ = 0.741, P = 0.709). There was no significant effect of sea water pH on (LMM: $F_{4,79.810}$=0.481, P = 0.749), or day (LMM: $F_{3,151.353}$ = 0.628, P = 0.598) on the latency to contact the cue. There was also no significant interaction effect between sea water pH and day (LMM: $F_{12,145.474}$ = 1.154, P = 0.322).

Haemolymph analysis

There were no significant differences between the treatments for the SID (ANOVA: $F_{3,24}$ = 0.075, P = 0.973), or for the individual ions Na^+ (ANOVA: $F_{3,24}$ = 0.924, P = 0.444), K^+ (ANOVA: $F_{3,24}$ = 0.384, P = 0.765) or Cl^- (ANOVA: $F_{3,24}$ = 0.964, P = 0.091). There was perhaps a trend towards a significant difference for Mg^{2+} (ANOVA: $F_{3,24}$ = 0.974, P = 0.074). There was, however, a significant difference between the treatments for Ca^{2+} (ANOVA: $F_{3,24}$ = 5.271, P = 0.006) (Figure 4.7.). *Post hoc* tests (Appendix 3 Table A3.6.) showed that the Ca^{2+} concentration was significantly higher in the haemolymph of the hermit crabs in the pH_{NBS} = 7.35 treatment than those in pH_{NBS} = 7.90 and pH_{NBS} = 8.00, and although the mean [Ca^{2+}] in pH_{NBS} = 7.70 was larger than pH_{NBS} = 8.00 and pH_{NBS} = 7.90, the difference was not significant; however there was also no significant difference between pH_{NBS} = 7.35 and pH_{NBS} = 7.70.

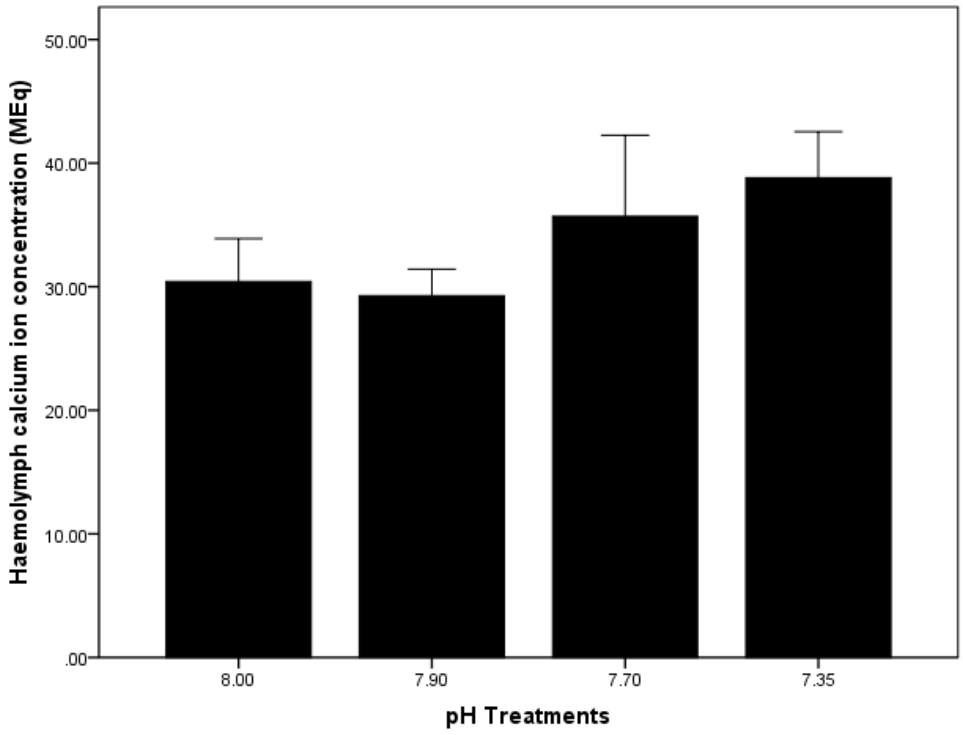

Figure 4.7. Mean concentration of calcium ions ($mEq.l^{-1}$) (± SE) in the haemolymph of hermit crabs on day 60. Note crabs in the pH_{NBS} = 6.80 treatment could not be included in the analysis due to high rates of mortality.

There was a significant negative correlation between the rate of antennular flicking and the [Ca^{2+}] (r_s = $^{-}$0.460, N = 28, P = 0.014) with flicking declining with increasing concentrations of haemolymph Ca^{2+} (Figure 4.8.). There was a trend for a negative correlation between the time spent in motion and the [Ca^{2+}] (r_s = $^{-}$0.352, N = 28, P = 0.066).

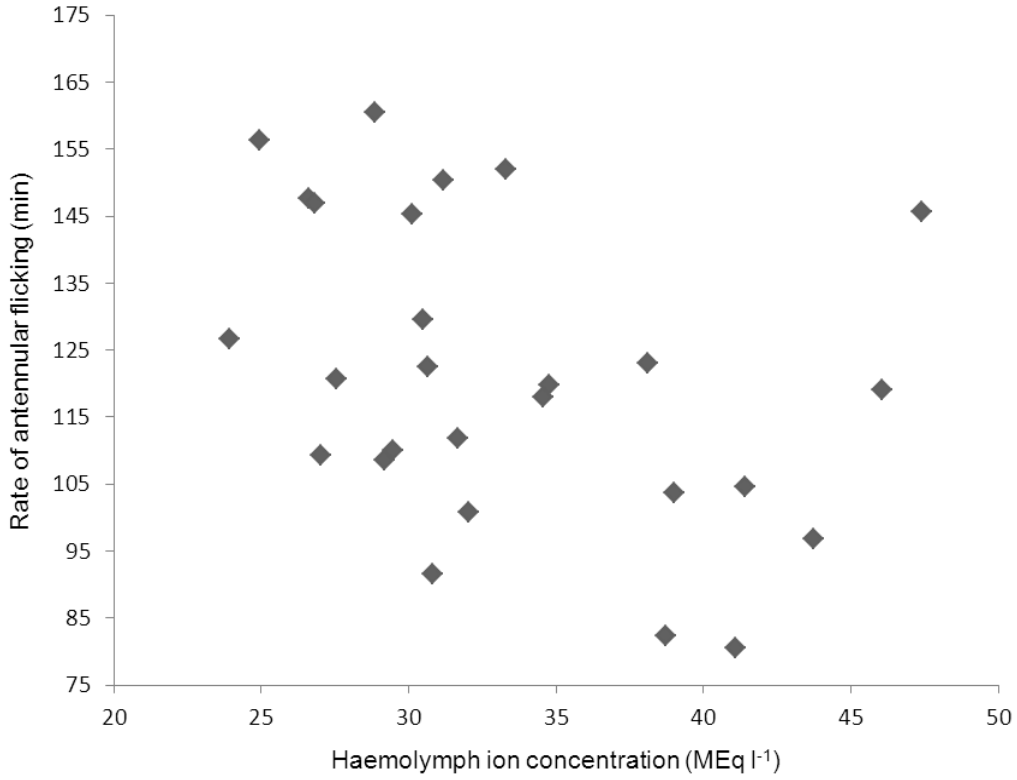

Figure 4.8. Rate of antennular flicking of the hermit crabs on day 60 expressed as a function of haemolymph Ca^{2+} concentration.

Survival analysis

There were significant differences in the survival rates between the 5 groups (Gehan's Generalized Wilcoxon:$\chi^2_4 = 28.424$, $P < 0.0001$). Pairwise comparisons are summarised in Appendix 3 Table A3.7., but revealed that the survival rate of hermit crabs in the treatment $pH_{NBS} = 6.80$ was significantly lower than crabs in each of the other 4 treatments. There was possibly a trend of lower survival rate for crabs in the treatment $pH_{NBS} = 7.35$ compared to treatments $pH_{NBS} = 8.00$ and $pH_{NBS} = 7.90$ but this was not significant ($\chi^2_1 = 3.268$, $P = 0.071$). The cumulative proportion surviving at the

end of each week shows that in treatments pH_{NBS} = 6.80 and pH_{NBS} = 7.35, 50 % and 83 % had survived to week 5 respectively (Figure 4.9.). Proportions surviving at the end of the experiment are shown in Table 4.1., and again reveal a gradient in response. Risk of dying was highest in weeks 5 and 7 for treatment pH_{NBS} = 6.80, and in week 5 for treatment pH_{NBS} = 7.35 (Figure 4.10.).

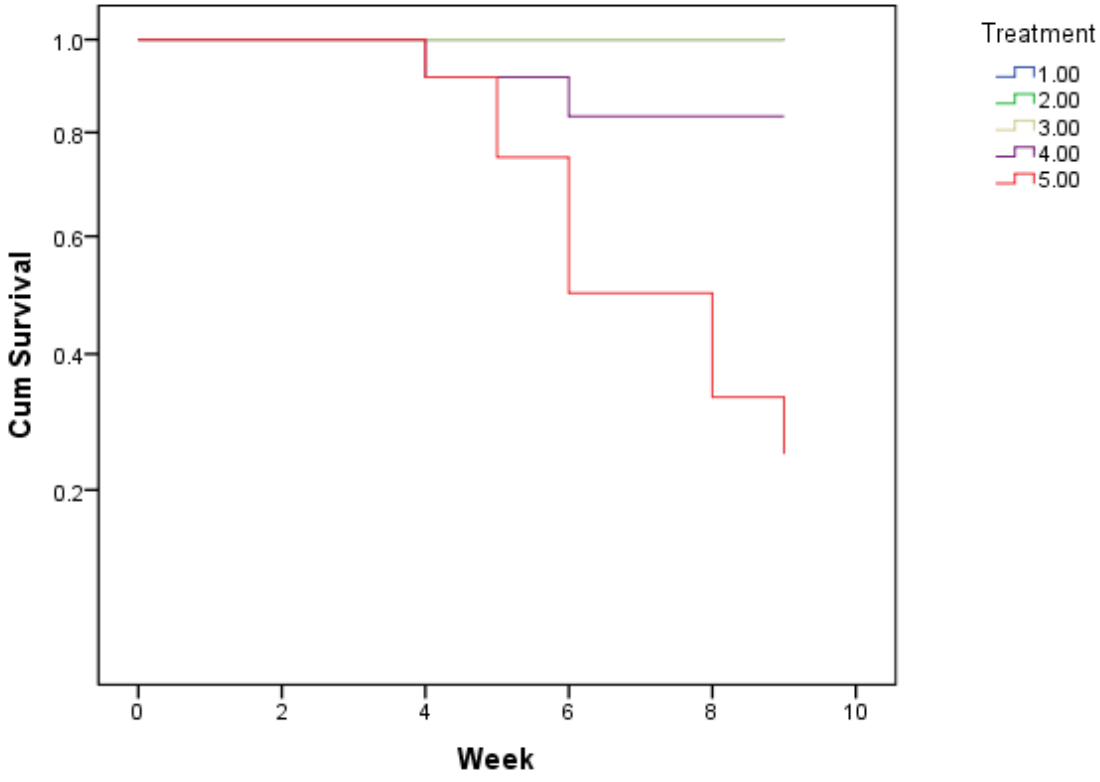

Figure 4.9. The cumulative proportion of hermit crabs surviving up to the end of each week during the 9 week (60 day) study.

Table 4.1. Percentage of crabs surviving at 5 weeks, and at the end of the 60 day (9 week) study.

Treatment (pH_{NBS})	Percentage of crabs surviving at week 5	Percentage of crabs surviving at the end of 9 week exposure
6.80	50	25
7.35	83	68
7.70	100	85
7.90	100	100
8.00	100	100

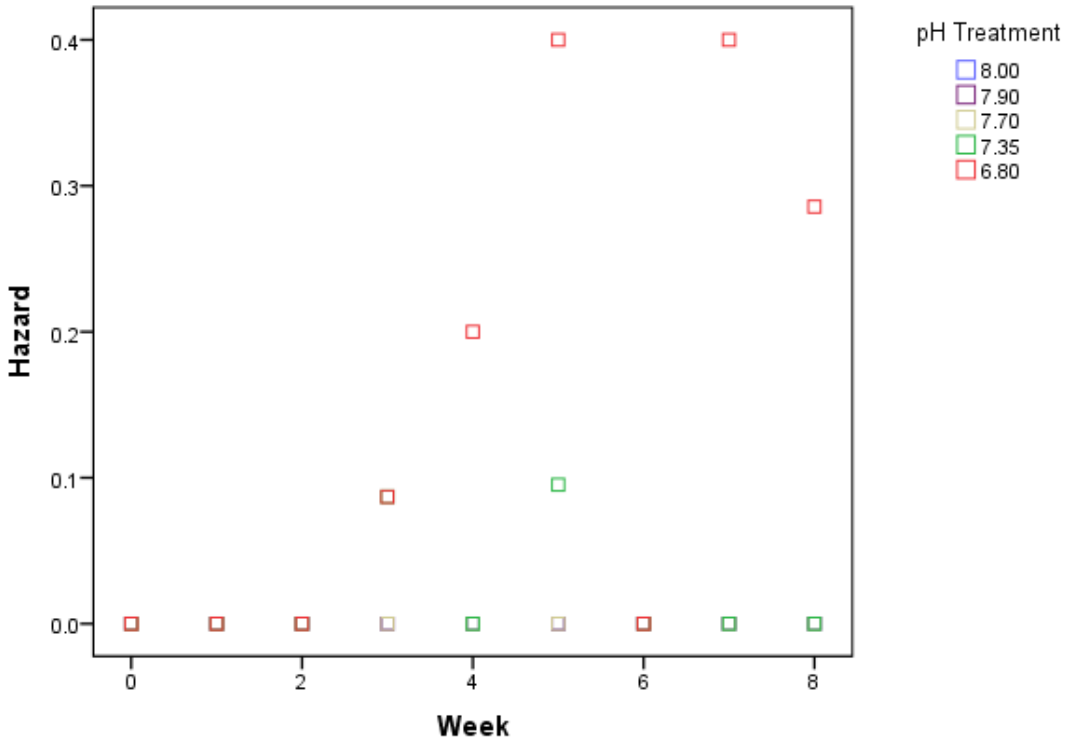

Figure 4.10. The proportion of hermit crabs that have survived up to the beginning of each week that are expected to die during that week.

Shell analysis

Shells in pH_{NBS} = 6.80 and pH_{NBS} = 7.35 showed visible effects of dissolution compared to the other treatments (Figure 4.11.), however, when tested with a crushing force there was no difference between treatments in the force required to produce an initial crack in the shells (ANOVA: $F_{1,29} = 1.174$, $P = 0.346$).

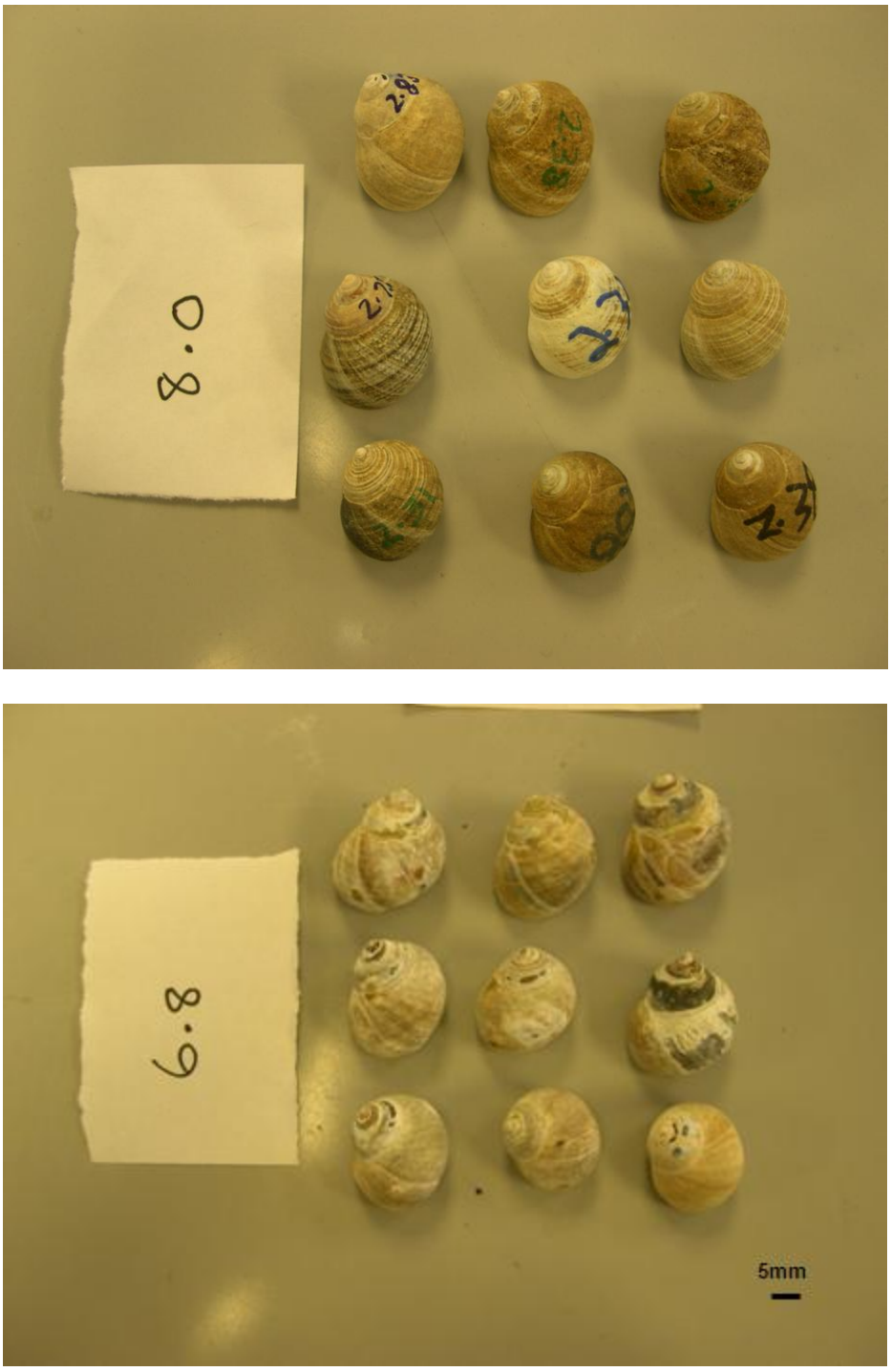

Figure 4.11. Visual comparison of dissolution of *L. littorea* shells from sea water pH treatments 8.00 and 6.80 after 60 days exposure.

Table 4.2. Mean ± SD for experimental aquaria sea water physical parameters measured weekly throughout the 60 day exposure period. The parameters were calculated with CO2sys (Pierrot et al 2006) using the pH and Alkalinity values with dissociation constants from Mehrbach et al (1973) refit by Dickson and Millero (1987) and KSO4 using Dickson (1990).

