Impacts of ocean acidification on fitness and chemical communication in a model marine invertebrate, *Nereis succinea*.

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Abstract

Oceanic uptake of anthropogenically produced atmospheric carbon dioxide (CO_2) alters carbonate chemistry and increases the acidity of seawater. Current forecasts estimate the pH of our oceans will drop by 0.3-0.4 pH units by the turn of the century. Past research has focused majorly on the impacts of ocean acidification (OA) upon calcifying organisms and few studies have incorporated long-term exposure to OA conditions. Additionally, the impacts associated with OA are variable between species.

The presented research shows long-term culture (4-6months) in near future OA conditions (CO_2 enriched to pH 7.8) delays metamorphosis in the marine polychaete, *Nereis succinea.* Culture in pH 7.8 seawater also caused interference with the reception of chemical cues in this species. Recognition of 3 feeding stimulants (glycine, taurine and fish food extract) was reduced in adult *N. succinea* cultured in pH 7.8 seawater for 32days (in comparison with pH 8.2 cultured control worms). The typical behavioural response of males to female sex pheromone, cysteine-glutathione disulfide (CSSG), was also impacted by culture in acidic conditions (pH 7.8, 4-6 months) with fewer males eliciting expected sexual behaviours and males also requiring a higher dose of pheromone to release gametes in comparison to control cultured males (pH 8.2, 4-6 months).

This thesis discusses how disruption to chemical communication may occur via pH driven conformational change in signal molecules and/or protein receptors, reducing receptor-ligand interactions. As the chemical sense is dominant in ocean environments, any reduction in its efficiency will likely have negative implications for the survival of marine organisms. This research highlights how near future OA will impact several important survival processes in *N. succinea* (metamorphosis, food detection, sexual behaviour) which in combination may potentially reduce species persistence.

To my parents, for always having faith in me and making me believe in myself.

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Chapter 1:
Introduction to ocean acidification and *Nereis Succinea*

1.1 Ocean Acidification

Prior to the industrial revolution in the late 1700's, atmospheric CO_2 was around 280parts per million (ppm) (Feely, *et al*., 2004). Practices associated with global industrialization, mainly the burning of fossil fuels, to a lesser extent deforestation (Siegenthaler and Sarimento, 1993) and cement production (Worrell, *et al.,* 2001), have caused CO_2levels to already rise significantly to 380-400ppm (Feely, *et al*., 2004). The impact of increasing carbon emissions on our planet's climate, biodiversity and ecosystem health is widely documented (Walther, *et al*., 2002), less so but rapidly becoming of interest is the impact such emissions have on the chemical balance of marine systems

Only half of anthropogenically released CO_2 is retained in the earth's atmosphere, while 20% is absorbed by the terrestrial biosphere and the remaining 30% is taken up by the oceans (Feely, *et al*., 2004). With their high buffering capacity, it was previously thought that the absorption of excess CO_2 by the oceans would have little effect on ocean health, however the growing body of evidence in recent years shows this not to be the case (Doney, 2006, Reibsell, 2008, Hoffmann, *et al.,* 2010, Ries, 2011). This phenomenon has recently been termed ocean acidification (OA), due to the reduction in pH (increase in acidity) associated with oceanic uptake of CO_2 (Doney, *et al.,* 2009). Increased carbon emissions have long been associated with warming of the earth's climate, however, OA presents the additional impact of alteration in ocean chemistry. An increase in dissolved inorganic carbon (DIC) caused by increased absorption of CO_2 gas results in decreased availability of free carbonate ions (CO_3^{2-}) in the ocean. This occurs as CO_2 reacts with water to form carbonic acid, which subsequently dissociates to form hydrogen ions $[H]^+$ and bicarbonate (HCO_3^-) (Doney, *et al*., 2009). Bicarbonate dissociates further into more $[H]^+$ and carbonate ions (CO_3^{2-}) as shown in the following equation and figure (1.1):

$$CO_{2(atmos)} \Leftrightarrow CO_{2(aq)} + H_2O \Leftrightarrow H_2CO_3 \Leftrightarrow H^+ + HCO_3^- \Leftrightarrow 2H^+ + CO_3^{2-}$$

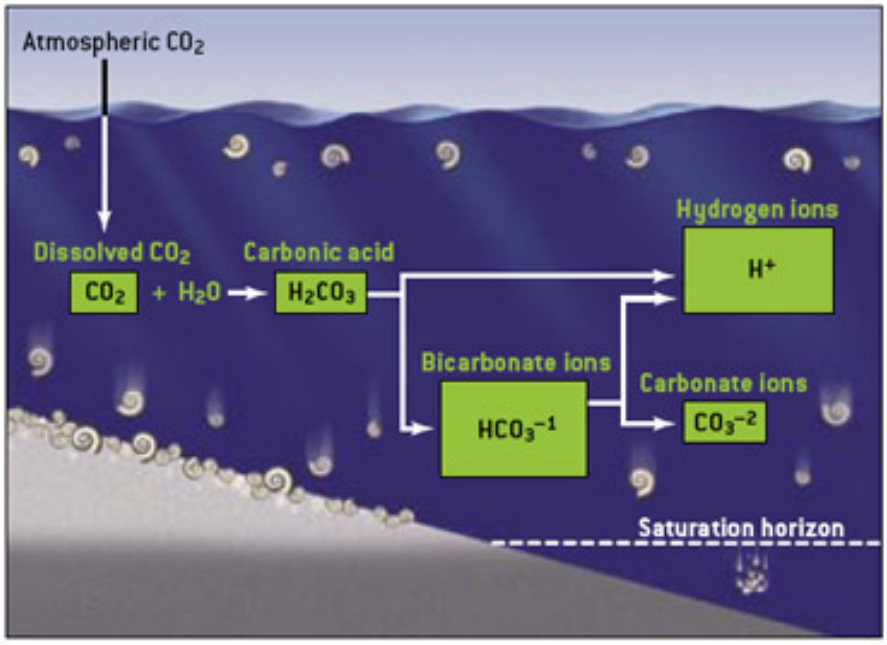

Figure 1.1: Carbonate chemistry of seawater showing molecular speciation when atmospheric CO_2 is added to the system (Doney, *et al.,* 2006).

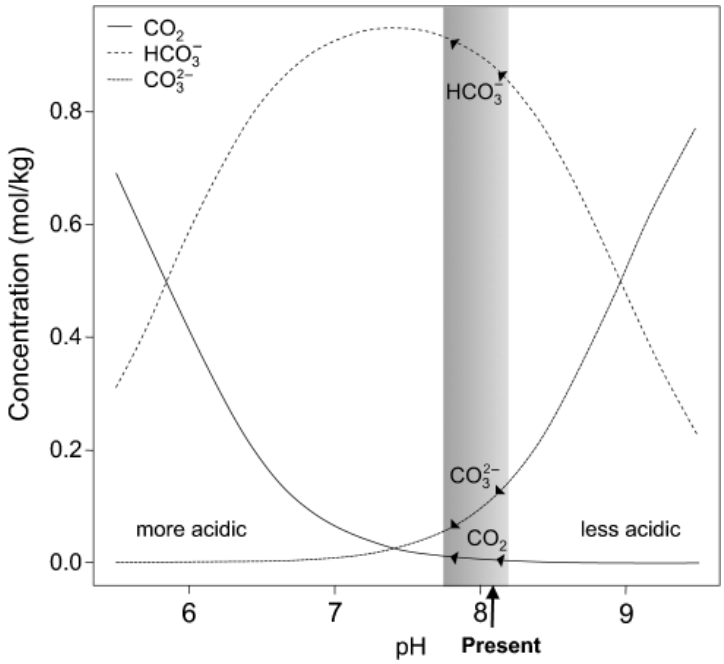

Figure 1.2: Carbonate speciation in relation to pH value of seawater. Increasing CO_2 leads to decreasing carbonate ions (CO_3^{2-}) and increasing H^+ and bicabonate ions (HCO_3^-) (Logan, 2010).

Seawater is currently maintained at a pH between 8.0 and 8.3 (Doney, 2006) by a natural buffering system whereby excess H^+ combine with free carbonate ions (CO_3^{2-}) to form HCO_3^- (Widdicombe and Spicer, 2008). However, when levels of [H]+ become too elevated, the availability of free carbonate ions becomes reduced as they are 'mopped up' by excess [H]+ to form bicarbonate. The result is an increase in excess [H]+ and subsequent reduction in pH (Figure 1.2).

The first studies on the impact of atmospheric CO_2 on carbonate speciation in sweater began the early 1900's (Stieglitz, 1909, Johnston and Williamson, 1916), but investigation into anthropogenic driven acidification of the oceans only gathered momentum around the 1950's when the first projects involving regular tracking of ocean chemistry began. This was pioneered by Roger Revelle and colleagues (Revelle and Suess, 1957), who developed the first data collection stations located in the South Pole and Hawaii to monitor changing ocean chemistry, which are still being used today. Data collected at these stations, along with indirect data from studying air bubbles in ice cores (Indermühle, *et al.,* 1999), have provided information on past levels of atmospheric CO_2 and the changing chemical state of the ocean to support the development of models to forecast future trends (Doney, 2006). Since the industrial revolution in the late 1700's, ocean pH has dropped by approximately 0.1pH units (Guinotte and Fabry, 2008). By the end of the 21st century, it is expected that ocean pH will decrease by a further 0.3 – 0.4 pH units (Orr, *et al.,* 2005), equivalent to a 100 -150% increase in acidity. Furthermore, the availability of free CO_3^{2-}in seawater will halve (Doney, *et al.,* 2009). Caldeira and Wickett (2003) predict that by 2300 levels of ocean CO_2 could potentially reach a maximum of 1900ppm, equivalent to a pH reduction of 0.7units from todays level (Zeebe, 2012). The conditions described in these forecasts have rarely been seen in the past 300million years and have certainly never occurred at such a rapid rate (Caldeira and Wickett, 2003). Quick environmental change has implications for the survival of single species and ecosystems alike. Are marine flora and fauna negatively impacted? If so, do they have the capacity to adapt? Such

questions are being increasingly addressed by the scientific community to assess the potential consequences of the aptly tilted, 'Other CO_2 Problem' (Doney, *et al.,* 2009).

1.2 Known Impacts of ocean acidification on marine life

1.2.1 Calcifying organisms

By far the most studied impact of OA on marine organisms is the negative effect it has on calcifying organisms including corals (*Oculina arbuscula*, Ries, 2009), molluscs (*Saccostrea glomerata,* Parker *et al.,* 2011) and pteropods (*Clio pyrimdata,* Orr, *et al.,* 2005). These species utilize free carbonate ions [CO_3^{2-}] within seawater to synthesize calcium carbonate ($CaCO_3$) in which their hard shells and skeletal structures are formed (Orr, *et al.,* 2005). Section 1.1 discussed how oceanic uptake of anthropogenic atmospheric CO_2 not only increases the concentration of hydrogen ions [H+] in seawater, resulting in lower pH, but also disrupts typical carbonate chemistry resulting in reduced carbonate saturation and less available [CO_3^{2-}] (Figures 1.1 and 1.2) (Orr, *et al*., 2005). The carbonate ion concentration within our oceans has declined by approximately 20-30% (Gaylord, *et al.,* 2011) since the late 1700's. This reduction translates almost exactly to reported rates of decline in coral calcification in the same time period, approximately 17-35% (Jokiel*, et al*., 2008). Forecasts predict a further decrease in oceanic concentration of [CO_3^{2-}] ions of 60% by the turn of the century (Feely, *et al*., 2004).