Variable	**Experimental Aquaria**				
Nominal pH:	8.00	7.90	7.70	7.35	6.80
Measured pH NBS Total	8.01 ± 0.02 7.90 ± 0.02	7.90 ± 0.02 7.80 ±0.02	7.70 ± 0.02 7.60 ± 0.02	7.35 ± 0.02 7.25 ± 0.02	6.85 ± 0.02 6.74 ± 0.02
TCO_2 (mmol l^{-1})	2.31 ± 0.02	2.34 ± 0.02	2.41 ± 0.02	2.56 ± 0.05	2.95 ± 0.08
Salinity	35.4 ± 0.28	35.5 ± 0.35	35.4 ± 0.36	35.5 ± 0.36	35.5 ± 0.35
Temperature (ºC)	10.83 ± 0.38	11.30 ± 0.38	11.30 ± 0.40	11.21 ± 0.51	11.05 ± 0.50
pCO_2 (µatm)	611.73 ± 31.49	807.56 ± 35.60	1317.88 ± 59.05	3083.85 ± 144.70	10230.35 ± 562.14
Alkalinity (µEq kg^{-1})	2447 ± 20.28	2442 ± 19.46	2446 ± 24.96	2476 ± 42.64	2534 ± 66.08
Calcite saturation	2.66 ± 0.12	2.15 ± 0.09	1.41 ± 0.05	0.66 ± 0.02	0.22 ± 0.01
Aragonite saturation	1.69 ± 0.08	1.37 ± 0.06	0.90 ± 0.03	0.42 ± 0.01	0.14 ± 0.01
HCO_3^- (mmol l^{-1})	2.17 ± 0.03	2.22 ± 0.02	2.30 ± 0.02	2.41 ± 0.04	2.51 ± 0.07
CO_3^{2-} (mmol l^{-1})	0.11 ± 0.00	0.09 ± 0.00	0.06 ± 0.00	0.03 ± 0.00	0.01 0.00

DISCUSSION

This study shows a gradient in disruption to the antennular flicking rate (the 'sniffing' response in a decapod crustacean) with the flicking rate waning with decreasing sea water pH. The threshold for such disruption appears at pH_{NBS}=7.70 with another significant step-change at pH_{NBS}=6.80. Perhaps surprisingly the hermit crabs' ability to locate the odour source was not affected in this instance. However, there was a correlation between the antennular flicking response and the concentration of Ca^{2+} in the haemolymph. The treatment-related disruption in both these behavioural and physiological measures is also echoed in the survival rates of the hermit crabs in those treatments, which also followed a gradient, with mortality increasing with declining pH levels. These linked patterns between behavioural and physiological measures, and mortality rates, allows the case to be made for a behavioural measure as a sensitive predictor of physiological status. This study also reveals differential survival rates between hermit crabs in the reduced pH treatments, and some evidence of possible acclimation to the lower pH conditions by surviving individuals that would have implications for population dynamics, and warrant further investigation.

After seven days of exposure there was an effect on the antennular flicking rate of the crabs (the 'sniffing response' in decapods), with a decline in flicking rate as the pH treatments got lower, revealing step changes in flicking rates at pH_{NBS}=7.70 and again at pH_{NBS}=6.80, suggesting some form of pH-induced disturbance to chemosensory behaviour. Crabs were also

shown to move significantly less in the pH_{NBS}=6.80 treatment than in all the other treatments, although there was no gradient in response as there was with the rate of flicking. These results are in agreement with the short-term experiments presented in Chapters 2, 3 and 5. In the study in Chapter 3, *P. bernhardus* was exposed to pH_{NBS}=6.80 for 5 days and showed a decreased flicking rate and decreased levels of activity. The lack of movement, combined with the reduced flicking rate, was thought to be due either to a lack of olfactory stimulation, or metabolic depression brought on by the energy required to regulate acid-base balance, or support enhanced metabolic activity and calcification in high-CO_2 conditions (Pörtner et al. 2004; Wood et al. 2008). However, in the present study the disruption to the crabs' ability to locate the cue was less marked than in the study in Chapter 3, and did not show any significant differences between the pH treatments. The difference in the present study may have been owing to differences in the experimental setup, for example: the sample sizes were smaller, possibly making it more difficult to detect an effect; natural sea water was used in this study, instead of artificial sea water, and although it was filtered it may not have been free from other biological cues; the crabs were also housed together in different treatment aquaria. Such differences in results reveal the wider problems with repeatability and consistency in laboratory experiments investigating the effects of reduced pH on behaviour, due to the natural variability in animal behaviour and the complexities of carbonate chemistry. However, although some aspects of the methodology may have been unavoidably altered for this study, the main thrust of the results is compatible.

The analyses of the study in its entirety revealed a similar pattern to the 7 day analysis for antennular flicking within treatments. The rate of antennular flicking was again shown to decrease with decreasing pH. These analyses also investigated the effects of the time of exposure on the crabs in different treatment groups, and the temporal data for the flicking response revealed that overall, crabs flicked their antennules at a significantly higher rate on day 60 than on the previous days (7, 14 and 30). The interaction effect between flicking and day showed that on days 7, 14 and 30 crabs demonstrated the same gradient in their flicking response with a decline in flicking with decreasing pH. However, on day 60 this effect disappeared and the crabs in all treatments flicked their antennules at a similarly higher rate. This might indicate an acclimation response in the crabs in the lower pH treatments, however, owing to high mortality in treatments pH_{NBS}=7.35 and pH_{NBS}=6.80, the sample sizes were dramatically reduced and these results have to be viewed with caution. Antennular flicking is an energetically costly activity and a previous experiment showed that *P. bernhardus* flicks its antennules at a significantly lower rate when there are no chemical cues present (Chapter 3: de la Haye et al. 2012). It has not yet been established whether a reduction in flicking under reduced pH conditions indicates a lack of olfactory stimulation or a conservation of energy owing to metabolic stress where energy demanding behaviours such as the sniffing response are down-regulated (Chapter 3). The analysis of the time spent in motion, like the 7 day analysis, showed that crabs in treatment pH_{NBS} = 6.80 moved significantly less than crabs in all the other treatments, and again may indicate either a lack of olfactory stimulation, or metabolic depression; there was also no significant

effect of sea water pH on the ability of the crabs to locate the odour cue. The length of exposure did not affect the crabs' abilities to locate the cue. Again, this may have been owing to the different experimental setup, lower samples sizes, and high mortality in the lower pH treatments, and needs further investigation.

Analysis of the hermit crabs' haemolymph on day 60 revealed that crabs in treatment pH_{NBS} = 7.35 had elevated Ca^{2+} concentrations compared to those in treatments pH_{NBS} = 8.00 and pH_{NBS} = 7.90, and no difference in Ca^{2+} concentrations to treatment pH_{NBS} = 7.70. These results differ from the study in the previous chapter where *P. bernhardus* exposed to pH_{NBS} = 6.80 for 5 days only showed elevated $[Cl^-]$ in their haemolymph. The differences are likely to be owing to the different lengths of the exposures. Other studies on lower shore intertidal/subtidal decapod crustaceans exposed to reduced sea water pH have yielded similar results. Dissanayake et al. (2010) found elevated concentrations of haemolymph Ca^{2+}, Na^+ and Cl^- in the prawn *Palaemon serratus* exposed to a pH of ~ 7.45 after 14 days, but after 30 days this effect disappeared, and they suggested this was due to an acclimation effect. Spicer et al. (2007) found elevated concentrations of Ca^{2+} and Mg^{2+} in the haemolymph of the velvet swimming crab *Necora puber* after 2-5 days exposure to a pH level of 7.59; and Small et al (2010) found significantly elevated Ca^{2+} concentrations, and a trend towards significantly elevated Mg^{2+} concentrations, in the haemolymph of the same species after 30 days exposure to pH levels of 6.69 and 7.26. They were shown to be effectively regulating their haemolymph pH, although such regulation over time was predicted to be costly. Decapods have been known to acquire HCO_3^- in order

to buffer extracellular acidosis, either from the dissolution of their calcium carbonate ($CaCO_3$) carapace, which could result in elevated Ca^{2+} in their haemolymph (Findlay et al. 2009), or from the surrounding sea water using ion exchange across the gills (Cameron 1985; Spicer et al. 2007). Calcifying marine invertebrates can actively regulate intracellular Ca^{2+}, along with other ions, in order to create an extracellular micro-environment that is saturated with respect to calcium carbonate, and allows calcification to continue (Pomar & Hallock 2008; Findlay et al. 2009; Nienhuis et al. 2010).The elevated Ca^{2+} concentration in the haemolymph of *P. bernhardus* on day 60 in the lower pH treatments in this study could be interpreted as some form of ion exchange activity taking place, in order to compensate for disruption to their acid-base balance, or related to maintaining calcification rates in the face of exoskeleton dissolution (Pomar & Hallock 208; Wood et al. 2008; Findlay et al. 2009; Nienhuis et al. 2010). The measured sea water parameters show that the sea water was under-saturated with respect to calcite and aragonite in treatments pH_{NBS} = 7.35 and pH_{NBS} = 6.80, which would have resulted in dissolution of the hermit crabs' carapaces, however, without additional data on haemolymph pH and [HCO_3^-] it is difficult to come to any firm conclusions about the cause of the elevated Ca^{2+} in the haemolymph of *P. bernhardus*. Either of the processes described above might account for it, but whichever is responsible, both are energetically costly, and likely to have longer-term consequences for the survival of individuals and populations. This cost is reflected in the results of the survival analysis that will be discussed later. What is also possible, as with the elevated Cl^- levels in crabs in the shorter-term experiment in Chapter 3, is that increased levels of extracellular Ca^{2+}

could block nerve transmission, as Ca^{2+} gates sodium channels during such processes (Clay 2005), and this could be another explanation for the effects observed causing disruption to perception or muscle control.

Scott and Sloman (2004) pointed out the importance of integrating behavioural and physiological indicators of toxicity in aquatic pollutants, and the need to look for more subtle effects at sub-lethal concentrations in order to gain a better understanding of their ecological relevance. Here I show a negative correlation between antennular flicking rate and Ca^{2+} concentration on day 60, with flicking decreasing with increasing concentrations of Ca^{2+} in the hermit crabs' haemolymph. No correlations were found in Chapter 3 between haemolymph Cl^- concentrations and the time spent in motion, or the flicking response. The exposure may not have been long enough, and the disruption to their acid-base status not severe enough, over such a short exposure to affect their energetic state in the same way. The link between a behavioural and physiological measure in this study possibly represents stronger evidence that flicking rate is influenced more, in this instance, by physiological condition than by a disruption to chemoreception, as appeared to be the case after the shorter term exposure in Chapter 3. It also shows that disruption to a behavioural response may be used as a sensitive indicator of underlying physiological condition. The results of the survival analysis support this conclusion.

The energetic costs to *P. bernhardus* of longer term exposure to reduced pH sea water implied by both the behavioural and physiological disruptions are also borne out in the survival data. By the end of the study there was no mortality in treatments $pH_{NBS} = 8.00$ and $pH_{NBS} = 7.90$, whereas

15 % of hermit crabs in pH_{NBS} = 7.70, 32 % in pH_{NBS} = 7.35 and 75 % of those in pH_{NBS} = 6.80 had died. This gradient in mortality with pH follows the same pattern as the behavioural and physiological responses. Mortality has been seen in some ocean acidification studies as a measure of an organism's sensitivity to acidified sea water, but it is hardly a subtle one (Widdicombe & Spicer 2008). The results in this study indicate that behavioural measures could be used as a more sensitive measure of underlying physiological condition. Essential behaviours can often be disrupted by pollutants at sub-lethal levels, with far reaching consequences for the health of populations (Scott & Sloman 2004; Widdicombe & Spicer 2008; Tuomainen & Candolin 2010). The fact that even 25 % of the hermit crabs in the pH_{NBS} = 6.80 treatment survived the 60 day exposure is a testament to their resilience, and the 68 % surviving in the pH_{NBS} = 7.35 treatment reveals interesting differences in tolerances between individuals, considered alongside the possible signs of acclimation in the recovery of their antennular flicking response on day 60. Such individual differences were not formally analysed here, but would be interesting to investigate in future studies. Ocean acidification is likely to exert a harsh selection pressure on populations, and some individuals within a species may be more resilient to its affects than others. Pistevos et al. (2011) found individual variation in the responses of four different genetic clones of the bryozoan species *Celleporella hyalina* to varying pH and temperature, and they concluded that such variation may enable future adaptation through natural selection. Differences between individuals may provide the mechanism for adaptation, but only if the traits of

interest are heritable within the timescale of predicted change, and populations are sustainable in the face of the mortality rates they suffer.

The *L. littorea* shells that *P. bernhardus* preferentially adopts to protect its soft abdomen exhibited visible signs of deterioration in treatments pH_{NBS} = 6.80 and pH_{NBS} = 7.35, compared to the shells in the other treatments (Figure 11). However, when the shells were tested with a crushing force there was no difference between treatments in the force required to produce an initial crack. This was surprising as the sea water parameters during the experiment show that it was under-saturated with respect to calcite and aragonite in these lower pH treatments (Table 4.2.). Bibby et al. (2007) found that *L. littorea* suffered significant thinning of their shells when exposed to pH_{NBS} = 6.45 over 15 days. Calcareous shells can be particularly vulnerable to dissolution in low pH conditions, even when the surrounding sea water is supersaturated with respect to calcite (Wood et al. 2008; Findlay et al. 2009, 2011; Nienhuis et al. 2010). Consequently, as demonstrated by Findlay et al. (2011) an empty shell will become progressively weaker in acidified sea water, than one with its molluscan contents, with serious consequences for hermit crabs, and other species that occupy such shells for protection against predators and environmental extremes (Nienhuis et al. 2010; de la Haye et al. 2011). However, *L. littorea* shells are composed predominantly of calcite interlaminated with aragonite and such structures have been shown to have biomechanical properties that make them resistant to cracking (Li et al. 2004; Ries 2011), and it is possible that the shells, despite exhibiting visual deterioration, required an exposure longer than 60 days for their integrity to be significantly compromised.

This study has identified a gradient in the responses of the hermit crab *P. bernhardus* to different sea water pH levels, and possible thresholds for disruption at pH_{NBS} = 7.70 (one of the predicted sea water pH levels for 2100) and another significant step change at pH_{NBS} = 6.80 (a level predicted for a high CO_2 CCS leakage scenario). Behavioural and physiological responses and mortality rates followed similar patterns in response to declining pH, and the correlation between the behavioural measure of antennular flicking, and the physiological measure of haemolymph Ca^{2+} concentration on day 60 indicates that behavioural measures could prove a sensitive predictor of underlying physiological condition. A mechanism for disruption to chemo responsive behaviour is also suggested by the elevated levels of Ca^{2+} as extracellular ionic changes, caused by the need for acid-base regulation in response to CO_2-induced hypercapnia, have recently been shown to alter fish behaviour *via* interference with neurotransmitter function (Nilsson et al. 2012). This study also reveals interesting differences in survival rates between individuals from the same treatments. The mortality suffered by crabs in pH_{NBS} = 6.80 was high, even after 30 days, and although the hermit crabs in this treatment showed resilience in surviving at all, populations are unlikely to recover from such losses, demonstrating the potential impact of a CO_2 CCS leak. Crabs in the pH_{NBS} = 7.70 treatment fared better but may still incur longer term costs that were not investigated here. There was evidence of a possible acclimation response in the lowest pH treatments with the recovery in their antennular flicking rates on day 60, although haemolymph calcium was still disrupted. More research is needed to establish whether such individual variation in responses is maintained over longer timescales, and

whether the ability to acclimate forms the basis for adaptations that can be achieved within the timescale of predicted change, or whether the costs of acclimation on other physiological functions are ultimately unsustainable. The effects of declining sea water pH on organisms are likely to be complex and unpredictable, as seen in the differences between the results in this experiment and the previous one. But behavioural responses may be an important area of research in relation to environmental disturbances, like reduced pH and other pollutants, not only because changes in behaviour can be sensitive indicators of overall condition, but because even subtle changes in an animal's behaviour can have consequences for its survival, and individual variations in behaviour may form the basis for adaptation to such change.

CHAPTER 5

Reduced sea water pH disrupts resource assessment and decision making in the hermit crab *Pagurus bernhardus*

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Abstract: The decisions that animals make are based on information gathered from their environment, and can have consequences for both their fitness and survival. Such processes can be disrupted by environmental change. Hermit crabs find and select the gastropod shells they inhabit using chemical and visual cues, and tactile assessment. The choice of an optimal shell is important since it provides shelter against environmental extremes and protection against predators; inhabiting a suboptimal shell can also reduce fecundity. Hermit crabs are subject to cyclical reductions in the pH of the water in the intertidal rock pools that they inhabit, and such reductions may be further exacerbated by ongoing climate change. Reduced sea water pH, a consequence of ocean acidification and leaks from geological storage sites, has already been shown to disrupt the behaviour of marine animals. Here I investigate the effects of reduced sea water pH on the shell assessment and selection behaviour of the hermit crab *Pagurus bernhardus*. Under highly reduced pH conditions (pH_{NBS} = 6.80) crabs were less likely to change from a suboptimal to an optimal shell than those in untreated sea water; and those that did change shells took longer to do so. Crabs in the reduced pH treatment also showed significantly lower antennular flicking rates (the 'sniffing' response in decapods) and reduced movement. Thus, a reduction in sea water pH disrupts the resource assessment and decision making processes of these hermit crabs, indicating that the ability to acquire a vital resource may be influenced by both naturally occurring environmental cycles and future anthropogenically induced environmental change.

INTRODUCTION

The decisions animals make can have far reaching consequences for both their fitness and survival (Dill 1987; Blumstein & Bouskila 1996; Schmidt et al. 2010). Animals continually gather and assess information about their environment, in order to make decisions such as where to look for food (Pyke et al. 1977), how to choose a potential mate (Jennions & Petrie 1997), which habitat to settle in (Johnson & Strathmann 1989), whether to hide to avoid predators (Lima & Dill 1990), or whether to enter into a fight (Arnott & Elwood 2009). The environment from which animals gather this information may be constant or variable. Some variability constitutes predictable change, like natural cycles, although these may still suspend or interfere with information gathering activities. Environmental variability is common and animals have therefore evolved strategies to cope with such 'predictable unpredictability' (Wells 2007). Information gathering, although energetically costly, can be used to reduce ecological uncertainty; and behavioural plasticity, an evolutionary strategy that anticipates environmental variability, allows animals to respond to change on the basis of the information that they gather (Dall et al. 2005; Donaldson-Matasci et al. 2008). However, uncertainty can never be entirely eliminated and both stochastic natural events and anthropogenic effects can disrupt the environment. Information must be both acquired and processed successfully in order to be useful, and a growing number of studies have shown how changes in the environment, particularly anthropogenically induced ones, can disrupt these processes (Scott & Sloman 2004; Zala & Penn 2004; Tuomainen & Candolin 2010). Such

changes can interfere with the information gathering process itself, by compromising sensory systems, or by altering the stimulus, and this has been termed ‘info-disruption’ (Lürling & Scheffer 2007). Environmental changes can also affect an animal’s physiology, thereby disrupting the processing of information and decision making owing to neurological disturbance (Dias-Ferreira et al. 2009; Graham et al. 2010); or they can induce metabolic stress, resulting in certain energy-demanding behaviours being allocated a lower priority in favour of maintaining homeostasis (Bernatis et al. 2007). Variability and disruption make decision making a more complex process for animals, where the consequences of their choices cannot be known *a priori*, introducing the possibility of maladaptive, as well as adaptive, responses (Dall et al. 2005; Miner et al. 2005; Schmidt et al. 2010). Many decisions, made within the context of environmental uncertainty and disruption, can involve animals in costly trade-offs that may simultaneously afford fitness benefits and incur fitness penalties (Domenici et al. 2007).

Environmental changes caused by anthropogenic effects have been shown to disrupt the information gathering and decision making of aquatic organisms. Changes in water pH, in particular, have altered the behaviour of crayfish (Allison et al. 1992), of freshwater and marine molluscs (Bibby et al. 2007; Turner & Chislock 2010) and fish (Leduc et al. 2004; Munday et al. 2009; Dixson et al. 2010). Reduced sea water pH has also been shown to disrupt the chemo-sensory behaviour of the hermit crab *Pagurus bernhardus* (Chapters 3 and 4). In intertidal rock pools pH fluctuates naturally, along with other physico-chemical characteristics (Truchot 1988). These small, intermittently isolated environments can experience enormous spatial and

temporal variability in pH, salinity, temperature, and oxygen concentrations, which are dependent upon weather conditions, the time of day, and the abundance of resident organisms (Huggett & Griffiths 1986). The pH in rock pools can rise and fall rapidly on a diurnal basis during emersion (eg., 9.5-6.5 pH units, Morris & Taylor 1983, and 10.16-7.29 pH units, Truchot 1988), owing to algal and animal respiration. As well as undergoing natural cyclical changes in rock pools, sea water pH is predicted to fall on a global scale owing to anthropogenically induced ocean acidification (Caldeira & Wickett 2003; Raven et al. 2005). Extreme and localised high-CO_2/low pH events could also occur as a result of leaks from seabed carbon dioxide (CO_2) sequestration sites, proposed as a mitigating strategy for CO_2 emissions (Hawkins 2004). The average pH in rock pools is therefore likely to be reduced, and perhaps even forced below its natural limits, in the future, owing to the additive effect of ocean acidification and potential point source leaks from CO_2 storage sites. This could have biological consequences for intertidal species many of which, although resilient, are already living at the threshold of their tolerance limits (Stillman 2002; Tomanek & Helmuth 2002). Any disruption to the information gathering and decision making activities of intertidal animals, even over diurnal cycles, could have overall consequences at the level of population health and, if there is a differential effect amongst individuals, fitness consequences.

The hermit crab *Pagurus bernhardus* is a decapod crustacean (Anomura), which for most of its early adult life is commonly found in intertidal rock pools. Unlike brachyuran crabs it has an asymmetrical, membranous abdomen that is protected by the adoption of an empty gastropod shell.

These gastropod shells are an important resource for hermit crabs, as they protect their soft bodies both from predation and from environmental stressors (Lancaster 1988). As hermit crabs grow, they constantly upgrade to a larger shell. They acquire shells either by locating sites where there are empty shells, or by competing with each other and engaging in contest behaviour to obtain a better shell (Hazlett 1981). Hermit crabs gather information about available gastropod shells using visual cues (Elwood & Neil 1992) and through the olfactory detection of calcium ions (Mesce 1982; Gravel et al. 2004), or of dead gastropods (Rittschof et al. 1990). Like other decapod crustaceans, hermit crabs possess short antennules that are used for distance chemoreception (Snow 1975). The antennules are flicked rapidly at the onset of a stimulus (the 'sniffing' response in decapods) to ascertain its nature, concentration and direction (Schmitt & Ache 1979; Koehl 2006). When hermit crabs locate a suitable shell they undertake characteristic shell investigation behaviour to assess the size and quality of the prospective shelter before deciding to exchange shells (Reese 1962; Jackson & Elwood 1989). Choosing an optimal shell can have important consequences for survival, growth and fitness (Bertness 1981). Such ritual assessment and decision making in hermit crabs also extends to contest behaviour (Briffa & Elwood 2000), and, to a lesser extent, to mate choice and anti-predator behaviours (Jackson & Elwood 1989; Briffa & Twyman 2011). *Pagurus bernhardus* is an excellent model organism for investigating the potential effects of reduced pH on shell investigation and chemo-sensory behaviour because the behavioural repertoire used for shell selection, under normal conditions, is already well understood (Jackson & Elwood 1989), such that

any changes owing to low pH may be readily identified, and its antennular flicking is a tractable measure of chemo-responsiveness.

In Chapters 3 and 4 reduced sea water pH was shown to disrupt the chemo-sensory behaviour of *P. bernhardus*, and its ability to detect food cues, therefore in this study, I determined the effects of exposure to reduced sea water pH on the ability of *P. bernhardus* to detect empty gastropod shells, either visually or chemically, and on the more complex behaviours of shell investigation and shell choice behaviour that involve decision-making. One possibility is that any apparent reduction in the ability to detect shells, and in shell investigation, could be owing to overall metabolic depression and a related lack of motivation to search for new shells, as a result of exposure to reduced pH. In this case I would expect the lower pH to cause a reduction in overall activity rates, which, as well as indicating a lack of olfactory stimulation, can also be an indicator of physiological condition. On the other hand, if the effects on the specific chemo-responsive behaviour (antennular flicking), and shell choice behaviour were more marked than the effects on non-specific activity rates, this would indicate either greater flexibility in these particular behaviours, or the possibility of a specific effect upon them.

METHODS

Study organisms

Hermit crabs, *P. bernhardus*, were collected by hand from mid shore rock pools at Hannafore Point, Looe, Cornwall, UK (50:20°N, 04:27°W), February to March 2009. Crab mass was standardised as much as possible (mass = 1.2 g ± 0.7g). Only crabs inhabiting *Littorina littorea* shells were collected, as this is the preferred shell species for crabs of this size (Briffa & Elwood 2007). In the laboratory, individuals were kept in holding aquaria (vol. = 80 l) supplied with aerated, filtered sea water (S = 34). Approximately 50 hermit crabs were held in each aquarium and were provided with numerous refuges to limit potentially harmful agonistic interactions and cannibalism. All aquaria were maintained under conditions of natural light and at a temperature of 15ºC. At least one week was allowed to elapse between collection and the start of the experiment to acclimate the hermit crabs to laboratory conditions. During the holding period crabs were fed with the white fish Coley (*Pollachius virens*). Two days before the start of the experiment crabs were randomly selected and placed in individual aquaria (vol = 1l). To standardise hunger levels all crabs were food deprived during this period, and twenty-four hours before the experiment the hermit crabs were carefully excised from their original shells using a bench vice, and their sex determined. Only males with a full complement of undamaged limbs, and free from obvious parasites were used, to control for intersexual differences in shell selection behaviour. Each crab was presented with an empty, sub-optimal shell (50 % of the mass of an

optimal shell, determined from a previous shell-selection experiment by Briffa & Elwood 2007), and allowed to recover for 24 h before sea water treatments commenced.

Experimental design

I used a repeated measures design where crabs that had been re-housed in sub-optimal shells were divided into two groups (A and B). In the first part of the experiment both groups were kept in untreated sea water (pH_{NBS} = 8.20) for five days and then underwent behavioural trials in which they were offered an optimal shell and their shell investigation behaviour recorded. This controlled for any effects of crabs being kept in the laboratory during the experimental period. In the second part of the experiment Group A continued in untreated sea water (pH_{NBS} = 8.20) for another five days and Group B was kept in reduced pH sea water (pH_{NBS} = 6.80) for the same period, after which both groups again underwent shell investigation trials. A 5-day exposure at a pH of 6.80 could mimic a CO_2 storage leak, however, owing to the diurnal fluctuations in rock pool pH, animals are unlikely to be exposed to such a low pH for extended periods of time (although pH can be depressed in rock pools for several hours during spring tides); therefore, the behavioural consequences shown in this study may be more marked than those that might occur under normal field conditions. Nevertheless, the approach of using a manipulation that is relatively short-term, but ecologically informed, is necessitated by the high degree of variance encountered in behavioural data. By taking this experimental approach, I am able to identify behavioural effects

that may be present, but would be difficult to discern following a brief exposure. A total of 74 individuals (46 in reduced pH sea water, 28 in untreated sea water) were used in the experiment.

Sea water treatments

Hermit crabs were kept in individual aquaria (vol = 1l) with either untreated (pH_{NBS} = 8.20) or reduced pH (pH_{NBS} = 6.80) artificial sea water (ASW) (Instant Ocean®) (S = 34_{PSU}, T = 15°C). Untreated ASW was aspirated with natural air, supplied under pressure ([CO_2] ~ 380 ppm). In the reduced pH treatments CO_2-enriched air ([CO_2] ~12000 ppm) was aspirated through the ASW using an air mixing system identical to that described by Findlay et al. (2008). In both cases actual [CO_2] was measured using a gas analyser (LI-7000, LI-COR Nebraska USA). Hermit crabs in the reduced pH treatments were introduced into aquaria with untreated ASW that was then gradually lowered to the nominated pH, to avoid shocking the crabs. The ASW pH_{NBS} (SevenEasy™ S20 Mettler Toledo pH Meter, Switzerland) and total CO_2 concentration (Ciba-Corning 965, Carbon Dioxide Analyser, England) was recorded daily (Appendix, Table 1.), and water changes were carried out every other day.

Behavioural observations

All behavioural observations were conducted on day six for each trial group in a temperature-controlled environment (15ºC). Hermit crabs were placed in a

behavioural arena comprising a crystallizing dish (diam. = 135 mm, height = 75 mm) filled with 1L of either untreated ASW (pH_{NBS} = 8.20) or reduced pH ASW (pH_{NBS} = 6.80). Artificial Sea water was used because it is assumed not to contain any biological cues. The dish was then placed behind the two-way mirror of an observation chamber, so that the crab could not see the observer. Trials were carried out in still water to mimic a rock pool environment. There is evidence that water currents stimulate antennular flicking, which could be a complicating factor (Snow 1975). Hermit crabs were placed in the centre of the arena, and were left undisturbed for 10 min. After this period an optimal gastropod shell was placed about 130 mm from the crab, the door of the observation chamber closed, and the behaviour observed for a period of 10 mins. Shell detection and investigatory behaviour was recorded using The Observer Mobile 5.0 Workabout mx handheld computer (Psion Teklogix Inc., Ontario, Canada). The behavioural measures recorded were: the number of antennular flicks, the duration of shell investigation (see Elwood & Neil 1992 for a detailed description), the duration of locomotion and the occurrence of shell exchange.

Statistical methods

Data were analysed with Statview 5.0 (SAS, Cary, NC, U.S.A.) using repeated measures ANOVAs and the chi-squared (χ^2) test for association. In order to determine the effects of 'trial number' ('one' or 'two') and 'treatment group' ('A' - normal pH in trial one followed by normal pH in trial two; or 'B' - normal pH in trial one followed by reduced pH in trial two) on the behavioural

measures, a series of one-within, one-between repeated measures ANOVAs were performed. The repeated measure was 'trial number' and the between-subjects factor was 'treatment group'. Thus, if reduced pH influences shell selection behaviour I would expect to see greater differences in behaviour between trials one and two for crabs in Group B, than for crabs in Group A. Such differences in the effects of trial number between groups A and B would be indicated by a significant interaction effect between trial number and group. In order to investigate which mean differences caused any significant interaction effects I performed post-hoc t-tests to make pair-wise comparisons between means (Briffa & Elwood 2010). I used paired tests for the effect of 'trial number' within treatment groups and unpaired tests for differences between 'treatment group' within trials. In order to compare the magnitude of the effects of reduced pH treatment on different behavioural measures, I calculated their effect size estimates, partial eta^2 (η^2_p) (Briffa et al. 2008), where appropriate.

Ethical note

No crabs were injured during the process of removing them from their shells using the bench vice. At the end of the experiment crabs were provided with an optimal shell and, following re-acclimation, released back to the sea.

RESULTS

There was no overall effect of pH treatment ($F_{1,72}$ = 0.824, P = 0.367), or trial ($F_{1,72}$ = 0.265, P = 0.608), and no significant interaction effect of pH treatment and trial ($F_{1,72}$ = 1.589, P = 0.212) on the latency to find the optimal shell. There was no effect of trial ($F_{1,72}$ = 0.041, P = 0.840) on the latency to change shells. There was, however, a significant effect of pH treatment ($F_{1,72}$ = 8.695, P = 0.0043) and a significant interaction effect ($F_{1,72}$ = 4.549, η^2_p = 0.06, P = 0.04) (Figure 5.1.) on the latency to change shells. *Post hoc* t-tests tests revealed that the time taken to change shells was longer for crabs in Group B than it was for crabs in Group A in trial two, whereas there was no difference between Groups A and B in trial one (Table 5.2.).

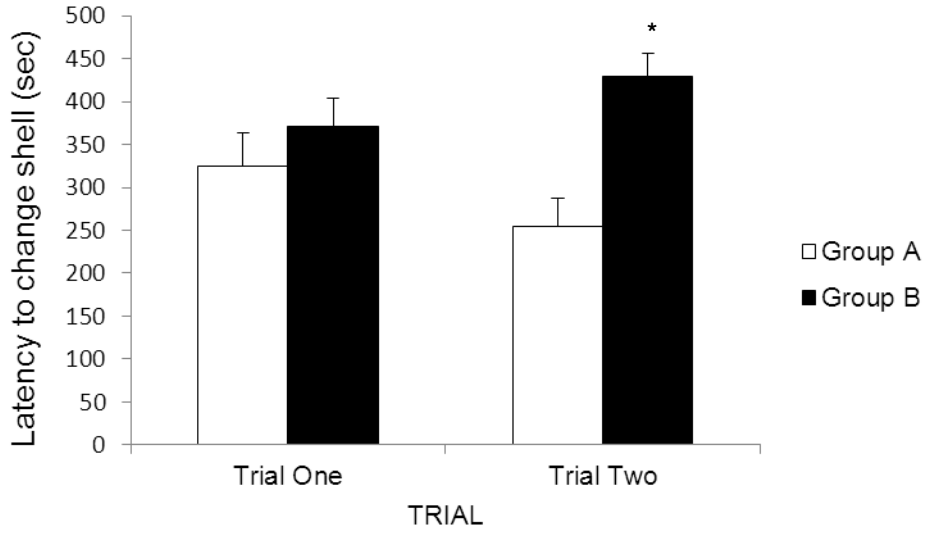

Figure 5.1. Time taken for hermit crabs to change shells across two trials, and between two groups. The first trial was conducted after both groups of hermit crabs had been kept in untreated sea water for five days. The second trial was conducted after Group A had been kept in untreated sea water (pH_{NBS} = 8.20) for five days, and Group B had been kept in reduced pH sea water (pH_{NBS} = 6.80) for the same period. Values are means (+ SE). * Asterisk indicates significant differences between Groups.

Crabs in group B were less likely to exchange shells than were crabs in group A during trial two (χ^2_1 = 9.695, P = 0.002). Of the 28 crabs in control sea water (Group A) only three failed to exchange shells (10.7%), whereas of the 46 crabs exposed to reduced pH sea water (Group B) 21 failed to exchange shells (45.7%).

Antennular flicking varied significantly with pH treatment ($F_{1,72}$ = 37.349, P < 0.0001), and trial ($F_{1,72}$ = 44.340, P < 0.0001), with a significant interaction effect ($F_{1,72}$ = 49.245, η^2_p = 0.41, P < 0.0001) (Figure 5.2.). *Post hoc* t-tests revealed that the flicking rate was lower for crabs in Group B than

it was for crabs in Group A in trial two, whereas there was no difference between Groups A and B in trial one (Table 5.2.).

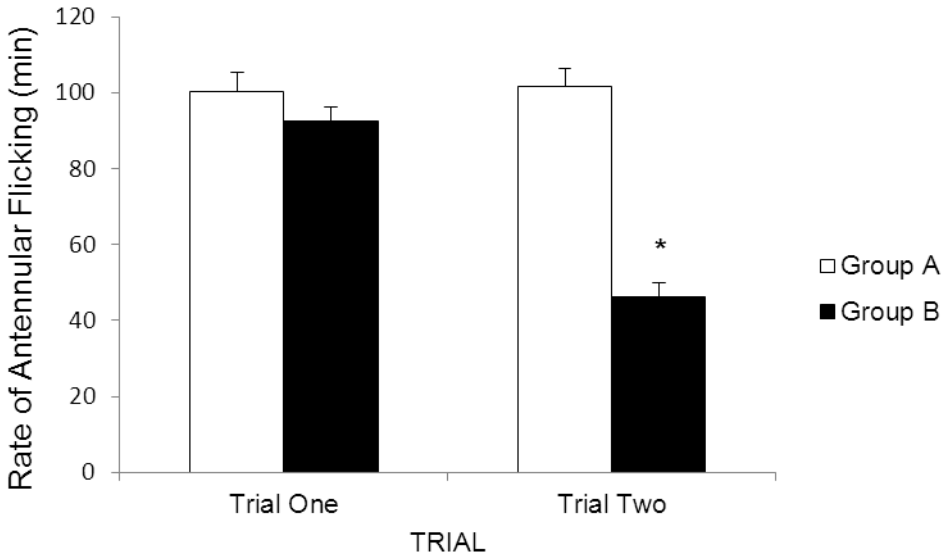

Figure 5.2. Rates of antennular flicking across two trials, and between two groups of hermit crabs. The first trial was conducted after both groups of hermit crabs had been kept in untreated sea water for five days. The second trial was conducted after Group A had been kept in untreated sea water (pH_{NBS} = 8.20) for five days, and Group B had been kept in reduced pH sea water (pH_{NBS} = 6.80) for the same period. Values are means (+ SE). * Asterisk indicates significant differences between Groups.

There was no effect of trial ($F_{1,72}$ = 2.951, P = 0.0901) on the time spent on locomotory activities, but there was a significant effect of pH treatment ($F_{1,72}$ = 9.769, P = 0.003) and a significant interaction effect between trial and pH treatment ($F_{1,72}$ = 8.703, η^2_p = 0.11, P = 0.004) (Figure 5.3.).

Post hoc tests revealed that the amount of time spent in motion was lower for crabs in Group B than it was for crabs in Group A in trial two, whereas there was no difference between Groups A and B in trial one (Table 5.1.).

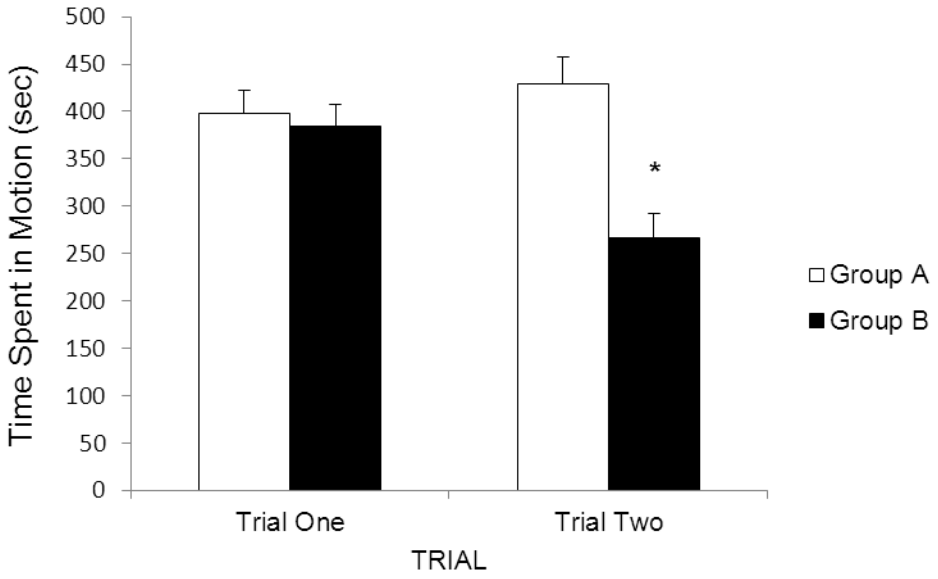

Figure 5.3. Time spent in motion across two trials and between two groups of hermit crabs. The first trial was conducted after both groups of hermit crabs had been kept in untreated sea water for five days. The second trial was conducted after Group A had been kept in untreated sea water (pH_{NBS} = 8.20) for five days, and Group B had been kept in reduced pH sea water (pH_{NBS} = 6.80) for the same period. Values are means (+ SE). * Asterisk indicates significant differences between Groups.