The extent to which calcifying organisms may be affected, however, is a complex subject. Although the general trend reveals deleterious effects, different organisms are affected to different extents (Hofmann*, et al*., 2010) and some not at all, such as the blue mussel, *Mytilus edulis* (Ries, *et al.,* 2009). Furthermore, different populations of the same species have shown inconsistent responses to acidified conditions as recorded in wild and

selectively bred populations of the Sydney rock oyster, *Saccostrea glomerata* (Parker*, et al.,* 2011).

Recent improvements in experimental design may also cause previous research to come under criticism. Rodolfo – Metalpa, *et al*. (2011), showed how overlooking the distinction between gross and net calcification rates can lead to the misinterpretation of calcifying activity of corals and mollusks. It has been shown that in conditions simulating future OA conditions, both in the lab and in the field at marine areas of volcanic activity (Ischia, Italy), rates of gross calcification in two coral species (*Cladocora caespitosa* and *Balanophillia europea*) and two mollusk species (*Mytilus galloprovincialis* and *Patella caerulea*) actually increase (Rodolfo – Metalpa, *et al.,* 2011). However, acidic conditions can cause the dissolution of external calcified parts (in some species), leading to a reduction in net calcification (gross calcification – dissolution) but not the physical ability of the organism to calcify overall (Rodolfo – Metalpa, *et al.,* 2011). This finding has implications for previous studies, which may not have taken this into account and reported a reduction in the ability of organisms to calcify successfully when it is in fact acidic dissolution causing degradation of hard parts, not a reduction in the ability to synthesize them (Ries, 2011).

A similar conclusion was reached by Wood, *et al.* (2007), who investigated the effect of OA in the regeneration of limbs in the calcifying brittle star *Amphiura filiformis.* The study found that in conditions acidified with CO_2, removed arms grew back to a greater length and contained more calcium than established ones, indicating OA conditions facilitated improved rates of calcification. However, as in the work of Rodolfo-Metalpa, *et al*. (2011), dissolution rates increased with acidification and it was further noted that muscle wastage in the limbs also occurred which may have implications for overall fitness, reducing efficiency of feeding and burrow construction and irrigation.

These studies emphasize that while OA may not have the negative effect on the calcification process as once thought, increased calcifying activity is counteracted by reduced pH driven dissolution and may also reduce fitness in other ways (Wood, *et al.,* 2007). Such conclusions, coupled with the knowledge that different species are impacted to differing degrees (Hoffmann, *et al.,* 2010), illustrate the complexities of assessing the impact of OA on marine organisms and support the need for further research and understanding into the subject as a whole.

1.2.2 Reproduction and early development

Early life stages in marine invertebrates are critical for successful recruitment (Hunt and Scheibling, 1997). The highest incidence of mortality is observed at the egg and larval stages (largely due to predation) and so it is imperative that the few remaining individuals are of adequate quality to enable the species to persist (Hunt and Scheibling, 1997). For this reason, much research on the effect of OA on development and survival is focused on assessing the effect at early life stages including fertilization and a number of parameters contributing to larval success.

It is presumed that invertebrates will be more sensitive to OA in the early stages of their life history (Ross, *et al*., 2011) due to their tendency to be broadcast spawning species, meaning both gametes and planktonic larvae come into direct contact with acidified seawater in the water column. Additionally, invertebrates tend to have less sophisticated mechanisms for acid-base regulation in comparison to more complex organisms, such as fish (Miles, *et al.,* 2007). For example, the sea urchin *Psammechius millaris,* is particularly sensitive to hypercapnia as the composition of the coelomic fluid varies according to that of the surrounding seawater (Miles, *et al.,* 2007). Many marine invertebrates, such as *P. millaris,* lack the physical ability to actively regulate intracellular pH levels via complex metabolic processes and active transport of ions across internal membranes that are commonplace in higher organisms such as fish (Claiborne, *et al.,* 2002). The

extent to which different organisms are affected seems is variable, however there insufficient evidence that the early life stages of a number of invertebrates from a wide range of taxa are negatively affected by conditions associated with reduction in ocean pH and elevation in pCO_2 (Ericson, *et al.,* 2010; Ross, *et al.,* 2011; Stumpp, *et al.,* 2011b). Despite evidence that OA negatively impacts sperm motility (Havenhand*, et al*., 2008) and increases the risk of polyspermy (Reuter, *et al.,* 2011), fertilization in invertebrates seems to be relatively unaffected by low pH unless values reach more extreme levels (< pH7.0) (Kurihara and Shirayama, 2004; Ericson, *et al.,* 2010). Fertilization success was however, found to be significantly reduced at pH levels expected in the near future in both the marine polychaete, *Nereis succinea* (<pH 8.0) and the sea urchin*, Heliocidaris erythrogramma* (<pH 7.7) (Havenhand, *et al*., 2008).