Table 5.1. Results of *post hoc* paired and unpaired t tests for pair-wise comparisons between means. Group A was exposed to normal pH in trial one and normal pH in trial two. Group B was exposed to normal pH in trial one and reduced pH in trial two. * significant results.

Behaviour	Within Groups Paired t Tests				Between Groups Unpaired t Tests			
	Group	t	df	P	Group	t	df	P
Latency	A1 v A2	1.427	27	0.165	A1 v B1	-0.848	73	0.399
	B1 v B2	1.606	45	0.115	A2 v B2	-3.968	72	<0.001*
Antennular Flicking	A1 v A2	-0.243	27	0.810	A1 v B1	-1.236	73	0.220
	B1 v B2	10.723	45	<0.001*	A2 v B2	9.112	72	<0.001*
Motion	A1 v A2	-0.965	27	0.343	A1 v B1	0.210	77	0.834
	B1 v B2	3.454	45	0.001*	A2 v B2	3.989	72	<0.001*

Table 5.2. Mean ± SD for experimental aquaria ASW physical parameters measured throughout the 5 day exposure period (80 measurements from each treatment group). The parameters were calculated with CO2sys (Pierrot et al. 2006) using the pH and pCO_2 values with dissociation constants from Mehrbach et al. (1973) refit by Dickson and Millero (1987) and KSO_4 using Dickson (1990).

Variable	**Experimental Aquaria**	
Nominal pH	pH 8.2	pH 6.8
Measured pH	8.22 ± 0.04	6.85 ± 0.04
TCO_2 (mmol l^{-1})	2.22 ± 0.20	3.30 ± 0.28
Salinity	34.0 ± 0.05	34.0 ± 0.04
Temperature (ºC)	15.1 ± 0.00	15.1 ± 0.00
pCO_2 (µatm)	375.49 ± 3.76	12191.48 ± 122.22
Alkalinity (µEq kg^{-1})	2480.21 ± 242.40	2860.02 ± 287.10
Calcite saturation	4.51 ± 0.74	0.27 ± 0.05
Aragonite saturation	2.89 ± 0.47	0.18 ± 0.03
HCO_3^- (mmol l^{-1})	2.02 ± 0.17	2.83 ± 0.28
CO_3^{2-} (mmol l^{-1})	0.19 ± 0.03	0.01 ± 0.00

DISCUSSION

Reduced sea water pH altered the normal shell assessment and selection behaviour of *P. bernhardus.* The crabs in the reduced pH treatment were less likely to change shells, and those crabs that did change took significantly longer to do so, wasting valuable time, and potentially energy, and prolonging an activity that makes them vulnerable to predation. Significantly higher numbers of hermit crabs in the reduced pH treatment failed to investigate and exchange shells altogether, remaining in inferior shells that gave inadequate protection. There was also a significant reduction in antennular flicking (the 'sniffing response' in decapods) in the reduced pH group, implying a possible disruption to chemo-sensory function; and a significant effect of reduced pH on a measure of overall activity, the duration of locomotion. Reduced locomotion has been demonstrated in response to physiological stress in a range of crustaceans (Taylor & Spicer 1988; Eriksson & Baden 1997; Taylor & Eggleston 2000; McAllen & Taylor 2001), but may also indicate a lack of olfactory stimulation (Reeder & Ache 1980; Chapter 3: de la Haye et al. 2012). Thus physiological stress and perhaps its effects on the metabolic and neurological functions of these crabs might be partly responsible for the disruption to decision making and reduced flicking frequency under low pH conditions. However, the effect size of low pH on antennular flicking behaviour was greater than that for locomotion, indicating that a change in overall physiological condition might not explain all of the changes observed, as in Chapter 3. The changes to the crabs' normal shell assessment and selection behaviour could reduce survival, as finding an optimal shell protects

them from predators and confers fitness advantages (Bertness 1981). Given the importance of sensory mediated assessment and decision-making processes in marine ecosystems, my study shows that the disruption to such activities, resulting from reduced pH, has the potential to affect the behavioural interactions between individuals and their environment in intertidal systems.

The hermit crabs in the reduced pH treatment took significantly longer to change shells. However, there was no difference between treatment groups in the latency to locate the shells; many were still able to find the shells but did not ultimately change to an optimal shell. The fact that some were able to locate the shells may have been a function of the experimental set-up where the shells were situated near enough for the crabs to be able to use visual cues. What is interesting is that the reduced pH affected their decision-making processes, and not all individuals were affected in the same way. As the results show, between the crabs in both groups, that found the optimal shell, a significantly lower number in the reduced pH sea water group made the decision to adopt the optimal shell than those crabs in the control sea water group, although many in the reduced pH group did make the decision to change. Such differential responses to an altered environment are likely to give some individuals an advantage over others, and this could have wide reaching implications for hermit crabs that also use such ritual assessment and decision making processes during contest behaviour (Elwood & Neil 1992). Physiological stress could be the cause of this disruption to their behaviour as it has been shown to alter neurological

functions and decision making in animals (Dias-Ferreira et al. 2009; Graham et al. 2010). However, animals are also able to alter their behavioural decisions in response to a changing environment (Inglis & Langton 2006). It has already been demonstrated that hermit crabs are sensitive to chemical context and are able to discriminate amongst complex sets of odours to help them make a decision appropriate to the current conditions (Gherardi & Atema 2005). Environmental factors such as hypoxia have been shown to alter the shell investigation and agonistic behaviours of hermit crabs (Cote et al. 1998; Briffa & Elwood 2000). Cote et al. (1998) showed that crabs kept in hypoxic conditions chose thinner, lighter, and therefore smaller, shells. This choice allowed them to cope better with the hypoxic conditions, because lighter shells are less energetically costly to carry. However, a cramped shell has been shown to have deleterious effects on growth and reproductive success, and increases vulnerability to predation (Bertness 1981). Behavioural plasticity can be a rapid, adaptive response to environmental change, but may also involve potentially costly trade-offs. In this study the decision of those crabs in the reduced pH sea water that chose not to move to an optimal shell, but remain in a smaller, lighter one, may have concealed an adaptive response to reduced pH conditions, rather than an inability to detect or accurately assess the new shell. A trade-off that mitigates environmental stressors like hypoxia and hypercapnia, but increases the risk of predation and reduces fitness, could be interpreted as simultaneously adaptive and maladaptive. Similar, potentially maladaptive, disruptions to behaviour were observed by Munday et al. (2009) and Dixson et al. (2010) in juvenile fish exposed to reduced pH sea water. The olfactory abilities of

Amphiprion percula larvae were confused under these conditions and they were attracted to odours that they would usually avoid, like predators, and were repelled by odours they should find attractive, like suitable settlement site cues. Bibby et al. (2007) also showed how reduced pH can disrupt induced defences in a marine gastropod, resulting in changes to anti-predator behaviour. *Littorina littorea* maintained in low pH sea water and conditioned with a predator cue were unable to express the induced defence of shell thickening owing to the reduced availability of carbonate ions, and shell dissolution under high CO_2 conditions. In subsequent behavioural trials, when re-exposed to the predator cue, these snails showed an increased avoidance response: able to perceive that their shells were thinner, and afforded less protection, they made the decision to leave the water. This decision could be seen as adaptive as it gives more protection from predation, however it involves a costly trade-off owing to the energy required, and the suspension of other activities such as grazing. Reductions in sea water pH are likely to create such complex problems for marine animals in the future, and differential responses within a population, as observed in this study, may increase some individuals' survivorship over others, affecting intra-specific interactions.

Hermit crabs in the reduced pH treatment flicked their antennules less than those in untreated sea water, as in Chapters 3 and 4. This may indicate a disruption to olfactory function, one of the senses used by hermit crabs to gather information about empty shells. The response was similar to that observed for the freshwater crayfish *Cambarus bartoni* (Allison et al. 1992) under reduced pH conditions. It is possible that the antennules may have

been flicked less rapidly owing to physical damage (Fabry et al. 2008), fatigue, or an inability to detect any odour. Typically the antennules are flicked rapidly through the water, allowing chemicals to bind repeatedly to receptor sites, and the greatest frequency of flicking occurs at the onset of the chemical stimulation (Schmitt & Ache 1979; Reeder & Ache 1980; Devine & Atema 1982; Allison et al. 1992), so the decreased flicking in this study implies an impaired ability to detect the chemical stimulus, especially as calcareous objects, such as the *L. littorea* shells used here, might be expected to be more detectable *via* chemical cues owing to increased shell dissolution (Orr et al. 2005; Hall-Spencer et al. 2008). In this study the crabs were able to use visual cues owing to the proximity of the shells, however in other studies by the authors the same reduced flicking response to a chemical cue under reduced pH conditions was observed in the absence of any visual cue (de la Haye et al. 2011, 2012). Mundy et al. (2009) showed that the olfactory senses of *A. percula* larvae were compromised after being reared and tested in reduced pH sea water, but they found no abnormalities in the gross morphology of the olfactory organs of these fish larvae examined under an electron microscope. The antennules of *P. bernhardus* examined under an electron microscope after 5 days of exposure to reduced pH also showed no visible signs of damage (Appendix 2). This may indicate that physical damage to receptor organs is not the cause of disruption to chemoreception. After finding no physical damage to their olfactory organs, Mundy et al. (2009) speculated that the effects they observed were more likely to stem from some kind of chemical disruption to the reception process. Such disruption could represent interference with the ionic bonding of odour

molecules to receptor cells, caused by the increase in the concentration of hydrogen ions ($[H^+]$) under reduced pH conditions (Tierney & Atema 1988). This hypothesis is not easy to investigate directly, but such disruption might be expected to have a fairly rapid onset. The structure and function of the olfactory organs of crustaceans, as a group, are highly congruent (Hallberg et al. 1992); therefore any observed effect in *P. bernhardus* has the potential to affect other marine crustaceans in a similar way. Given the importance of chemical cues in the marine environment, any disruption to chemo-sensory behaviour could have serious consequences for community dynamics (Atema 1995; Hay 2009).

The reduction in locomotory activity observed could have a number of causes. Firstly, the effect could be owing to reduced ability to detect the empty shell, as stillness has been shown to be a response to a lack of olfactory stimulation in *P. bernhardus* (Chapter 3: de la Haye et al. 2011). However a significant number of crabs in the reduced pH sea water were able to locate the shell, in this case perhaps using visual cues alone. On the other, hand, this effect could represent some form of energetic stress, for example owing to the cost of maintaining extracellular acid-base balance, or supporting enhanced metabolic activity and calcification under such reduced pH conditions (Pörtner et al. 2004; Wood et al. 2008). Under physiological stress responsive behaviour is likely to be allocated a lower priority and energetically costly activities suspended. Allison et al. (1992) conceded that this could have been the mechanism for the reduced antennular flicking rate of their freshwater crayfish in low pH conditions. However, a study by Tierney

and Atema (1986) on two species of crayfish found that activity levels were unaffected by reduced pH, and they attributed a reduction in feeding to an inability to detect the food odour, rather than any lassitude brought on by the stresses of maintaining acid-base balance. The previous studies on *P. bernhardus* in Chapters 3 and 4 found elevated chloride and calcium ions in samples of the crabs' haemolymph after both short and medium term exposure to reduced pH respectively, indicating that disruptions to chemo-sensory behaviour may be due to physiological stress, but also the possibility that extracellular ionic changes might interfere with nerve transmission, as has recently been shown in reef fish (Nilsson et al. 2012), and thus affect functions such as perception and muscular control. However, more work is needed to investigate these hypotheses.

The study of behavioural responses to environmental disruption is important as behaviour can link physiological function with ecological processes (Scott & Sloman 2004). Behavioural plasticity can also allow animals to respond rapidly to environmental disruption where physical acclimation might take longer to achieve, or be too costly (Tuomainen & Candolin 2010). However, any behaviour adopted in response to such disruption may not be ultimately sustainable. Ongoing anthropogenic pollution is likely to create complex problems for animals by disrupting their behaviour and decision making abilities, or forcing them to make costly trade-offs in an attempt to mitigate such changes to their environment. The inhabitants of rock pools may experience disruptions to their normal behaviour for extended periods during tidal cycles owing to a reduction in sea water pH, and such

reductions are likely to be exacerbated in the future by ocean acidification and potential CO_2 sequestration leaks. This study indicates that reduced sea water pH may affect sensory-mediated information gathering activities, and demonstrates disruption to the resource assessment and behavioural decisions of an intertidal species, potentially leaving it more vulnerable to predators and affecting its reproductive success, or forcing it to make costly trade-offs. Such changes to normal behaviour could have serious consequences for marine animals that rely so heavily on the gathering of information in order to make crucial decisions impacting on their fitness and survival. The likelihood for reduced pH to produce differential effects upon different species, and upon individuals from the same species, may have wider implications in a future, more acidic ocean, with the potential to alter intertidal community dynamics and structure.

CHAPTER 6

Discussion

Summary

The aim of this thesis was to investigate the effects of reduced sea water pH on the behaviour of intertidal decapod crustaceans within the context of natural variations in the pH of rock pool habitats, and in relation to predicted changes in sea water pH resulting from ocean acidification (OA) and CO_2 carbon capture storage (CCS) leakage scenarios. The main focus was on pH-induced info-disruption, including disruptions to the information gathering and chemo-sensory behaviour of crustaceans, with a final study on a more complex behaviour, resource assessment that necessitates the use of chemical and tactile cues, but also involves decision making processes. Previous studies had identified pH-induced disruptions to the olfactory behaviour of the freshwater fish, *Oncorhynchus mykiss* (Hara 1976) and the freshwater decapod *Cambarus bartoni* (Hara 1976; Alison et al. 1996), and more recently in the marine fish *Amphiprion percula* (Munday et al. 2009). The initial study in this thesis was to see if a similar disruption would be observed in a marine decapod. The aim was to test an extremely tolerant marine species, the prawn *P. elegans*, with a short-term exposure to highly reduced pH. A highly reduced pH level was used in order to be able to detect any effects in a species already well adapted to the extreme variability of the sea water pH of high shore rock pools. When reduced pH was shown to disrupt the 'sniffing' response in *P. elegans*, the study that followed was designed to build on this initial discovery by using the same protocol but with a less tolerant species, the hermit crab *P. bernhardus*, to determine whether its chemo-sensory behaviour would be more seriously disrupted. In addition,

in order to gain an insight into some of the possible underlying mechanisms for any disruption, I focussed on measuring haemolymph ion concentrations, a method that has been used to investigate the acid-base status of crustaceans. When the chemo-sensory behaviour of *P. bernhardus* was found to be more severely affected than *P. elegans* to a short-term exposure to highly reduced pH, the subsequent study investigated this species' responses to a range of pH levels to attempt to identify the thresholds for that disruption, and also observed the effects over time by subjecting the hermit crabs to a longer exposure of 60 days and testing their responses at regular intervals during that period. Their survival was also monitored and the physiological measure of haemolymph ion concentrations was taken once again, at the end of the study, in order to investigate any disruptions to acid-base balance regulation, alongside their behavioural responses. The aim of the final study in this thesis was to look at more complex behaviours involving decision making, to see how they were affected by reduced pH; therefore the shell assessment and selection behaviour of *P. bernhardus* was used as a model behavioural system to investigate this question.

General findings

Overall the chemo-sensory behaviour of *P. elegans* and *P. bernhardus* were shown to be disrupted by reduced sea water pH. The findings also show that species inhabiting rock pools may experience natural disruptions to their behaviour and physiology over short time periods when pH falls rapidly during tidal cycles. Possible thresholds for such disruption were detected that matched OA and CO_2 CCS leakage scenario predictions. Similar patterns in response were revealed between behavioural and physiological measures in the longer term study, indicating that behaviour could be a sensitive indicator of underlying physiological status. Finally the more complex behaviour of resource assessment, and the decision making abilities it requires, were also significantly affected by a reduction in sea water pH in *P. bernhardus*, with implications for a range of activities involving decision making, across diverse behavioural contexts. Such sub-lethal effects of low pH (i.e. disruption of information gathering and decision making) could therefore impact on the fitness of individuals and, ultimately, on the health and viability of populations.

Chemo-sensory behaviour in both *P. elegans* and *P. bernhardus* were shown to be significantly affected by a short-term exposure to highly reduced pH (Chapters 2 and 3). In Chapter 2 reduced pH sea water had a significant impact on the antennular flicking rate ('sniffing' response) of *P. elegans*, implying an effect upon their chemo-responsiveness, although, contrary to predictions, they were still able to locate the food cue. This effect on their sniffing response was consistent across both experiments, and agrees with the findings of Allison et al. (1992) where the rate of antennular flicking of the

crayfish *C. bartoni* significantly decreased under low pH conditions in freshwater. The disruption was not immediate, and only manifested itself on day five, with no significant change in the behaviour of the prawns on day one, therefore in subsequent studies behavioural trials were carried out after at least five days of exposure. A similar response was found in *P. bernhardus* in Chapter 3 using the same protocol of a five day exposure to highly reduced sea water pH. The crabs in the reduced pH groups showed a significantly lower antennular flicking rate, as *P. elegans* had done, under similar conditions. Such reduced rates of flicking are either owing to a disruption to the chemo-sensory function of the antennules, or because flicking is an energy-costly activity (Mellon 1997) and may be down-regulated under stressful environmental conditions. The chemo-responsiveness of the hermit crabs was more severely affected by the reduced pH than that of the prawns, as they also took significantly longer to locate the odour source, and spent less time in contact with it, compared to both those crabs in untreated sea water and to the prawns in the previous study. There did not appear to be any permanent degradation of the cue under reduced pH conditions in this study, as crabs in the control sea water were still able to detect a cue that had been acidified.

The activity levels of the two species also differed between studies with *P. elegans* showing equal levels of activity under both reduced pH and control conditions, and *P. bernhardus* showing less locomotory activity under reduced pH conditions. Species-specific behaviours may have been responsible for these differences because, even though they are in the same taxonomic group, these two species are not closely related. Prawns are more

mobile, swimming decapods and have been previously shown to exhibit escape responses to hypoxic conditions (Taylor & Spicer 1987, 1988), they are also a more resilient species from a niche higher up the shore, and therefore may not have been as vulnerable to the effects of reduced pH as *P. bernhardus* as they are accustomed to the wider and more prolonged variations in the sea water pH of high shore rock pools, and are efficient iono-regulators (Dissanayake et al. 2010). The lower rates of activity in *P. bernhardus* in response to the reduced pH could have been a sign, as with antennular flicking, of either a lack of stimulation, owing to their inability to detect the cue, or of a metabolic depression brought on by the increased concentrations of CO_2 in the water. The analysis of *P. bernhardus*' haemolymph in Chapter 3 revealed an elevated concentration of chloride ions (Cl^-) in crabs from the reduced pH group, suggesting some disruption to their iono-regulatory ability and/or acid-base balance. This disruption may have been the cause of their lack of activity, as iono-regulation is an energy-costly activity (Kurihara et al. 2008; Small et al 2010). However, the effect of low pH on the specific chemo-responsive behaviour, antennular flicking, was greater than that for activity levels. Moreover, there was no correlation between [Cl^-] and activity levels or antennular flicking rates, providing a weight of evidence to suggest that there may also be a direct effect of low pH on the chemoreception process itself.

The studies in Chapters 2 and 3 established disruption to the chemo-sensory behaviour of two species of intertidal decapod crustaceans; however, the pH exposure was highly reduced and over a short time period, and although it is relevant to the potential damaging effects of a CO_2 CCS leakage

scenario, and to the natural variation that already exists in rock pool habitats, there was no indication of whether such effects would still be observed with the more modest pH levels predicted by ocean acidification models. Pörtner et al. (2004) and Widdicombe & Spicer (2008) stressed the importance of establishing the points of onset of disruptions caused by reduced sea water pH and of observing such effects over longer time periods to see whether there would be any signs of acclimation to such conditions. Chapter 4 addressed these questions by taking *P. bernhardus*, the species most affected by reduced pH, and carrying out a longer exposure of 60 days using five different pH levels, including levels predicted for future ocean acidification and CO_2 CCS leakage scenarios. The results revealed a very clear gradient in the disruption to the antennular flicking rate ('sniffing' response) of *P. bernhardus*, with the flicking response waning with decreasing sea water pH. This disruption appeared at pH_{NBS}=7.70 (a realistic value for near-surface temperate waters by the year 2100 (Meehl et al. 2007)), with another significant step-change in the flicking rate at pH_{NBS}=6.80 (representing a high CO_2 CCS leakage scenario (Blackford et al. 2009)). The significant disruptions at these levels may indicate thresholds for olfactory dysfunction, or for the onset of physiological stress. However, perhaps surprisingly, the hermit crabs' ability to locate the odour source was not affected in this instance, even in the lowest treatment, and these results differed to the previous study (Chapter 3) and may have been owing to differences in the protocol, or to smaller sample sizes which made an effect more difficult to detect. However, despite the differences, the main tenor of the results is still compatible.

In this longer term study the hermit crabs in the lower pH treatments had higher concentrations of Ca^{2+} in their haemolymph after the 60 day exposure, compared with the short-term (five day) exposure where Cl^- concentrations were elevated. This time there was a negative correlation between the antennular flicking response and the concentration of haemolymph Ca^{2+}, with the flicking rate declining with increasing haemolymph Ca^{2+}. The treatment-related disruption in both these behavioural and physiological measures was also echoed in the survival rates of the hermit crabs in those treatments, which also followed a gradient, with mortality increasing with declining pH levels. These linked patterns between behavioural and physiological measures, and mortality rates, suggest that the physiological effects are more prevalent after a longer term exposure to reduced pH and may account more for the disruptions to the crabs' chemo-sensory behaviour than after a short term exposure, where disruptions to the chemoreception process itself appear to dominate. The relationship between the behavioural and physiological measures and survival also allows the case to be made for a behavioural measure as a sensitive predictor of physiological status. This study also revealed differential survival rates between hermit crabs in the reduced pH treatments, and some evidence of possible acclimation to low pH conditions by surviving individuals where they showed an increase in both their flicking response and movement on day 60, relative to the other days. If there were a differential acclimation response amongst individuals of a species this would have implications for population dynamics, and warrants further investigation.

The aim of the final study in Chapter 5 was to take a more complex behaviour, reliant on a sequence of decisions, and determine whether this was affected by reduced sea water pH in a similar way to the more simple olfactory behaviour described above. The shell assessment and selection behaviour of *P. bernhardus* was used as a model system, as this behaviour is already well understood and entails a sequence of assessment-led decisions (Reese 1962; Jackson & Elwood 1989). The results revealed that reduced sea water pH disrupted the normal shell assessment and selection behaviour of *P. bernhardus.* The crabs in the reduced pH treatment were less likely to change from a shell that was too small for them into an optimal shell. Those crabs that did change shells took longer to make the exchange, using more energy and prolonging an activity that makes them vulnerable to predation. Significantly higher numbers of hermit crabs in the reduced pH treatment failed to investigate and exchange shells altogether, remaining in the smaller shells that gave inadequate protection. So their decision making also appeared to be adversely affected, however, it was not clear whether the reduced pH was affecting their decision making *per se*, or whether the crabs remaining in smaller shells made a decision to stay in them based on a response to the high CO_2 conditions, where carrying a lighter shell might require less energy in a physiologically stressful environment. In addition to the disruptions to their shell selection behaviour there was also a significant reduction in antennular flicking and activity levels in the crabs in the reduced pH treatment, consistent with the other studies. Thus physiological stress and perhaps its effects on the metabolic or neurological functions of these crabs might be partly responsible for the disruption to decision making and reduced

flicking frequency under low pH conditions. In addition to choosing between available resource units, decision-making plays a key role during hermit crab contests (Briffa & Elwood 2000), and in mate selection and anti-predator behaviour (Jackson & Elwood 1989; Briffa & Twyman 2011); therefore, my results indicate the possibility of far-reaching behavioural consequences for hermit crabs, and for decapod crustaceans in general, resulting from ocean acidification. A recent study by Domenici et al. (2012) shows that reduced pH causes disruption to cognitive function in reef fish. They found that reduced pH disrupts behavioural lateralization (the tendency to favour the left or right side during behavioural activities) in coral reef fish larvae. This behaviour helps to minimise decision times when faced with decisions requiring a directional response, like the avoidance of a predator. A follow-up study by Nilsson et al. (2012) proposed the possible mechanism for such dysfunction as interference to neurotransmitter function caused by changes in extracellular ionic gradients, resulting from acid-base regulation. It seems likely that a similar mechanism could be responsible for the disruptions observed in hermit crab behaviour as extracellular ionic imbalances were detected in both the short and longer term studies. Many marine species control their acid-base balance *via* iono-regulation, and therefore rising CO_2 levels could cause widespread sensory and behavioural disruption if this process impairs neurotransmitter function.

Conclusions and future directions

The data presented in this thesis provide evidence that reduced sea water pH causes info-disruption and alters decision-making processes in intertidal crustaceans after a short-term exposure of five days. There is also evidence for disruption over a longer term exposure of 60 days with specific thresholds for these behavioural disruptions that coincide with near future OA predictions and models for pH levels in CO_2 CCS leakage scenarios. Information gathering and decision making are crucial processes that determine the fitness and survival of all marine animals. Disruptions to such processes could have far reaching effects on both populations and on intra- and interspecific species interactions. From these studies on intertidal crustaceans, along with those on reef fish, it is concluded that the mechanism for such disruptions is unlikely to be due to a change in the state of the odour molecule, or to physical damage to sensory organs (although this may be more relevant after long term exposure). Rather, the effects observed could be caused by (a) changes in ionic gradients resulting from acid-base regulation (Pörtner et al. 2004; Nilsson et al. 2012) or (b) changes in the charge distribution at receptor sites (Tierney & Atema 1988), but additional work is required in order to test these hypotheses. Future studies could attempt to untangle whether info-disruption is due to metabolic stress, as a result of hypercapnia (Pörtner et al. 2004), or caused by changes in extracellular ionic gradients interfering with the functioning of neurotransmitters (Nilsson et al 2012). This could be achieved by

manipulating the concentrations of specific ions in sea water. Another important focus for future research is to take into account the potential for other environmental factors, such as rising temperature, to have a synergistic effect upon the functioning and behaviour of marine animals, as many physiological processes are temperature, as well as pH, dependent. Rising temperatures could also have a knock-on effect on parameters such as salinity in intertidal environments.

The implications drawn from this thesis are that climate change related reductions to ocean pH may have far reaching effects on information gathering and decision-making activities in marine animals. The correlation observed between behavioural and physiological responses makes a case for behavioural measures as sensitive indicators of underlying physiological condition. The evidence of a possible acclimation response by some of the hermit crabs in the lower pH treatments by day 60, as well as differences in survival, may have implications for future adaptation to changing environmental conditions. The individual differences in the responses of the hermit crabs in both Chapters 4 and 5 may prove a fruitful line of future enquiry. It will be important, with anthropogenically-induced environmental changes, to establish whether any acclimation responses observed in organisms to such changes are sustainable over longer timescales, and what costs might be incurred. The individual differences within and between species in their behavioural and physiological responses to environmental perturbations could provide the basis for selection and perhaps eventual adaptation to such changes.

APPENDICES

APPENDIX 1

Rock pool Survey Hannafore Point, Looe, Cornwall

METHODS

A 24-hour rock pool survey was carried out at Hannafore Point, Looe, Cornwall, UK (50:20N, 04:27W) on 14-15 July 2010. The aim was to measure water parameters (pH, temperature and salinity) at regular intervals during the day and night. Local conditions and high and low water times, with low water tidal heights, are given in Table A1.1.

Table A1.1. Tidal data and weather conditions for 14-15 July 2010. Tidal data from Admiralty EasyTide©,meteorological data from the Meteorological Archive, Casella Automatic Weather Station, School of Marine Science and Engineering, Plymouth University.

Date	Tidal Times and Height (m)	Windspeed (ms^{-1})	Temperature (^{o}C)	Total Sunshine (hrs)	Total Rainfall (mm)
14 July 2010	HW 08.30 LW 14.38 (0.5) HW 20.30	Max 22.34	Max 18.8 Min 15.5	9.00	7.00
15 July 2010	LW 02.30 (0.4) HW 08.30	Max 33.03	Max 18.6 Min 13.6	7.33	7.60

Six rock pools were randomly selected at each level of the shore (high, middle and low shore); heights on the shore were estimated using chart datum. The size of the pools was standardised as far as possible (mean length = 181.17 ± 70.84 cm; mean width = 69.17 ± 23.91 cm; mean depth =

12.36 ± 3.64 cm). Pools were marked using drilled brackets and glow sticks (6 inch taper glow sticks, Glow Sticks Direct, Newport, UK), that were attached to the metal brackets with cable ties. An additional 'control pool' was set up at the high and mid shore. This consisted of a bucket filled with seawater as emersion took place. Each bucket had no visible algae or animals present and was placed at the same height as the other rock pools. This was to give some idea of the modifying effects of macro organisms present in the other pools. Time constraints meant that there was only one control for the high and mid shore pools, which was not enough to be used in a formal statistical analysis.

Seawater pH, temperature and salinity were recorded using a handheld multimeter (YSI 63 pH Meter); and water samples were taken from each pool in brown borosilicate glass bottles poisoned with mercuric chloride ($HgCl_2$) according to Dickson et al. (2007) then analysed using pentiometric titration (Apollo SciTech Alkalinity Titrator Model AS-ALK2 and Batch 100 certified reference materials from Andrew Dickson). Sea water parameters for the study are given in Table A1.3. Recordings from each pool were taken every two hours over a 24 hr period. The number of recordings at each pool was subject to time constraints, owing to the tidal cycle and manpower. We took two readings for the lower shore (one day, one night); five readings for the mid shore (two day, three night); and eight readings for the upper shore (four day, four night).

RESULTS

The pH_{NBS} in the rock pools went up during the daytime and dropped overnight (Fig. A1.1.). Minimum and maximum values for pH_{NBS}, temperature and salinity for the rock pools and the two controls are given in Table A1.2. Sea water parameters for the rock pools and controls are given in Table A1.3.

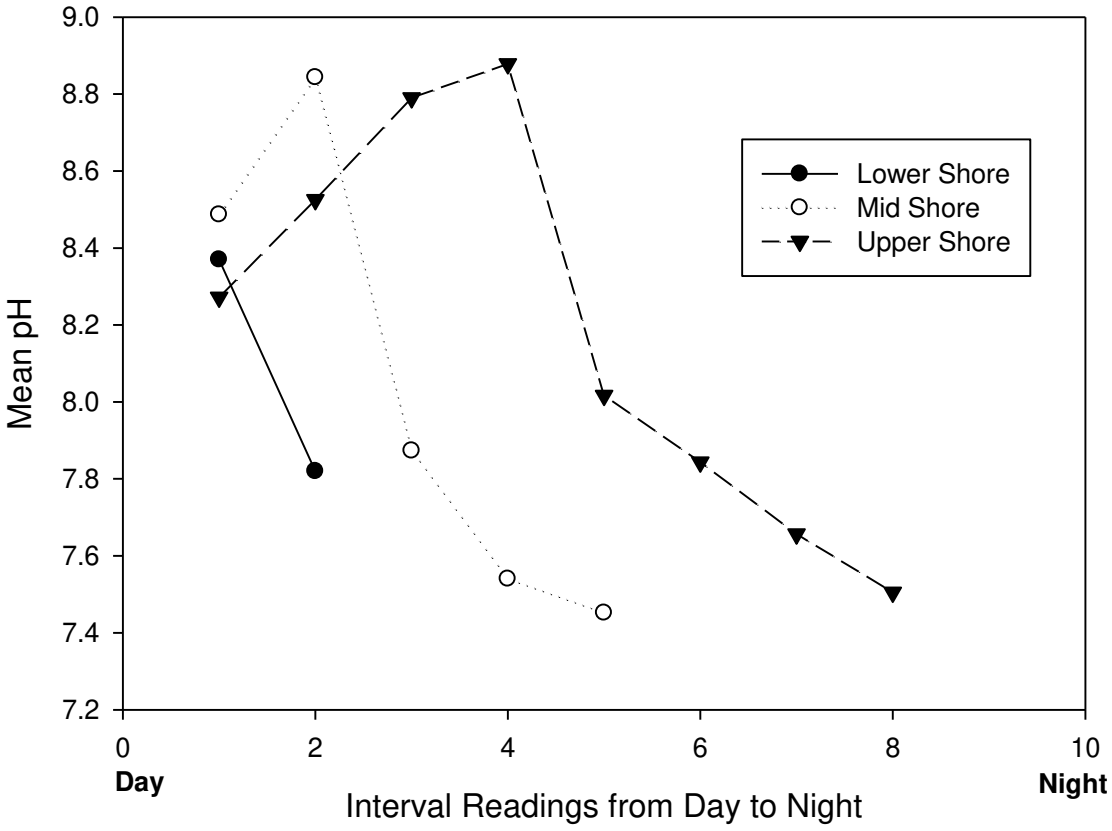

Fig A1.1. pH_{NBS} readings from rock pools at three heights on the shore taken over a 24 hour period through the day and night.

Table A1.2. Minimum and maximum values of pH, temperature and salinity recorded in rock pools at each height on the shore during the day and night (14-15 July 2010).

Parameters	Upper Shore		Mid Shore		Lower Shore		Upper Shore Control		Mid Shore Control	
	Day max	Night min	Day max	Night min	Day max	Night min	Day max	Night min	Day max	Night min
pH_{NBS}	9.10	7.29	8.96	7.35	8.70	7.76	8.27	8.02	8.54	7.88
Temperature ºC	23.9	15.2	23.1	14.9	22.5	15.8	21.4	15.2	20.4	14.9
$Salinity_{PSU}$	35.2	32.6	35.1	34.5	35.4	34.5	34.8	34.2	35.0	34.3

Table A1.3. Mean ± SD for rock pool sea water parameters measured throughout the night and day. The parameters were calculated with CO2sys (Pierrot et al. 2006) using the pH and pCO_2 values with dissociation constants from Mehrbach et al. (1973) refit by Dickson and Millero (1987) and KSO_4 using Dickson (1990).

Variable	Height on the shore																											
	Low Shore parameters		Mid shore parameters (mean + SD and control sample)										High shore parameters (mean + SD and control samples)															
Time	14.00	02.00	13.05		16.00		01.15		03.00		04.30		10.30		12.30		14.30		16.30		23.00		00.45		03.00		05.00	
Sample	S1	S2	S1	Con	S2	Con	S3	Con	S4	Con	S5	Con	S1	Con	S2	Con	S3	Con	S4	Con	S5	Con	S6	Con	S7	Con	S8	con
pH_{NBS}	8.37 ± 0.23	7.82 ± 0.05	8.49 ± 0.10	8.35	8.84 ± 0.14	8.54	7.87 ± 0.05	7.90	7.54 ± 0.08	7.88	7.45 ± 0.07	7.89	8.27 ± 0.06	8.25	8.53 ± 0.07	8.23	8.79 ± 0.08	8.24	8.88 ± 0.19	8.27	8.02 ± 0.01	8.07	7.84 ± 0.08	8.10	7.66 ± 0.11	8.07	7.51 ± 0.15	8.02
TCO_2 (mmol l^{-1})	8.77 ± 5.25	37.19 ± 4.15	5.40 ± 1.57	8.22	1.52 ± 1.06	4.43	31.99 ± 3.43	30.09	75.12 ± 13.31	32.02	95.44 ± 18.23	31.39	10.26 ± 1.66	11.06	4.50 ± 0.95	11.38	1.67 ± 0.44	11.39	1.50 ±1.23	10.05	22.30 ± 1.31	18.88	34.25 ± 7.74	17.76	55.36 ± 15.85	19.28	82.65 ± 29.67	22.19
Salinity	33.00 ± 1.63	34.88 ± 0.22	33.85 ± 0.97	34.8	33.88 ± 0.37	34.8	34.87 ± 0.19	34.2	34.68 ± 0.15	34.6	34.85 ± 0.14	34.6	34.93 ± 0.24	34.9	34.98 ± 0.25	35.00	33.97 ± 0.40	34.30	33.87 ± 0.26	34.60	34.95 ± 0.15	35.10	33.17 ± 0.42	34.30	32.98 ± 0.28	34.30	33.13 ± 0.34	34.60
Temp (ºC)	20.07 ± 1.36	15.83 ± 0.05	18.73 ± 0.71	18.60	22.08 ± 0.72	20.40	15.92 ± 0.08	16.00	15.62 ± 0.10	15.20	15.37 ± 0.10	14.90	18.95 ± 0.34	18.10	20.92 ± 0.82	19.10	23.55 ± 0.40	21.40	21.77 ± 0.50	19.60	16.38 ± 0.08	16.40	16.13 ± 0.10	15.80	15.93 ± 0.19	15.60	15.50 ± 0.09	15.20
pCO_2 (µatm)	264.66 ± 153.12	1621.51 ± 113.79	160.21 ± 46.16	244.36	49.41 ± 32.44	138.48	880.31 ± 93.98	827.00	2046.61 ± 362.46	861.16	2583.56 ± 494.14	836.61	308.60 ± 52.14	324.37	143.48 ± 33.17	343.75	56.61 ± 15.10	364.98	48.61 ± 40.37	307.11	622.60 ± 36.59	527.78	939.95 ± 214.39	485.55	1508.78 ± 433.59	523.87	2225.84 ± 801.65	596.69
Alkalinity (µEq kg^{-1})	2233.72 ± 35.53	2368.43 ± 12.72	2208.03 ± 66.31	2336.60	2073.18 ± 98.04	2345.60	2361.87 ± 21.21	2368.60	2396.80 ± 23.53	2369.70	2454.53 ± 94.82	2367.20	2333.82 ± 13.23	2339.30	2285.27 ± 57.68	2336.0	2153.10 ± 45.62	2495.80	2130.38 ± 24.03	2319.80	2413.83 ± 71.84	2360.90	2269.77 ± 45.36	2341.60	2273.27 ± 45.71	2337.30	2286.45 ± 54.48	2350.00
Calcite sat.	6.17 ± 2.56	1.99 ± 0.19	6.97 ± 0.99	5.94	10.88 ± 1.33	8.39	2.25 ± 0.23	2.35	1.11 ± 0.18	2.22	0.93 ± 0.13	2.24	5.25 ± 0.51	4.93	8.09 ± 0.65	4.88	11.04 ± 0.86	5.61	11.57 ± 1.98	5.25	3.11 ± 0.07	3.39	1.99 ± 0.29	3.46	1.34 ± 0.31	3.24	0.98 ± 0.33	2.93
Aragonite sat.	4.00 ± 1.68	1.28 ± 0.12	4.50 ± 0.63	3.84	7.10 ± 0.86	5.46	1.45 ± 0.15	1.51	0.71 ± 0.12	1.42	0.60 ± 0.08	1.44	3.40 ± 0.33	3.19	5.27 ± 0.41	3.17	7.24 ± 0.56	3.65	7.54 ± 1.29	3.41	2.00 ± 0.04	2.19	1.28 ± 0.19	2.22	0.86 ± 0.20	2.08	0.63 ± 0.21	1.88
HCO_3^- (mmol l^{-1})	1607.04 ± 260.87	2163.23 ± 18.06	1490.79 ± 113.44	1725.84	930.58 ± 202.14	1483.80	2130.24 ± 28.28	2127.94	2282.90 ± 41.95	2141.54	2359.44 ± 101.27	2136.48	1793.71 ± 51.29	1831.40	1448.98 ± 94.70	1833.25	1007.19 ± 104.39	1932.11	921.72 ± 230.74	1781.31	2094.17 ± 70.18	2010.59	2066.60 ± 54.92	1986.03	2136.34 ± 30.69	2004.43	2186.16 ± 36.06	2048.29
CO_3^{2-} (mmol l^{-1})	254.06 ± 105.56	83.41 ± 8.17	289.12 ± 39.33	248.18	450.11 ± 54.91	350.25	94.12 ± 9.69	98.08	46.42 ± 7.65	92.76	38.88 ± 5.40	93.75	219.43 ± 21.81	206.35	337.83 ± 27.67	204.32	456.14 ± 36.84	232.93	478.68 ± 82.27	218.92	130.44 ± 2.81	142.28	82.45 ± 12.05	144.50	55.63 ± 12.71	135.20	40.77 ± 13.76	122.47

APPENDIX 2

Electron Microscopy – the antennules of *P. bernhardus*

METHODS

It is possible that the disruptions to the behaviour of *P. bernhardus* were owing to physical damage (dissolution) to their antennules as a result of a five day exposure to reduced pH sea water. To investigate this possibility a single antennule was removed from 10 of the hermit crabs, five from the control treatment (pH_{NBS} = 8.20) and five from the reduced pH (pH_{NBS} = 6.80) treatment at the end of the study in Chapter 5.