Taxa with high metabolic rates, such as fish and cephalopods, are expected to show higher tolerance to elevated pCO_2 with respect to development due to them having more sophisticated mechanisms for acid-base regulation (Franke and Clemmensen, 2011). However, these taxa may still be susceptible during very early life stages before such mechanisms are fully developed. In recent years, research has become more focused on both ecologically and economically important fish species and how OA may affect key development processes in the early stages of life. Munday, *et al*. (2009a; 2009b; 2010; 2011), have carried out extensive research on the impact of OA on tropical marine fishes. Several of these studies indicate that elevated CO_2 and reduced pH has relatively little negative effect on early development both in the orange clown fish (*Amphiprion percula*) (Munday, *et al.,* 2009a) and the spiny damselfish (*Acanthochromis polyacanthus*) (Munday, *et al.,* 2011). In pH 7.8 conditions, embryonic duration, egg survival, and size at hatching was not affected in *A. percula*. Similarly, *A. polyacanthus* was not adversely affected, with no negative effect on growth, survival, skeletal development or ootilith size when exposed to near future elevations in seawater acidity. However, it was noted that a proportion of *A. percula* exposed to relatively high levels of pCO_2 (1030ppm, approx. pH 7.8)

were positively affected (Munday, *et al.,* 2009a), having longer overall lengths and weights in comparison to control animals after 11 days of development. It is suggested by the authors that the species may exhibit increased feeding habits as a compensatory mechanism of pH and hypercapnic stress resulting in increased growth rate (Munday, *et al.,* 2011).

Evidence that non tropical fishes are also relatively robust to near future levels of OA at the juvenile stage is shown in the somewhat economically important species the Atlantic herring (*Clupea harengus*) (Franke and Clemmensen, 2011). When exposed to acidified seawater during incubation and hatching, this species also showed no reduction in hatch rate, weight or length and no increase in egg mortality or embryonic duration. However, levels of RNA were less in hatchlings from low pH conditions indicating a reduction in protein synthesis and subsequently a possible reduced capacity for growth (Franke and Clemmensen, 2011).

It is clear to see that the impact of OA on early life stages in marine organisms is highly variable. Overall, fishes appear more robust than invertebrates probably due to their capacity to better regulate acids and bases within the body (Franke and Clemmensen, 2011; Munday, *et al.,* 2009a and 2011). However, even in such higher organisms there is some evidence of negative impacts to development potential (Franke and Clemmensen, 2011).

1.3 Emerging research into impacts ocean acidification on chemical communication in marine systems

1.3.1 Previous research on chemical communication

Research into chemical signals began with and has continued to focus primarily on the identification and isolation of pheromones in insects due to the economical benefit of application to pest management. The term 'pheromone' was introduced by Karlson and Luscher in 1959, to describe chemical signaling between organisms (Karlson and Luscher, 1959). The first of these compounds to be isolated was the silk moth's (*Bombyx mori*) sex pheromone Bombykol, also in 1959 by Adolf Butenandt. Pheromones are chemical signals that consist of a single or specific combination of molecules in defined amounts (Wyatt, 2010). These externally released compounds are detected via olfaction and elicit a specific behavioural or physiological response in the receiver (Wyatt, 2009). Behavioural responses to pheromones are generally innate and do not need to be learnt. This, along with defined ratios of the chemicals involved, helps distinguish between true pheromone mixtures and 'signature mixtures' (an individual's smell) which are chemical mixtures unique to the individual and are only recognized once encountered and memorised (Wyatt, 2010). Pheromone treatments involved in pest control include mass trapping of insects and the interference of reproductive behaviours (Wyatt, 2009). Such techniques have grown in popularity as they are deemed a more 'environmentally friendly' alternative to traditional pesticides. Pheromonal bio control agents may be used in isolation or in conjunction with pesticides (Integrated Pest Management) to eliminate or reduce environmental exposure to harmful chemicals, which may accumulate in higher trophic levels. The majority of pheromone research has therefore involved the identification and exploitation of sex and aggregation pheromones in insects and other pest species (Pickett, *et al*., 1997) such as in the successful management of bark

beetles that rely heavily on pheromonal coordination to decimate large areas of forest (Borden, 1989).

1.3.2 Chemical signaling in the marine environment

In recent years, the effect of OA on the ability of organisms to communicate by chemical means in the marine environment has emerged as a key research area. Light transmission and visibility can be greatly reduced in marine environments (Bronmark, *et al*., 2000), particularly in deep waters or areas with high sediment loading. Many organisms, therefore, rely heavily on the reception of chemical signals to sense and interpret to surrounding environment (Wisenden, 2000). Locating food, detecting predators and identifying a suitable place to settle are all critical survival behaviours mediated by olfaction (smell), the reception of chemical cues (Wyatt, 2003). Pheromones also play important roles in transmitting information between conspecifics, coordinating animal behaviour and inducing developmental processes at critical stages in the life history, such as maturation and reproduction (Wyatt, 2009). Disruption to this method of communication could therefore potentially reduce the efficiency of many behaviours that are key to the survival of many marine organisms.

1.3.3 Known impacts of ocean acidification on chemical communication in the marine environment

Examples of pH driven disturbance to chemical communication in marine systems are now beginning to emerge. In the last few years, there have been studies which describe how seawater with pH reduced by CO_2 to levels expected in the next 100 years can have a negative effect on homing, settlement and predator detection in an number of fish species, but primarily in the orange clownfish, *Amphiprion percula* (Munday, *et al*., 2009b). In natural systems, clownfish larvae choose a suitable host species of anemone to settle in using olfaction. These anemones offer the fish cover

and protection from predators and so choosing the appropriate site to settle is vital to maximize survival. It was found that larvae reared in pH 7.8 were less attracted to host species favoured by the control group and more worryingly became strongly attracted to odour from species that were previously avoided in un-acidified (pH 8.2) conditions (Munday, *et al.*, 2009b). This study also found that larval recognition of parental odour was affected whereby larvae raised in acidified conditions (pH 7.8) were unable to discriminate between their own parents and other adults (Munday, *et al.,* 2009b). Clownfish larvae usually use olfactory cues to avoid their parents and natal habitat to minimize the occurrence of inbreeding. These findings have implications for gene flow within natural populations under pressure from OA.