Antennules were removed using surgical scissors and the area locally anaesthetised with clove oil. The antennules were fixed and stored in 2.5% glutaraldehyde. Samples were rinsed in buffer (sodium cacodylate 0.1 M pH 7.2), dehydrated with ethanol, critical point dried (Emitech K850) then mounted and gold sputter coated (Emitech k550). They were then examined under a JEOL SEM 5600LV scanning electron microscope.

RESULTS

There was no evidence of visible damage to the structure of the antennules from the reduced sea water pH treatment, compared to the controls, which might account for the disruption to their behaviour (Fig. A2.1.). Images were also taken of the aesthetasc hairs on the antennules, showing the gross structure of the hairs (Fig. A2.2.), and hairs in section (Fig. A2.3.) showing

their internal structure and what are possibly bundles of dendrites. Such structures observed in other hermit crab antennules have been thought to strongly support the hypothesis that aesthetascs are chemoreceptors (Ghiradella et al. 1968a, 1968b).

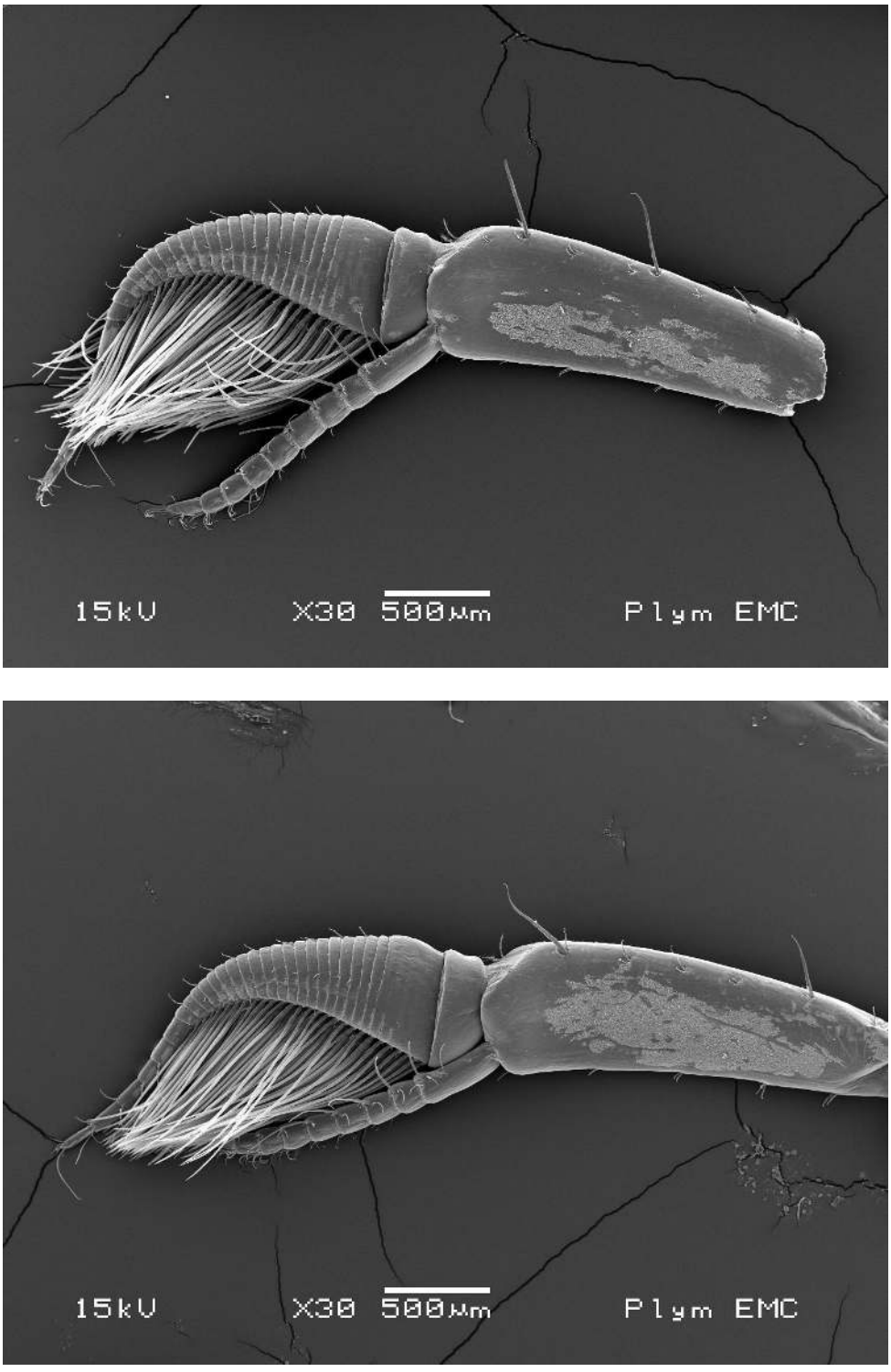

Fig. A2.1. Electron microscopy images showing a visual comparison of a single antennule of *P. bernhardus* from sea water treatments pH_{NBS} = 8.20 (top) and pH_{NBS} = 6.80 (bottom) after a five day exposure.

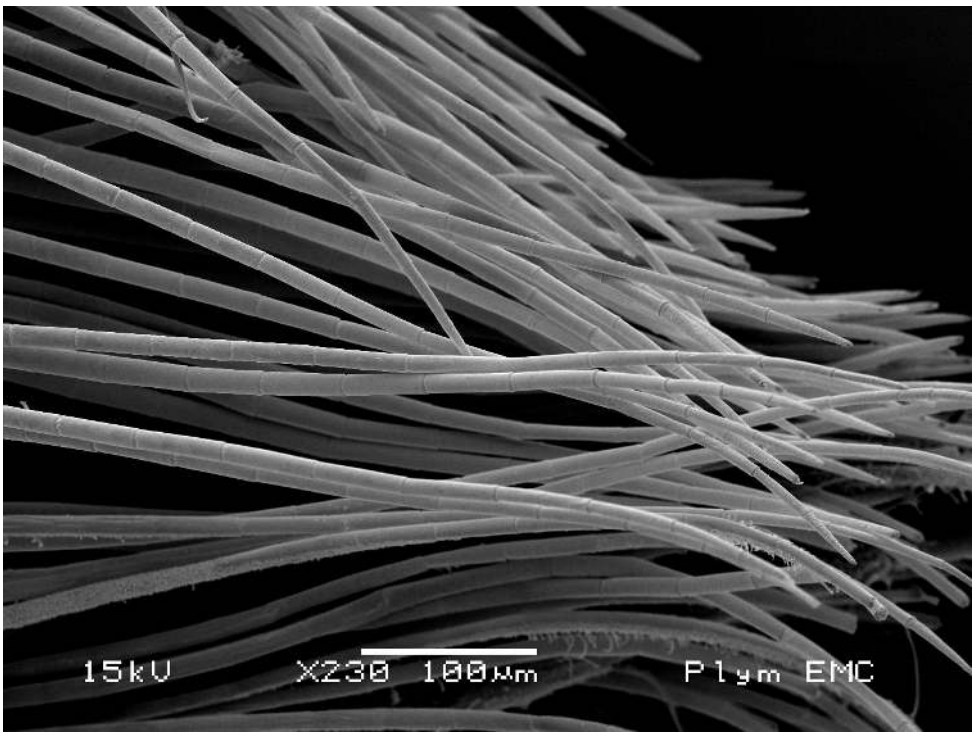

Fig. A2.2. Electron microscopy image of aesthetasc hairs

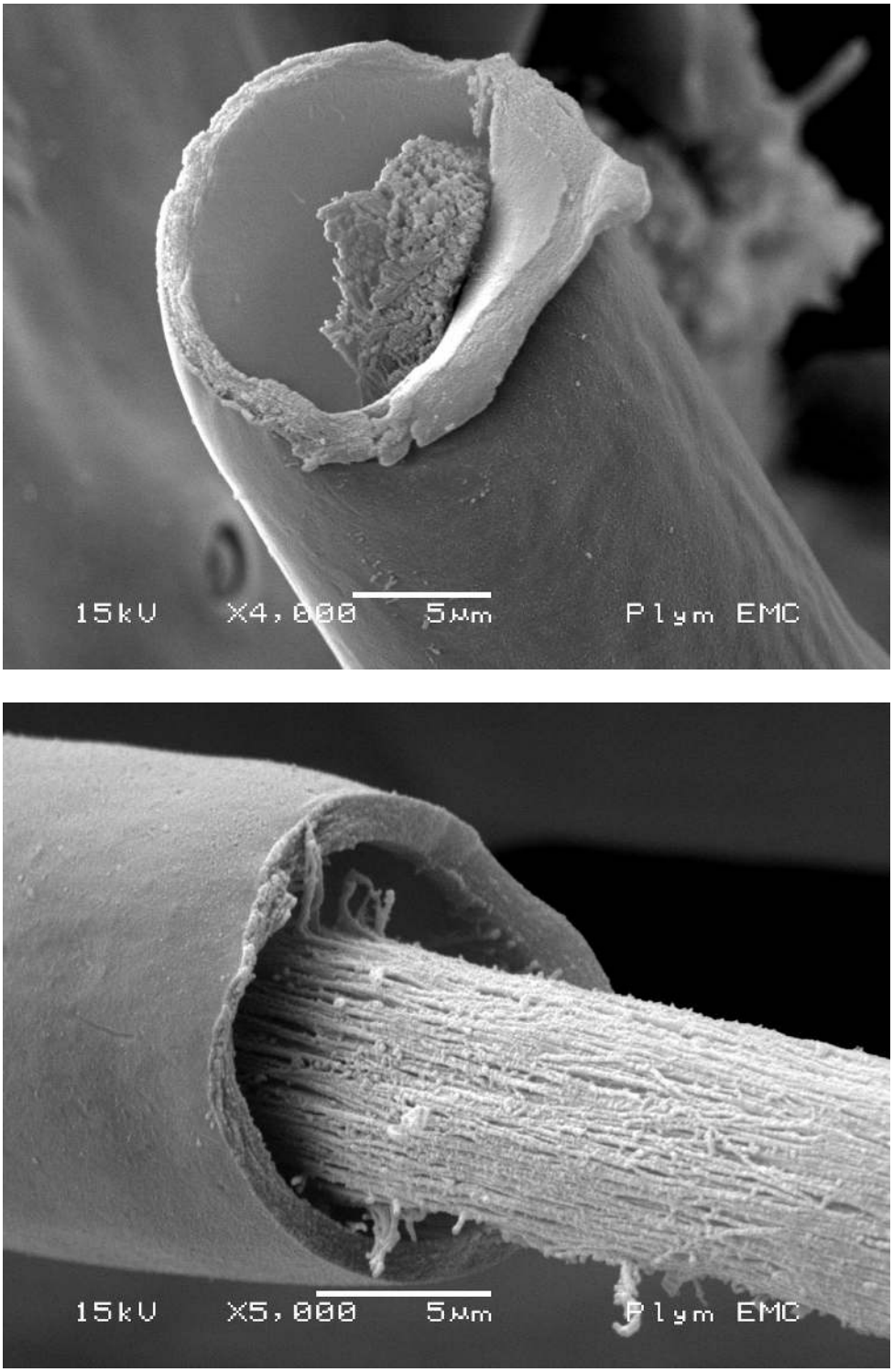

Fig. A2.3. Electron microscopy images of an aesthetasc hair in section from a *P. bernhardus* antennule, showing the internal structure.

APPENDIX 3

Tables for *post hoc* pairwise comparisons for the analyses in Chapter 4.

Table A3.1. Pairwise comparisons (Tukey's HSD) for the rate of antennular flicking across the 5 pH treatments (A = 8.00, B = 7.90, C = 7.70, D = 7.35, E = 6.80) after seven days of exposure.

Multiple Comparisons

Flicking

Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A	B	15.5833	9.70930	.501	-11.8001	42.9667
	C	41.3750*	9.70930	.001	13.9916	68.7584
	D	52.2833*	9.70930	.000	24.8999	79.6667
	E	85.4250*	9.70930	.000	58.0416	112.8084
B	A	-15.5833	9.70930	.501	-42.9667	11.8001
	C	25.7917	9.70930	.074	-1.5917	53.1751
	D	36.7000*	9.70930	.003	9.3166	64.0834
	E	69.8417*	9.70930	.000	42.4583	97.2251
C	A	-41.3750*	9.70930	.001	-68.7584	-13.9916
	B	-25.7917	9.70930	.074	-53.1751	1.5917
	D	10.9083	9.70930	.793	-16.4751	38.2917
	E	44.0500*	9.70930	.000	16.6666	71.4334
D	A	-52.2833*	9.70930	.000	-79.6667	-24.8999
	B	-36.7000*	9.70930	.003	-64.0834	-9.3166
	C	-10.9083	9.70930	.793	-38.2917	16.4751
	E	33.1417*	9.70930	.010	5.7583	60.5251
E	A	-85.4250*	9.70930	.000	-112.8084	-58.0416
	B	-69.8417*	9.70930	.000	-97.2251	-42.4583
	C	-44.0500*	9.70930	.000	-71.4334	-16.6666
	D	-33.1417*	9.70930	.010	-60.5251	-5.7583

Based on observed means.

The error term is Mean Square (Error) = 565.623.

*. The mean difference is significant at the 0.05 level.

Table A3.2. *Post hoc* pairwise comparisons on the time spent in motion by the hermit crabs after seven days of exposure, between the different sea water pH treatments using a Mann-Whitney *U*-test.

pH Group Comparisons	*U* (test statistic)	*P*
6.80 and 7.35	29.000	0.013*
6.80 and 7.70	24.000	0.006*
6.80 and 7.90	50.000	0.204
6.80 and 8.0	33.000	0.024*
7.35 and 7.70	66.000	0.729
7.35 and 7.90	47.000	0.149
7.35 and 8.0	72.000	1.00
7.70 and 7.90	35.500	0.035*
7.70 and 8.0	63.500	0.624
7.90 and 8.0	50.000	0.204

Table A3.3. *Post hoc* pairwise comparisons (Bonferroni corrected) for the rate of antennular flicking between the five pH treatments (A = 8.00, B = 7.90, C = 7.70, D = 7.35, E = 6.80) during the 60 day study.

Pairwise Comparisons[b]

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Df	Sig.[a]	95% Confidence Interval for Difference[a] Lower Bound	Upper Bound
A	B	8.004	6.260	61.020	1.000	-10.230	26.238
	C	30.393*	6.284	61.715	.000	12.097	48.688
	D	40.072*	6.464	63.639	.000	21.272	58.871
	E	65.777*	7.390	82.334	.000	44.459	87.094
B	A	-8.004	6.260	61.020	1.000	-26.238	10.230
	C	22.388*	6.284	61.715	.007	4.093	40.684
	D	32.068*	6.464	63.639	.000	13.268	50.867
	E	57.773*	7.390	82.334	.000	36.455	79.090
C	A	-30.393*	6.284	61.715	.000	-48.688	-12.097
	B	-22.388*	6.284	61.715	.007	-40.684	-4.093
	D	9.679	6.487	64.307	1.000	-9.181	28.539
	E	35.384*	7.410	82.911	.000	14.012	56.756
D	A	-40.072*	6.464	63.639	.000	-58.871	-21.272
	B	-32.068*	6.464	63.639	.000	-50.867	-13.268
	C	-9.679	6.487	64.307	1.000	-28.539	9.181
	E	25.705*	7.564	83.878	.010	3.898	47.512
E	A	-65.777*	7.390	82.334	.000	-87.094	-44.459
	B	-57.773*	7.390	82.334	.000	-79.090	-36.455
	C	-35.384*	7.410	82.911	.000	-56.756	-14.012
	D	-25.705*	7.564	83.878	.010	-47.512	-3.898

Based on estimated marginal means

a. Adjustment for multiple comparisons: Bonferroni.

*. The mean difference is significant at the .05 level.

b. Dependent Variable: Flicking.

Table A3.4. *Post hoc* pairwise comparisons (Bonferroni corrected) for the rate of antennular flicking between behavioural trials on day 7, 14, 30 and 60 during the 60 day study.

Pairwise Comparisons[b]

(I) Day	(J) Day	Mean Difference (I-J)	Std. Error	df	Sig.[a]	95% Confidence Interval for Difference[a] Lower Bound	Upper Bound
7.00	14.00	-.903	4.197	123.518	1.000	-12.157	10.350
	30.00	4.915	4.872	194.819	1.000	-8.073	17.902
	60.00	-19.165*	5.429	194.077	.003	-33.636	-4.694
14.00	7.00	.903	4.197	123.518	1.000	-10.350	12.157
	30.00	5.818	4.472	130.079	1.000	-6.164	17.799
	60.00	-18.262*	5.377	195.481	.005	-32.593	-3.931
30.00	7.00	-4.915	4.872	194.819	1.000	-17.902	8.073
	14.00	-5.818	4.472	130.079	1.000	-17.799	6.164
	60.00	-24.080*	5.099	134.574	.000	-37.734	-10.425
60.00	7.00	19.165*	5.429	194.077	.003	4.694	33.636
	14.00	18.262*	5.377	195.481	.005	3.931	32.593
	30.00	24.080*	5.099	134.574	.000	10.425	37.734

Based on estimated marginal means

a. Adjustment for multiple comparisons: Bonferroni.

*. The mean difference is significant at the .05 level.

b. Dependent Variable: Flicking.

Table A3.5. *Post hoc* pairwise comparisons for time spent in motion (log1+) by the hermit crabs between the 5 pH treatments (A = 8.00, B = 7.90, C = 7.70, D = 7.35, E = 6.80) during the sixty day study.

Pairwise Comparisons[b]

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Df	Sig.[a]	95% Confidence Interval for Difference[a] Lower Bound	Upper Bound
A	B	-.013	.118	61.027	1.000	-.355	.329
	C	-.004	.118	61.892	1.000	-.348	.340
	D	.009	.122	63.504	1.000	-.346	.363
	E	.435*	.142	83.284	.030	.024	.846
B	A	.013	.118	61.027	1.000	-.329	.355
	C	.009	.118	61.892	1.000	-.335	.353
	D	.022	.122	63.504	1.000	-.332	.376
	E	.448*	.142	83.284	.023	.038	.859
C	A	.004	.118	61.892	1.000	-.340	.348
	B	-.009	.118	61.892	1.000	-.353	.335
	D	.013	.122	64.327	1.000	-.343	.369
	E	.440*	.143	83.960	.028	.027	.852
D	A	-.009	.122	63.504	1.000	-.363	.346
	B	-.022	.122	63.504	1.000	-.376	.332
	C	-.013	.122	64.327	1.000	-.369	.343
	E	.427*	.146	84.461	.045	.006	.848
E	A	-.435*	.142	83.284	.030	-.846	-.024
	B	-.448*	.142	83.284	.023	-.859	-.038
	C	-.440*	.143	83.960	.028	-.852	-.027
	D	-.427*	.146	84.461	.045	-.848	-.006

Based on estimated marginal means

a. Adjustment for multiple comparisons: Bonferroni.

*. The mean difference is significant at the .05 level.

b. Dependent Variable: log_moving.

Table A3.6. *Post hoc* pairwise comparisons for haemolymph Ca^{2+} concentration between four of the pH_{NBS} treatments (A = 8.00, B = 7.90, C = 7.70, D = 7.35) after 60 days exposure.

Multiple Comparisons

Calcium

Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
A	B	1.1479	2.71299	.974	-6.3362	8.6319
	C	-5.2738	2.91638	.294	-13.3190	2.7713
	D	-8.3871*	2.80197	.030	-16.1167	-.6576
B	A	-1.1479	2.71299	.974	-8.6319	6.3362
	C	-6.4217	2.83100	.134	-14.2313	1.3880
	D	-9.5350*	2.71299	.009	-17.0191	-2.0509
C	A	5.2738	2.91638	.294	-2.7713	13.3190
	B	6.4217	2.83100	.134	-1.3880	14.2313
	D	-3.1133	2.91638	.712	-11.1585	4.9318
D	A	8.3871*	2.80197	.030	.6576	16.1167
	B	9.5350*	2.71299	.009	2.0509	17.0191
	C	3.1133	2.91638	.712	-4.9318	11.1585

Based on observed means.

The error term is Mean Square(Error) = 27.479.

*. The mean difference is significant at the 0.05 level.

Table A3.7. *Post hoc* pairwise comparisons for the survival of hermit crabs in 5 different pH_{NBS} treatments (1 = 8.0, 2 = 7.90, 3 = 7.70, = 7.35, 5 = 6.80) over the 60 day (9 week) study.

Pairwise Comparisons[a]				
		Wilcoxon		
(I) Treatment	(J) Treatment	(Gehan) Statistic	Df	Sig.
1	3	1.000	1	.317
	4	3.268	1	.071
	5	12.893	1	.000
2	3	1.000	1	.317
	4	3.268	1	.071
	5	12.893	1	.000
3	1	1.000	1	.317
	2	1.000	1	.317
	4	1.337	1	.248
	5	11.491	1	.001
4	1	3.268	1	.071
	2	3.268	1	.071
	3	1.337	1	.248
	5	5.104	1	.024
5	1	12.893	1	.000
	2	12.893	1	.000
	3	11.491	1	.001
	4	5.104	1	.024

a. Comparisons are exact.

REFERENCES

Agnew, D. J. & Taylor, A. C. 1986. Effects of oxygen tension, temperature, salinity, and humidity on the survival of two intertidal Gammarid amphipods. *Marine Ecology-Progress Series*, 32, 27-33.

Alcock, J. 2005. *Animal Behaviour*. 8th edn. Sunderland: Sinauer Associates Inc

Allison, V., Dunham, D. W. & Harvey, H. H. 1992. Low pH alters response to food in the crayfish *Camarus bartoni*. *Canadian Journal of Zoology-Revue Canadienne De Zoologie*, 70, 2416-2420.

Arnott, G. & Elwood, R. W. 2009. Assessment of fighting ability in animal contests. *Animal Behaviour*, 77, 991-1004.

Atema, J. 1995. Chemical signals in the marine environment: dispersal, detection, and temporal signal analysis. *Proceedings of the National Academy of Sciences of the United States of America*, 92, 62-66.

Bernatis, J. L., Gerstenberger, S. L. & McGaw, I. J. 2007. Behavioural responses of the Dungeness crab, *Cancer magister*, during feeding and digestion in hypoxic conditions. *Marine Biology*, 150, 941-951.

Bertness, M. D. 1981. Conflicting advantages in resource utilization: the hermit crab housing dilemma. *American Naturalist*, 118, 432-437.

Bibby, R., Cleall-Harding, P., Rundle, S., Widdicombe, S. & Spicer, J. 2007. Ocean acidification disrupts induced defences in the intertidal gastropod *Littorina littorea*. *Biology Letters*, 3, 699-701.

Blackford, J., Jones, N., Proctor, R., Holt, J., Widdicombe, S., Lowe, D. & Rees, A. 2009. An initial assessment of the potential environmental impact of CO_2 escape from marine carbon capture and storage systems. *Proceedings of the Institution of Mechanical Engineers Part a-Journal of Power and Energy*, 223, 269-280.

Bliss, D. E. (Ed.) 1982-1983. *The Biology of Crustacea (Vols 1-10)*. New York, USA: Academic Press Inc.

Blumstein, D. T. & Bouskila, A. 1996. Assessment and decision making in animals: A mechanistic model underlying behavioural flexibility can prevent ambiguity. *Oikos*, 77, 569-576.

Bradbury, J. W. & Vehrencamp, S. L. 1998. *Principles of Animal Communication*. Sunderland: Sinauer Associates Inc.

Breithaupt, T. & Eger, P. 2002. Urine makes the difference: chemical communication in fighting crayfish made visible. *Journal of Experimental Biology*, 205, 1221-1231.

Briffa, M. & Elwood, R. W. 2000. Cumulative or sequential assessment during hermit crab shell fights: effects of oxygen on decision rules. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 267, 2445-2452.

Briffa, M. & Elwood, R. W. 2007. Monoamines and decision making during contests in the hermit crab *Pagurus bernhardus*. *Animal Behaviour*, 73, 605-612.

Briffa, M., Rundle, S. D. & Fryer, A. 2008. Comparing the strength of behavioural plasticity and consistency across situations: animal personalities in the hermit crab *Pagurus bernhardus*. *Proceedings of the Royal Society B-Biological Sciences*, 275, 1305-1311.

Briffa, M. & Elwood, R. W. 2010. Repeated measures analysis of contests and other dyadic interactions: problems of semantics, not statistical validity. *Animal Behaviour*, 80, 583-588.

Briffa, M. & Twyman, C. 2011. Do I stand out or blend in? Conspicuousness awareness and consistent behavioural differences in hermit crabs. *Biology Letters*, 7, 330-332.

Brooks, W. R. 1991. Chemical recognition by hermit crabs of their symbiotic sea-anemones and a predatory octopus. *Hydrobiologia*, 216, 291-295.

Brown, G. E., Adrian, J. C., Lewis, M. G. & Tower, J. M. 2002. The effects of reduced pH on chemical alarm signalling in ostariophysan fishes. *Canadian Journal of Fisheries and Aquatic Sciences*, 59, 1331-1338.

Caldeira, K. & Wickett, M. E. 2003. Anthropogenic carbon and ocean pH. *Nature*, 425, 365-365.

Cameron, J. N. 1985. Compensation of hypercapnic acidosis in the aquatic blue crab *Callinectes sapidus*: the predominance of external sea water over carapace carbonate as the proton sink. *Journal of Experimental Biology*, 114, 197-206.

Cameron, J. N. & Iwama, G. K. 1987. Compensation of progressive hypercapnia in channel catfish and blue crabs. *Journal of Experimental Biology*, 133, 183-197.

Cerri, R. D. 1983. The effect of light intensity on predator and prey behavior in Cyprinid fish: factors that influence prey risk. *Animal Behaviour*, 31, 736-742.

Cheal, M. 1975. Social olfaction: review of ontogeny of olfactory influences on vertebrate behavior. *Behavioral Biology*, 15, 1-25.

Chester, R. 2000. *Marine Geochemistry* (2nd ed.), Oxford, UK: Blackwell Science Ltd.

Chivers, D. P. & Smith, R. J. F. 1998. Chemical alarm signalling in aquatic predator-prey systems: A review and prospectus. *Ecoscience*, 5, 338-352.

Clay, J. R. 2005. Review: axon excitability revisited. *Progress in Biophysics & Molecular Biology*, 88, 59-90.

Cote, I. M., Reverdy, B. & Cooke, P. K. 1998. Less choosy or different preference? Impact of hypoxia on hermit crab shell assessment and selection. *Animal Behaviour*, 56, 867-873.

Dall, S. R. X., Giraldeau, L. A., Olsson, O., McNamara, J. M. & Stephens, D. W. 2005. Information and its use by animals in evolutionary ecology. *Trends in Ecology & Evolution*, 20, 187-193.

Daniel, M. J. & Boyden, C. R. 1975. Diurnal variations in physico-chemical conditions within intertidal rockpools. *Field Studies*, 4, 161-176.

de la Haye, K. L., Spicer, J. I., Widdicombe, S. & Briffa, M. 2011. Reduced sea water pH disrupts resource assessment and decision making in the hermit crab *Pagurus bernhardus*. *Animal Behaviour*, 82, 495-501.

de la Haye, K. L., Spicer, J. I., Widdicombe, S. & Briffa, M. 2012. Reduced pH sea water disrupts chemo-responsive behaviour in an intertidal crustacean. *Journal of Experimental Marine Biology and Ecology*, 412, 134-140.

Derby, C. D. & Sorensen, P. W. 2008. Neural processing, perception, and behavioral responses to natural chemical stimuli by fish and crustaceans. *Journal of Chemical Ecology*, 34, 898-914.

Devine, D. V. & Atema, J. 1982. Function of chemoreceptor organs in spatial orientation of the lobster, *Homarus americanus*: differences and overlap. *Biological Bulletin*, 163, 144-153.

Dias-Ferreira, E., Sousa, J. C., Melo, I., Morgado, P., Mesquita, A. R., Cerqueira, J. J., Costa, R. M. & Sousa, N. 2009. Chronic stress causes frontostriatal reorganization and affects decision-making. *Science*, 325, 621-625.

Dickson, A. G. 1990. Thermodynamics of the dissociation of boric acid in synthetic seawater from 273.15 K to 318.15 K. *Deep-Sea Research Part a-Oceanographic Research Papers*, 37, 755-766.

Dickson, A. G. & Millero, F. J. 1987. A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. *Deep-Sea Research Part a-Oceanographic Research Papers*, 34, 1733-1743.

Dill, L. M. 1987. Animal decision-making and its ecological consequences: the future of aquatic ecology and behavior. *Canadian Journal of Zoology-Revue Canadienne De Zoologie*, 65, 803-811.

Dissanayake, A., Clough, R., Spicer, J. I. & Jones, M. B. 2010. Effects of hypercapnia on acid-base balance and osmo-/iono-regulation in prawns (Decapoda: Palaemonidae). *Aquatic Biology*, 11, 27-36.

Dixson, D. L., Munday, P. L. & Jones, G. P. 2010. Ocean acidification disrupts the innate ability of fish to detect predator olfactory cues. *Ecology Letters*, 13, 68-75.

Domenici, P., Allan, B., McCormick, M. I. & Munday, P. L. 2012. Elevated CO_2 affects behavioural lateralization in a coral reef fish. *Biology Letters*. 8, 78-81.

Domenici, P., Lefrancois, C. & Shingles, A. 2007. Hypoxia and the antipredator behaviours of fishes. *Philosophical Transactions of the Royal Society B-Biological Sciences*, 362, 2105-2121.

Donaldson-Matasci, M. C., Lachmann, M. & Bergstrom, C. T. 2008. Phenotypic diversity as an adaptation to environmental uncertainty. *Evolutionary Ecology Research*, 10, 493-515.

Elwood, R. W. & Neil, S J. . 1992. *Assessments and decisions: a study of information gathering by hermit crabs*, 1st edn. London: Chapman & Hall.

Eriksson, S. P. & Baden, S. P. 1997. Behaviour and tolerance to hypoxia in juvenile Norway lobster (*Nephrops norvegicus*) of different ages. *Marine Biology*, 128, 49-54.

Fabry, V. J., Seibel, B. A., Feely, R. A. & Orr, J. C. 2008. Impacts of ocean acidification on marine fauna and ecosystem processes. *Ices Journal of Marine Science*, 65, 414-432.

Feely, R. A., Sabine, C. L., Lee, K., Berelson, W., Kleypas, J., Fabry, V. J. & Millero, F. J. 2004. Impact of anthropogenic CO_2 on the $CaCO_3$ system in the oceans. *Science*, 305, 362-366.

Feely, R. A., Sabine, C. L., Hernandez-Ayon, J. M., Ianson, D. & Hales, B. 2008. Evidence for upwelling of corrosive "acidified" water onto the continental shelf. *Science*, 320, 1490-1492.

Findlay, H. S., Kendall, M. A., Spicer, J. I., Turley, C. & Widdicombe, S. 2008. Novel microcosm system for investigating the effects of elevated carbon dioxide and temperature on intertidal organisms. *Aquatic Biology*, 3, 51-62.

Findlay, H. S., Kendall, M. A., Spicer, J. I. & Widdicombe, S. 2009. Future high CO_2 in the intertidal may compromise adult barnacle *Semibalanus balanoides* survival and embryonic development rate. *Marine Ecology-Progress Series*, 389, 193-202.

Findlay, H. S., Kendall, M. A., Spicer, J. I. & Widdicombe, S. 2010. Post-larval development of two intertidal barnacles at elevated CO_2 and temperature. *Marine Biology*, 157, 725-735.

Findlay, H. S., Wood, H. L., Kendall, M. A., Spicer, J. I., Twitchett, R. J. & Widdicombe, S. 2011. Comparing the impact of high CO_2 on calcium carbonate structures in different marine organisms. *Marine Biology*, 7, 565-575.

Fisher, H. S., Wong, B. B. M. & Rosenthal, G. G. 2006. Alteration of the chemical environment disrupts communication in a freshwater fish. *Proceedings of the Royal Society B-Biological Sciences*, 273, 1187-1193.

Forster, G. R. 1951a. The biology of the Common Prawn, *Leander serratus* (Pennant). *Journal of the Marine Biological Association of the United Kingdom*, 30, 333-360.

Forster, G. R. 1951b. Notes on *Leander squilla* L. *Journal of the Marine Biological Association of the United Kingdom*, 30, 361-367.

Gazeau, F., Quiblier, C., Jansen, J. M., Gattuso, J. P., Middelburg, J. J. & Heip, C. H. R. 2007. Impact of elevated CO_2 on shellfish calcification. *Geophysical Research Letters*, 34.

Gehan, E. A. 1965. A generalized Wilcoxon test for comparing arbitrarily singly censored samples. *Biometrika*, 52, 203-213.

Gherardi, F. & Atema, J. 2005. Effects of chemical context on shell investigation behavior in hermit crabs. *Journal of Experimental Marine Biology and Ecology*, 320, 1-7.

Ghiradella, H., Case, J. & Cronshaw, J. 1968a. Fine structure of the aesthetasc hairs of *Coenobita compressus* edwards. *Journal of Morphology*, 124, 361-385.

Ghiradella, H., Cronshaw, J. & Case, J. 1968b. Fine structure of the aesthetasc hairs of *Pagurus hirsutiusculus* dana. *Protoplasma*, 66, 1-20.

Graham, L. K., Yoon, T. & Kim, J. J. 2010. Stress impairs optimal behavior in a water foraging choice task in rats. *Learning & Memory*, 17, 790-793.

Gravel, B. E., Wong, P. Y., Starks, P. T. & Pechenik, J. A. 2004. The use of artificial shells for exploring shell preference in the marine hermit crab *Pagurus longicarpus* (Say). *Annales Zoologici Fennici*, 41, 477-485.

Hallberg, E., Johansson, K. U. I. & Elofsson, R. 1992. The aesthetasc concept: structural variations of putative olfactory receptor cell complexes in crustacea. *Microscopy Research and Technique*, 22, 325-335.

Hall-Spencer, J. M., Rodolfo-Metalpa, R., Martin, S., Ransome, E., Fine, M., Turner, S. M., Rowley, S. J., Tedesco, D. & Buia, M. C. 2008. Volcanic carbon dioxide vents show ecosystem effects of ocean acidification. *Nature*, 454, 96-99.

Hara, T. J. 1976. Effects of pH on olfactory responses to amino acids in Rainbow Trout *Salmo gairdneri*. *Comparative Biochemistry and Physiology a-Physiology*, 54, 37-39.

Hardege, J. D., Jennings, A., Hayden, D., Muller, C. T., Pascoe, D., Bentley, M. G. & Clare, A. S. 2002. Novel behavioural assay and partial purification of a female-derived sex pheromone in *Carcinus maenas*. *Marine Ecology-Progress Series*, 244, 179-189.

Hawkins, D. G. 2004. No exit: thinking about leakage from geologic carbon storage sites. *Energy*, 29, 1571-1578.

Hay, M. E. 2009. Marine chemical ecology: chemical signals and cues structure marine populations, communities, and ecosystems. *Annual Review of Marine Science*, 1, 193-212.

Hazlett, B. A. 1981. The behavioral ecology of hermit crabs. *Annual Review of Ecology and Systematics*, 12, 1-22.

Henry, R. P. & Cameron, J. N. 1982. Acid-base balance in *Callinectes sapidus* during acclimation from high to low salinity. *Journal of Experimental Biology*, 101, 255-264.

Henry, R. P. & Wheatly, M. G. 1992. Interaction of respiration, ion regulation, and acid-base balance in the everyday life of aquatic crustaceans. *American Zoologist*, 32, 407-416.

Hoover, K. C. 2010. Smell with inspiration: the evolutionary significance of olfaction. *American Journal of Physical Anthropology*, 63-74.