It has also been documented that future levels of OA may impact odour-mediated detection of predators in *A. percula* (Dixson, *et al.,* 2010). In similar experiments to Munday, *et al.,* (2009b), whereby two way choice flumes were used to allow *A. percula* larvae reared in control or acidified conditions to show preference for presented odourants, it was found that settlement stage larvae spent more time in water treated with predator odourant (from predatory fish species *Cephalopolis cyanostigma* and *Pseudochromis fucus*) than in water with no odour present. For the control group, reared in normal seawater conditions (pH 8.2), the opposite was true. Larvae reared in reduced pH conditions (pH 7.8) also had difficulty distinguishing between the odour of non-predatory vs. predatory fish, spending equal amounts of time with each cue, whereas control specimens almost always associated themselves with odour of non-predatory species when given the choice (Dixson, *et al.,* 2010). Predation in early life stages (during hatching, settlement and pelagic larval stages) is a huge mortality bottleneck in the life history of marine species (Hunt and Scheibling, 1997). Olfactory mechanisms to enable juveniles to detect the correct, safest settlement sites and innately be able to identify and avoid predators are crucial to ensure adequate recruitment within populations. These two key studies show the capacity for OA to disrupt such mechanisms and

potentially reduce the survival of marine fish species, increasing mortality rate in conditions acidified by CO_2 by between 5 and 9 times (Munday, *et al.,* 2010).

It is not only prey species that may be negatively impacted by OA. As prey species use olfactory cues to avoid predation, predators use the same technique to locate their prey and are therefore susceptible to the same challenges OA may present with regards to disruption of the olfactory sense. Again using the method of two-way choice flumes, a study using the predatory fish species *Pseudochromis fucus*, showed that the ability to detect prey through olfaction was reduced in conditions simulating future levels of OA (Cripps, *et al.*, 2011). Using skin extracts of the prey species, *Pomacentrus moluccensis* as prey odour, *P. fucus* was given the choice between untreated seawater and prey odour. *P. fucus* that had been maintained in CO_2 enriched seawater (pH 7.9) for 7 days prior to experimentation spent 20% less time in water streams containing prey odour compared to control individuals that had not been exposed to low pH conditions. Although predators use a combination of visual and chemical cues to ultimately locate and capture prey, visual cues can become unreliable in complex habitats, when levels of suspended sediments are high and when light penetration is low (i.e. deep water/at night) (Wisenden, 2000). This emphasizes the critical importance of being able to successfully locate food sources through olfactory means and demonstrates how reduction in the efficiency of olfaction may have negative consequences for survival.

1.4 Acclimatization and adaptation to a changing ocean

The question of whether some species may be able to acclimatize to the changing state of our oceans is also widely debated and difficult to predict due to a relative lack of long-term studies (Hoffmann, *et al.,* 2010). Scientists

are now beginning to take advantage of naturally occurring volcanic activity and upwellings of acidic groundwater to investigate much more long term effects of ocean acidification on single species and whole communities (Rodolfo-Metalpa, *et al.,* 2011, Crook, *et al*., 2012). Sites such as these have maintained acidic conditions for thousands of years and studying the success of organisms present in these may provide insight into how marine ecosystems may respond to future OA.

1.5 Using polychaetes to assess the risk of future levels of ocean acidification

1.5.1 Scientific basis for selecting *Nereis succinea* as a study organism

A suitable marine organism was required to study the impact of future levels of OA on chemical signaling systems. Requirements for such an organism include availability, ease of culture, ecological relevance and the presence of a well-defined chemical communication system. The polychaete worm *Nereis succinea* fulfilled these requirements and was deemed an ideal candidate to study the impacts of OA on both overall fitness and chemical communication in marine systems. The characteristic sexual behaviour (described in 1.5.2) of this species is extremely predictable, allowing for any changes that occur to be easily observed and recorded. Unlike most other polychaete species and the majority of marine invertebrates, all pheromones involved in the sexual behaviour of *N. succinea* have been successfully isolated and are also commercially available in synthetic forms (Zeeck, *et al.,* 1998a). This allows for the development and practice of controlled bioassays to study the potential impacts of OA. The organism itself is relatively widespread and well-established populations are not only known but have been used previously to establish cultures within the laboratory (Hardege, 1999). They are easily collected in large numbers, travel well and can be transported simply in cool, damp sediment without

adverse effects to health. Procedures for laboratory culture, including diet and culture conditions are also known and have proven to be successful in the past (Hardege, 1999). Initial studies have already identified a sensitivity of this organism to reduced pH levels, showing that levels of OA expected in the near future have negative effects on several aspects of the life cycle including pheromone reception, growth, fertilization success and larval development. *N. succinea* is of ecological importance in benthic communities as the species plays an important role in the lower food web. They consume algae and small invertebrates and also serve as food for larger invertebrates and bottom-feeding fish. Negative impacts on *N. succinea* may therefore feedback and impact other species within or reliant on benthic communities for food.

1.5.2 Ecology of chosen test species: *Nereis succinea*

The life cycle of the marine polychaete *N. succinea* is split into two main stages: the immature benthic stage and the mature free swimming stage (Hardege, 1999). Juveniles and immature adults are found in benthic sediment and are completely non-swimming. During maturation, towards the end of the life cycle, the body undergoes metamorphosis whereby the morphology develops to carry out reproduction (Hardege, *et al.,* 1990). As well as the expected production of gametes during the maturation process, the parapodia of the mid section become modified for swimming and those of the anterior and posterior sections for sex pheromone reception (Hardege, 1999). Sex pheromones are only synthesized during the mature 'heteronereid' stage (Hardege, *et al*., 2004). As with all Nereid polychaetes, *N. succinea* has extremely well defined and closely controlled reproductive behaviour (Hardege, *et al.,* 1990). Both environmental and chemical cues play important roles in synchronizing the reproductive event and ensuring adequate recruitment within populations (Hardege*, et al.,* 1998). OA has been shown to disrupt chemical reception in the marine environment (Munday, *et al.,* 2009b; Dixson, *et al*., 2010) and therefore may potentially

reduce the efficiency and success of chemically coordinated reproduction. As a semelparous, broadcast spawning species, it is imperative that gamete release in *N. succinea* is timed correctly and occurs only when sexual partners are fully mature and in close proximity to one another (Hardege, *et al.,* 2004). Environmental factors including temperature, photoperiod and lunar phase ensure that maturation of individuals within a population is fully synchronized (Ram, *et al*., 2008), whereas the final reproduction event is controlled fully by chemical cues. Both the male and female sex pheromones responsible for inducing gamete release in the opposite sex have been identified (Zeeck, *et al.,* 1998a and 1998b).