Horner, A. J., Weissburg, M. J. & Derby, C. D. 2004. Dual antennular chemosensory pathways can mediate orientation by Caribbean spiny lobsters in naturalistic flow conditions. *Journal of Experimental Biology*, 207, 3785-3796.

Huggett, J. & Griffiths, C. L. 1986. Some relationships between elevation, physicochemical variables and biota of intertidal rock pools. *Marine Ecology-Progress Series*, 29, 189-197.

Inglis, I. R. & Langton, S. 2006. How an animal's behavioural repertoire changes in response to a changing environment: a stochastic model. *Behaviour*, 143, 1563-1596.

Jackson, N. W. & Elwood, R. W. 1989. How animals make assessments: information gathering by the hermit crab *Pagurus bernhardus*. *Animal Behaviour*, 38, 951-957.

Jacobsen, H. P. & Stabell, O. B. 2004. Antipredator behaviour mediated by chemical cues: the role of conspecific alarm signalling and predator labelling in the avoidance response of a marine gastropod. *Oikos*, 104, 43-50.

Jennions, M. D. & Petrie, M. 1997. Variation in mate choice and mating preferences: A review of causes and consequences. *Biological Reviews of the Cambridge Philosophical Society*, 72, 283-327.

Johnson, L. E. & Strathmann, R. R. 1989. Settling barnacle larvae avoid substrata previously occupied by a mobile predator. *Journal of Experimental Marine Biology and Ecology*, 128, 87-103.

Koehl, M. A. R. 2006. The fluid mechanics of arthropod sniffing in turbulent odor plumes. *Chemical Senses*, 31, 93-105.

Krebs, J. R. & Davies, N. B. 1997. *Behavioural Ecology: An Evolutionary Approach*. 4th edn. Oxford: Blackwell Publishing.

Kurihara, H., Matsui, M., Furukawa, H., Hayashi, M. & Ishimatsu, A. 2008. Long-term effects of predicted future seawater CO_2 conditions on the survival and growth of the marine shrimp Palaemon pacificus. *Journal of Experimental Marine Biology and Ecology*, 367, 41-46.

Lancaster, I. 1988. *Pagurus bernhardus* (L.) – An introduction to the natural history of hermit crabs. *Field Studies*, 7, 189-238.

Leduc, A., Kelly, J. M. & Brown, G. E. 2004. Detection of conspecific alarm cues by juvenile salmonids under neutral and weakly acidic conditions: laboratory and field tests. *Oecologia*, 139, 318-324.

Li, X. D., Chang, W. C., Chao, Y. J., Wang, R. Z. & Chang, M. 2004. Nanoscale structural and mechanical characterization of a natural nanocomposite material: The shell of red abalone. *Nano Letters*, 4, 613-617.

Lima, S. L. & Dill, L. M. 1990. Behavioral decisions made under the risk of predation: a review and prospectus. *Canadian Journal of Zoology-Revue Canadienne De Zoologie*, 68, 619-640.

Luquet, C. M. & Ansaldo, M. 1997. Acid-base balance and ionic regulation during emersion in the estuarine intertidal crab Chasmagnathus granulata Dana (Decapoda Grapsidae). *Comparative Biochemistry and Physiology a-Physiology*, 117, 407-410.

Lürling, M. & Scheffer, M. 2007. Info-disruption: pollution and the transfer of chemical information between organisms. *Trends in Ecology & Evolution*, 22, 374-379.

McAllen, R. & Taylor, A. 2001. The effect of salinity change on the oxygen consumption and swimming activity of the high-shore rockpool copepod *Tigriopus brevicornis*. *Journal of Experimental Marine Biology and Ecology*, 263, 227-240.

Meehl, G. A., Covey, C., Delworth, T., Latif, M., McAvaney, B., Mitchell, J. F. B., Stouffer, R. J. & Taylor, K. E. 2007. The WCRP CMIP3 multimodel dataset - A new era in climate change research. *Bulletin of the American Meteorological Society*, 88, 1383-+.

Mehrbach, C., Culberso.Ch, Hawley, J. E. & Pytkowic.Rm. 1973. Measurement of apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. *Limnology and Oceanography*, 18, 897-907.

Mellon, D. 1997. Physiological characterization of antennular flicking reflexes in the crayfish. *Journal of Comparative Physiology a-Sensory Neural and Behavioral Physiology*, 180, 553-565.

Mesce, K. A. 1982. Calcium-bearing objects elicit shell selection behavior in a hermit crab. *Science*, 215, 993-995.

Miner, B. G., Sultan, S. E., Morgan, S. G., Padilla, D. K. & Relyea, R. A. 2005. Ecological consequences of phenotypic plasticity. *Trends in Ecology & Evolution*, 20, 685-692.

Moore, A. 1994. An electrophysiological study on the effects of pH on olfaction in mature male Atlantic salmon *Salmo salar* Parr. *Journal of Fish Biology*, 45, 493-502.

Morris, S. & Taylor, A. C. 1983. Diurnal and seasonal variation in physicochemical conditions within intertidal rock pools. *Estuarine Coastal and Shelf Science*, 17, 339-355.

Morris, S. & Taylor, A. C. 1985. The respiratory response of the intertidal prawn *Palaemon elegans* (Rathke) to hypoxia and hyperoxia. *Comparative Biochemistry and Physiology a-Physiology*, 81, 633-639.

Munday, P. L., Dixson, D. L., Donelson, J. M., Jones, G. P., Pratchett, M. S., Devitsina, G. V., Doving, K. B. 2009. Ocean acidification impairs olfactory discrimination and homing ability of a marine fish. *Proceedings of the National Academy of Sciences*, 106, 1848-1852.

Munday, P. L., Dixson, D. L., McCormick, M. I., Meekan, M., Ferrari, M. C. O. & Chivers, D. P. 2010. Replenishment of fish populations is threatened by ocean acidification. *Proceedings of the National Academy of Sciences of the United States of America*, 107, 12930-12934.

Nienhuis, S., Palmer, A. R. & Harley, C. D. G. 2010. Elevated CO_2 affects shell dissolution rate but not calcification rate in a marine snail. *Proceedings of the Royal Society B-Biological Sciences*, 277, 2553-2558.

Nilsson, G. E., Dixson, D. L., Domenici, P., McCormick, M. I., Sørensen, C., Watson, S.-A., & Munday, P. L. 2012. Near-future carbon dioxide levels alter fish behaviour by interfering with neurotransmitter function. *Nature Climate Change*, 2, 1-4.

Orr, J. C., Fabry, V. J., Aumont, O., Bopp, L., Doney, S. C., Feely, R. A., Gnanadesikan, A., Gruber, N., Ishida, A., Joos, F., Key, R. M., Lindsay, K., Maier-Reimer, E., Matear, R., Monfray, P., Mouchet, A., Najjar, R. G., Plattner, G. K., Rodgers, K. B., Sabine, C. L., Sarmiento, J. L., Schlitzer, R., Slater, R. D., Totterdell, I. J., Weirig, M. F., Yamanaka, Y. & Yool, A. 2005. Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature*, 437, 681-686.

Parry, G. 1954. Ionic regulation in the palaemonid prawn *Palaemon* (=Leander) *serratus*. *Journal of Experimental Biology*, 31, 601-613.

Pierrot, D., Lewis, E. & Wallace, D. W. R. . 2006. CO_2SYS DOS p. program developed for CO2 system calculations. Oak Ridge: Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, U. S. Department of Energy.

Pistevos, J. C. A., Calosi, P., Widdicombe, S. & Bishop, J. D. D. 2011. Will variation among genetic individuals influence species responses to global climate change? *Oikos*, 120, 675-689.

Pomar, L. & Hallock, P. 2008. Carbonate factories: A conundrum in sedimentary geology. *Earth-Science Reviews*, 87, 134-169.

Pörtner, H. O., Langenbuch, M. & Reipschlager, A. 2004. Biological impact of elevated ocean CO_2 concentrations: Lessons from animal physiology and earth history. *Journal of Oceanography*, 60, 705-718.

Pyke, G. H., Pulliam, H. R. & Charnov, E. L. 1977. Optimal foraging: selective review of theory and tests. *Quarterly Review of Biology*, 52, 137-154.

Raabe, D., Romano, P., Sachs, C., Fabritius, H., Al-Sawalmih, A., Yi, S., Servos, G. & Hartwig, H. G. 2006. Microstructure and crystallographic texture of the chitin-protein network in the biological composite material of the exoskeleton of the lobster *Homarus americanus*. *Materials Science and Engineering a-Structural Materials Properties Microstructure and Processing*, 421, 143-153.

Raven, J. A., Caldeira, K., Elderfield, H., Hoegh-Guldberg, O., Liss, P., Riebesell, U., Shepherd, J., Turley, C. & Watson, A. . 2005. Ocean acidification due to increasing atmospheric carbon dioxide. Cardiff: The Royal Society.

Reeder, P. B. & Ache, B. W. 1980. Chemotaxis in the Florida spiny lobster, *Panulirus argus*. *Animal Behaviour*, 28, 831-839.

Reese, E. S. 1962. Shell selection behaviour of hermit crabs. *Animal Behaviour*, 10, 347-360.

Riebesell, U., Zondervan, I., Rost, B., Tortell, P. D., Zeebe, R. E. & Morel, F. M. M. 2000. Reduced calcification of marine plankton in response to increased atmospheric CO_2. *Nature*, 407, 364-367.

Ries, J. B. 2011. Skeletal mineralogy in a high-CO_2 world. *Journal of Experimental Marine Biology and Ecology*, 403, 54-64.

Rittschof, D. 1992. Chemosensation in the daily life of crabs. *American Zoologist*, 32, 363-369.

Rittschof, D., Kratt, C. M. & Clare, A. S. 1990. Gastropod predation sites: the role of predator and prey in chemical attraction of the hermit crab *Clibanarius vittatus*. *Journal of the Marine Biological Association of the United Kingdom*, 70, 583-596.

Royce-Malmgren, C. H. & Watson, W. H. 1987. Modification of olfactory-related behavior in juvenile Atlantic salmon by changes in pH. *Journal of Chemical Ecology*, 13, 533-546.

Sandeman, D. C. 1989. Physical properties, sensory receptors and tactile reflexes of the antenna of the Australian freshwater crayfish *Cherax destructor*. *Journal of Experimental Biology*, 141, 197-217.

Schindler, D. W. 1988. Effects of acid rain on freshwater ecosystems. *Science*, 239, 149-157.

Schmidt, K. A., Dall, S. R. X. & van Gils, J. A. 2010. The ecology of information: an overview on the ecological significance of making informed decisions. *Oikos*, 119, 304-316.

Schmidt, M. & Ache, B. W. 1996. Processing of antennular input in the brain of the spiny lobster, *Panulirus argus* .2. The olfactory pathway. *Journal of Comparative Physiology a-Sensory Neural and Behavioral Physiology*, 178, 605-628.

Schmitt, B. C. & Ache, B. W. 1979. Olfaction: responses of a decapod crustacean are enhanced by flicking. *Science*, 205, 204-206.

Scott, G. R. & Sloman, K. A. 2004. The effects of environmental pollutants on complex fish behaviour: integrating behavioural and physiological indicators of toxicity. *Aquatic Toxicology*, 68, 369-392.

Seibel, B. A. & Walsh, P. J. 2001. Carbon cycle - Potential, impacts of CO_2 injection on deep-sea biota. *Science*, 294, 319-320.

Shumway, S. E. 1978. Osmotic balance and respiration in the hermit crab, *Pagurus bernhardus*, exposed to fluctuating salinities. *Journal of the Marine Biological Association of the United Kingdom*, 58, 869-876.

Sih, A., Ferrari, M. C. O. & Harris, D. J. 2011. Evolution and behavioural responses to human-induced rapid environmental change. *Evolutionary Applications*, 4, 367-387.

Smaldon, G., Holthuis, L. B. & Fransen, C. H. J. M. 1993. *Coastal Shrimps and Prawns*, Revised edn. edn. Shrewsbury: Field Studies Council.

Small, D., Calosi, P., White, D., Spicer, J. I. & Widdicombe, S. 2010. Impact of medium-term exposure to CO_2-enriched seawater on the physiological functions of the velvet swimming crab *Necora puber*. *Aquatic Biology*, 10, 11-21.

Snow, P. J. 1975. Central patterning and reflex control of antennular flicking in the hermit crab *Pagurus alaskensis* (Benedict). *Journal of Experimental Biology*, 63, 17-32.

Spicer, J. I., Raffo, A. & Widdicombe, S. 2007. Influence of CO_2-related seawater acidification on extracellular acid-base balance in the velvet swimming crab *Necora puber*. *Marine Biology*, 151, 1117-1125.

Stacey, M. T., Mead, K. S. & Koehl, M. A. R. 2002. Molecule capture by olfactory antennules: Mantis shrimp. *Journal of Mathematical Biology*, 44, 1-30.

Steullet, P., Cate, H. S., Michel, W. C. & Derby, C. D. 2000. Functional units of a compound nose: aesthetasc sensilla house similar populations of olfactory receptor neurons on the crustacean antennule. *Journal of Comparative Neurology*, 418, 270-280.

Steullet, P., Krutzfeldt, D. R., Hamidani, G., Flavus, T., Ngo, V. & Derby, C. D. 2002. Dual antennular chemosensory pathways mediate odor-associative learning and odor discrimination in the Caribbean spiny lobster *Panulirus argus*. *Journal of Experimental Biology*, 205, 851-867.

Stewart, P. A. 1981. *How to understand acid-base*. New York: Elsevier.

Stillman, J. H. 2002. Causes and consequences of thermal tolerance limits in rocky intertidal porcelain crabs, genus *Petrolisthes*. *Integrative and Comparative Biology*, 42, 790-796.

Taylor, A. C. & Spicer, J. I. 1987. Metabolic responses of the prawns *Palaemon elegans* and *Palaemon serratus* (Crustacea, Decapoda) to acute hypoxia and anoxia. *Marine Biology*, 95, 521-530.

Taylor, A. C. & Spicer, J. I. 1988. Functional significance of a partial emersion response in the intertidal prawn *Palaemon elegans* (Crustacea, Palaemonidae) during environmental hypoxia. *Marine Ecology-Progress Series*, 44, 141-147.

Taylor, A. C. & Spicer, J. I. 1991. Acid-base disturbances in the hemolymph of the prawns *Palaemon elegans* (Rathke) and *P. serratus* (Pennant) (Crustacea, Decapoda) during exposure to hypoxia. *Comparative Biochemistry and Physiology a-Physiology*, 98, 445-452.

Taylor, D. L. & Eggleston, D. B. 2000. Effects of hypoxia on an estuarine predator-prey interaction: foraging behavior and mutual interference in the blue crab *Callinectes sapidus* and the infaunal clam prey *Mya arenaria*. *Marine Ecology-Progress Series*, 196, 221-237.

Tembo, R. N. 2009. The sublethal effects of low-pH exposure on the chemoreception of *Poecilia sphenops*. *Archives of Environmental Contamination and Toxicology*, 57, 157-163.

Thomsen, J., Gutowska, M. A., Saphorster, J., Heinemann, A., Trubenbach, K., Fietzke, J., Hiebenthal, C., Eisenhauer, A., Kortzinger, A., Wahl, M. & Melzner, F. 2010. Calcifying invertebrates succeed in a naturally CO_2-rich coastal habitat but are threatened by high levels of future acidification. *Biogeosciences*, 7, 3879-3891.

Tierney, A. J. & Atema, J. 1986. Effects of acidification on the behavioral response of crayfishes (O*rconectes virilis* and *Procambarus acutus*) to chemical stimuli. *Aquatic Toxicology*, 9, 1-11.

Tierney, A. J. & Atema, J. 1988. Amino acid chemoreception: effects of pH on receptors and stimuli. *Journal of Chemical Ecology*, 14, 135-141.

Tomanek, L. & Helmuth, B. 2002. Physiological ecology of rocky intertidal organisms: a synergy of concepts. *Integrative and Comparative Biology*, 42, 771-775.

Truchot, J. P. 1976. Carbon dioxide combining properties of blood of shore crab *Carinus maenas* L.: carbon dioxide solubility coefficient and carbonic acid dissociation constants. *Journal of Experimental Biology*, 64, 45-57.

Truchot, J. P. 1979. Mechanisms of the compensation of blood respiratory acid-base disturbances in the shore crab, *Carinus maenas* (L.). *Journal of Experimental Zoology*, 210, 407–416.

Truchot, J. P. 1983. Regulation of acid-base balance. In: *The Biology of Crustacea, Vol 5: Internal Anatomy and Physiological Regulation* (Ed. by L. H. Mantel), pp. 431-457. New York: Academic Press Inc.

Truchot, J. P. 1988. Problems of acid-base balance in rapidly changing intertidal environments. *American Zoologist*, 28, 55-64.

Truchot, J. P. & Duhameljouve, A. 1980. Oxygen and carbon dioxide in the marine intertidal environment: diurnal and tidal changes in rockpools. *Respiration Physiology*, 39, 241-254.

Tuomainen, U. C., U. . 2010. Behavioural responses to human-induced environmental change. *Biological Reviews*, published online Oct 2010, doi:10.1111/j.1469-185X.2010.00164.x.

Turner, A. M. & Chislock, M. F. 2010. Blinded by the stink: nutrient enrichment impairs the perception of predation risk by freshwater snails. *Ecological Applications*, 20, 2089-2095.

Wells, J. C. K. 2007. The thrifty phenotype as an adaptive maternal effect. *Biological Reviews*, 82, 143-172.

Whiteley, N. M. & Taylor, E. W. 1992. Oxygen and acid-base disturbances in the hemolymph of the lobster *Homarus gammarus* during commercial transport and storage. *Journal of Crustacean Biology*, 12, 19-30.

Whiteley, N. M., Scott, J. L., Breeze, S. J. & McCann, L. 2001. Effects of water salinity on acid-base balance in decapod crustaceans. *Journal of Experimental Biology*, 204, 1003-1011.

Widdicombe, S. & Spicer, J. I. 2008. Predicting the impact of ocean acidification on benthic biodiversity: What can animal physiology tell us? *Journal of Experimental Marine Biology and Ecology*, 366, 187-197.

Wisenden, B. D. 2000. Olfactory assessment of predation risk in the aquatic environment. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences*, 355, 1205-1208.

Wood, H. L., Spicer, J. I. & Widdicombe, S. 2008. Ocean acidification may increase calcification rates, but at a cost. *Proceedings of the Royal Society B-Biological Sciences*, 275, 1767-1773.

Zachos, J. C., Röhl, U., Schellenberg, S. A., Sluijs, A., Hodell, D. A., Kelly, D. C., Thomas, E., Nicolo, M., Raffi, I., Lourens, L. J., McCarren, H., Kroon, D. 2005. Rapid acidification of the ocean during the Paleocene-Eocene thermal maximum. *Science*, 308, 1611-1615.

Zala, S. M. & Penn, D. J. 2004. Abnormal behaviours induced by chemical pollution: a review of the evidence and new challenges. *Animal Behaviour*, 68, 649-664.

Zhang, D. & Lin, J. D. 2006. Mate recognition in a simultaneous hermaphroditic shrimp, *Lysmata wurdemanni* (Caridea : Hippolytidae). *Animal Behaviour*, 71, 1191-1196.

Zimmerfaust, R. K. 1987. Crustacean chemical perception: towards a theory on optimal chemoreception. *Biological Bulletin*, 172, 10-29.