N. succinea participate in a mass spawning event (Hardege, *et al.,* 1998) generally at the new or full moon in the evening, depending upon the population location (Ram*, et al.,* 1999). Both sexes emerge from the benthos and swim to the ocean surface to engage in characteristic reproductive behaviour termed the 'nuptial dance' (Hardege, 1999). Males are generally more active in their search for a partner and will cover larger distances in search of a mate (Ram and Hardege, 2005). As they swim, females release the sex pheromone cysteine-glutathione di-sulphide (CSSG) on a constant basis. At low concentrations ($10^{-6/7}$ M), this pheromone acts as a mate attractant (Figure 1.3), increasing swimming activity in males (Ram, *et al.,* 2008) to increase the likelihood that sexual partners will meet (Fei, *et al.,* 2008). Once a male and female arrive in close proximity to one another, the 'nuptial dance' is initiated whereby the two swim in tight circles around one another whilst releasing gametes (Hardege, *et al.,* 1990). This process is closely coordinated via chemical cues as the male responds to female CSSG in higher concentration ($10^{-4/5}$ M) by releasing a small amount of sperm and a bouquet of chemicals that induce the female to release eggs (Zeeck, *et al.,* 1998b). The bouquet of male emitted chemical compounds involved in this process were first isolated by Zeeck, *et al.,* (1996 and 1998b) and were identified as a combination of inosine, glutamic acid and glutamine. The female response is to release eggs, along with coelomic fluid containing large amounts of CSSG (Hardege, 1999; Hardege, *et al.,* 2004). This triggers

the final step in the process whereby the male releases large amounts of sperm and fertilization occurs (Hardege, *et al.,* 1998). As a semelparous organism, the spawned females proceed to die following the reproductive event although males may continue to release sperm several more times and over a few consecutive nights prior to death (Ram and Hardege, 2005).

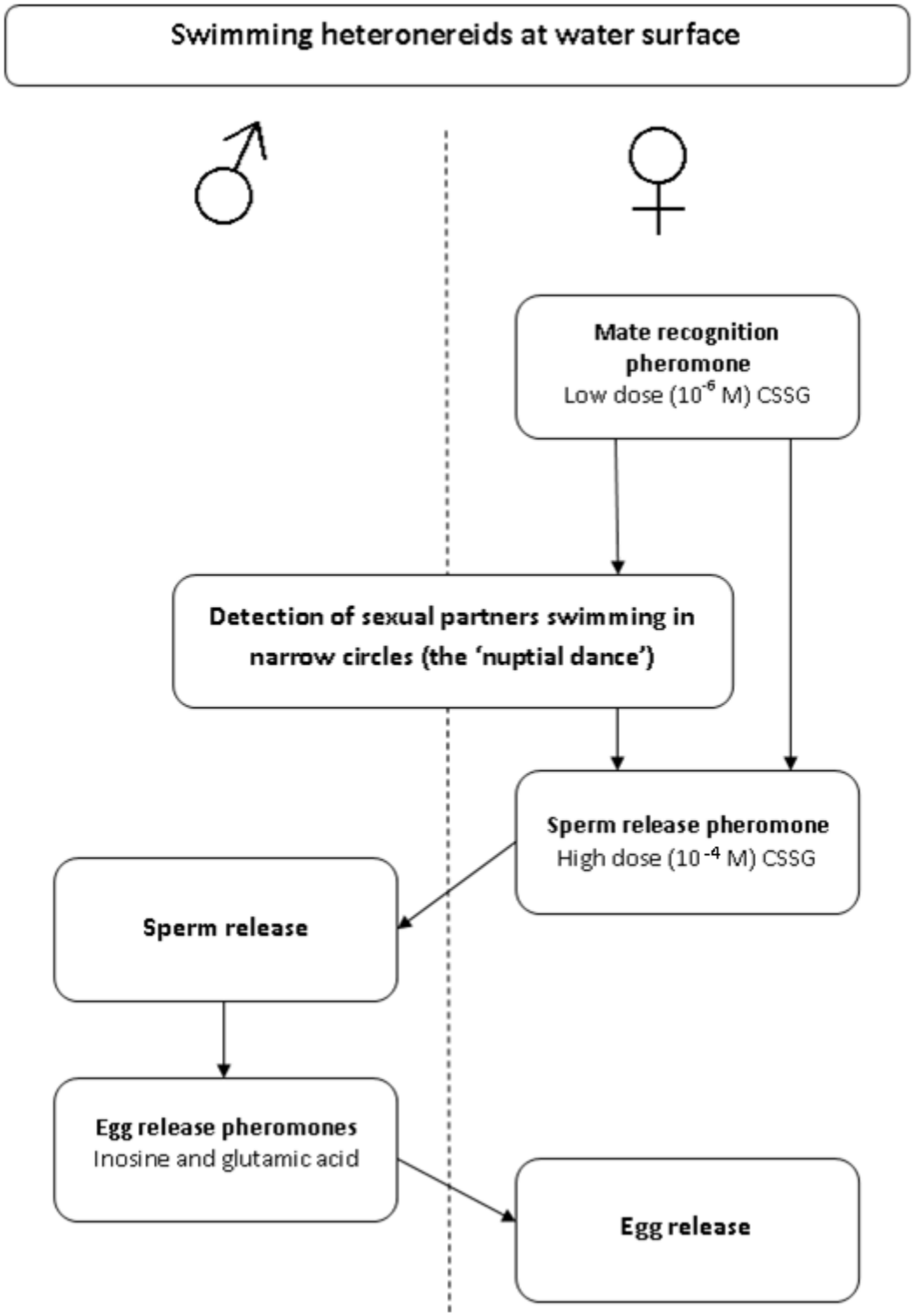

Figure 1.3: Schematic diagram of pheromone coordination of the reproductive event in *N. succinea* (adapted from Hardege, et al. 1999).

1.6 Project aim

The aim of the project is to assess the impact of OA on key survival processes of the model marine invertebrate, *N. succinea,* paying particular attention to how chemical communication may be disrupted. The experiments and results presented will explore how both long and short term exposure levels of OA forecast to occur in the next 100 years will impact physical development, response to environmental cues (food sources) and male response to female sex pheromones. Each chapter will focus on a different aspect of this overall aim as outlined below:

1. Assessment of how long term exposure to near future levels of OA may impact the overall health and fitness of *N. succinea*, using a range of physiological (length, weight, maturation, levels of intracellular GSH) and behavioural (burrowing activity) biomarkers (focus of Chapter 2).

2. Assessment of how acute exposure to near future levels of OA may impact the ability of *N. succinea* to respond to chemical cues including feeding stimulants (glycine, taurine and fish food extract) and the female sex pheromone cysteine-glutathione disulphide (focus of Chapter 3).

3. Assessment of whether *N. succinea* has the capacity to acclimatize to near future levels of OA with respect to chemical communication (using feeding stimulants and female sex pheromone as in chapter 3) when exposed to such conditions for an extended period (focus of Chapter 4).

Chapter 2:
The impact of ocean acidification on the general fitness and survival of *Nereis succinea*

2.1 Introduction

The main focus of this study is to investigate how CO_2 enriched conditions associated with OA may impact the reception of chemical feeding and pheromonal stimuli in *N. succinea*. However, prior to this it is necessary to carry out a more comprehensive assessment of species health in response to OA to correctly interpret the potential impacts OA may have on species persistence. For example, chemical communication systems may remain robust to changing ocean chemistry with individuals still capable of detecting chemical signals but unable to respond appropriately due to poor fitness, leading to potential misinterpretation of results. For this reason, this chapter will compare the overall health and performance of control specimens (cultured in normal seawater conditions, pH 8.2) and test specimens (cultured in CO_2 enriched seawater, pH 7.8) prior to behavioural studies on chemical signaling in subsequent chapters (3 and 4). Included are basic developmental comparisons between cultured worms (length/weight/maturation success/time to maturation), tissue content of intracellular detoxifying compounds (glutathione) and also performance in critical survival behaviours (digging activity).

2.2: The use of biomarkers to assess fitness

Biomarkers are defined as detectable biochemical, cellular, physiological or behavioural variations that can be measured in tissue or body fluid samples or at the level of whole organisms to provide evidence of exposure to and /or effects of, chemical pollutants including xenobiotics and environmentally harmful chemicals (Depledge, *et al.,* 1995). Such markers can be both analytical, assessing molecular content of cells and tissues or behavioural, using change in typical behaviours as an indicator of exposure to stressful environmental factors (Deplege, *et al.,* 1995). Assessing parameters such as these in chosen species within ecological systems is an extensively used technique to monitor the health status of marine habitats particularly in coastal areas where ecosystem exposure to industrial and

agricultural effluent is high (Durou, *et al*., 2007). Biomarkers can also be extremely useful to identify whether an organism has been under environmental stress by comparing results from a reference of healthy, unstressed individuals with conspecifics subjected to known stressors (Depledge, *et al.,* 1995). In the case of OA, increased absorption of anthropogenic CO_2 gas disrupts the buffering capacity of seawater, lowering pH and affecting the availability and structural nature of chemical compounds within the system (Feely, *et al.,* 2009). Such changes are considered stressful and could potentially impact fitness and provide challenges to survival. Particular processes that may be affected are growth and development, maturation, acid/base regulation (Miles, *et al*., 2007), tolerance to toxicants (Franklin*, et al*., 2000) and investment in reproductive processes (Kurihara, 2008).

2.3 Choice of biomarkers to assess health in *Nereis succinea*

2.3.1 Development

Organism size can affect individual success in many ways. Mortality in juvenile marine invertebrates often exceeds levels of 90% (Moran, 1999) due to reduced size increasing vulnerability to factors such as predation and desiccation. Furthermore, reduced mortality in later life stages can be attributed to increased individual body size associated with age (Moran, 1999). Inadequate adult size may also affect important survival behaviours. For example, in *N. succinea* (Ram and Hardege, 2005), it was found that males with larger body size were able to swim faster (an advantageous behavior with respect to reproduction) and were therefore more likely to successfully locate a mate and produce offspring. Poor growth and reduced maximum size as a result of OA stress may therefore have implications to recruitment and ultimately the successful persistence of *N. succinea* populations. Fecundity is also described to be dependent on size and weight in the closely related polychaete *Nereis diversicolor* (Durou, *et al.,* 2008),

where body size correlates directly to the total gamete capacity with larger specimens containing more gametes to contribute to the next generation. *N. succinea* has a similar life history and ecology to *N. diversicolor* indicating variation in such parameters may affect the fecundity of *N. succinea* in a similar way.

Calcifying species with carbonate skeletons are assumed to be more negatively impacted by OA with respect to growth and development (Stumpp*, et al*., 2011a) due to the challenges caused by alterations in seawater carbonate chemistry. However, OA may not significantly impact maximum adult size but cause a delay in development in the early stages of life (Stumpp, *et al.,* 2011b). Therefore, although adequate size may be attainable, it may take longer to achieve under pH stressed conditions. Recording size measurements during larval stages only and not taking into account size of fully grown adults may therefore lead to the false conclusion that maximum organism size is negatively impacted by OA. The experimental designs in this project have taken this into account by taking size measurements (length and weight) at the fully mature stage of the life cycle and also recording the length of time (days) taken to reach this life stage.

Not all studies report that OA has negative implications for growth and development processes (Wood, *et al.,* 2008) and the sensitivity of organisms that are negatively affected is variable between taxa (Kroeker, *et al*., 2010). For example, hatchlings of the marine fish *A. percula* reared in CO_2 enriched conditions were noted to be unaffected in respect to growth and in some cases positively affected (longer and heavier) (Munday, *et al.* 2009b). However, this study ceased at the larval stage of the life cycle and therefore may have overlooked possible impacts on later life stages.

Inconsistent conclusions about the impact of OA on growth and development between species and taxa support the requirement to carry out basic assessment of its impact on physiological parameters when using

novel study organisms. Both positive, negative and neutral impacts of OA have been recorded across a varied range of organisms making it difficult to make assumptions and generalizations (Ross, *et al.*, 2011) about the effects of OA on growth and development even in closely related taxonomic groups.

2.3.2 Maturation

As with all organisms, maturation is a key component to reproduction in *N. succinea* (Hardege, 1990). The complex process of metamorphosis in *N. succinea* is vital to prepare the body for reproduction involving the production of gametes, the development of physical features which transform the benthic adult to the free swimming mature heteronereid, the production of sex pheromones and the structures required to receive these signals (Hardege, 1999). Near future levels of OA have been described to significantly increase the time to metamorphosis in other marine invertebrates, namely the bay scallop, *Argopecten irradians* and the hard clam, *Mercenaria mercenaria* (Talmage and Gobler, 2009). Additionally, when exposed to CO_2 concentrations analogous with pre-industrialization, these two shellfish species showed more rapid growth rates and the time to metamorphosis was reduced (Talmage and Gobler, 2010), supporting the conclusion that OA is directly impacting these developmental processes. The life cycle of *N. succinea* is closely coordinated by environmental factors such as temperature, lunar phase and photoperiod (Hardege, 1990), ensuring mature individuals emerge to surface waters simultaneously and in the optimum conditions for the reproductive event (Hardege, 1999). It has previously been discussed (section 2.3.1) that OA may cause delay in organism development, particularly in early life stages (Stumpp, *et al,* 2011b), leading to the question of whether OA may affect the time taken for *N. succinea* to complete metamorphosis and become sexually mature or prevent this process from occurring completely. Even if individuals are reproductively competent, delay in metamorphosis could potentially disrupt the timing of the reproductive event, negatively impacting recruitment.

2.3.3 Intracellular glutathione

Glutathione (γ-glutamyl-cysteinyl-glycine) is a thiol containing tripeptide found in almost all plant and animal tissues, including all mammalian tissues, in millimolar quantities (Pastore, *et al*., 2003). Often concentrated in liver cells (Lu, 2009), it plays a number of roles in cell biology and experimentally is well established as an indicator of cell viability and functionality. The molecule itself consists of cysteine, glutamic acid and glycine and has two active areas, a γ -glutamyl linkage and sulphydryl group, responsible for the molecule's wide range of functions. The γ-glutamyl group linkage is associated with the transport of amino acids and peptides while the sulphydryl group is involved in detoxification, the biological role this molecule is best known for (Pastore*, et al.,* 2003). It is proposed that both the amount of GSH in cells along with the proportion existing in the oxidized state (GSSG) (the GSH/GSSG redox pair) acts as a trigger for detoxifying enzymes to be transcribed and expressed to aid the detoxifying process (Kirlin*, et al.,* 1999). Glutathione is important for the detoxification of peroxide molecules, free radicals and xenobiotics (Pastore, *et al.,* 2003) and plays important roles nutrient metabolism, and regulation of cellular events including gene expression, DNA and protein synthesis, cell proliferation and apoptosis (Wu, *et al*., 2004)

Glutathione is a crucial biological molecule in *N. succinea* as along with the role it plays in detoxification it is also a vital precursor for the synthesis of the female sex pheromone cysteine-glutathione disulfide (CSSG) (Hardege, *et al.,* 2004). It is therefore highly imperative in this species that adequate levels of GSH are maintained (particularly in females) in order to both maximize cell oxidative status and produce the pheromone that is crucial for inducing the reproductive event (Hardege, *et al.,* 1998).

CSSG, commonly known as 'Nereithione' (Hardege, *et al.,* 2004), is the peptide pheromone (Figure 2.1) released by female *N. succinea* when swimming. It acts as both a mate attractant pheromone inducing males to

significantly increase swimming activity and speed in low doses ($10^{-6/7}$ M) and as a sperm release pheromone in high doses ($10^{-4/5}$M)(Ram, *et al.,* 1999 and 2008). CSSG is synthesised from GSH present in the body fluid and the amino acid cysteine and is made on demand in the mature stage of the life cycle exclusively (Hardege, *et al.,* 2004). In stressed conditions (such as reduced pH level or in the presence of heavy metals) there may arise a choice for female *N. succinea* of whether to prioritise the use of GSH for detoxification, maintenance of healthy eggs or pheromone production, leading to potential negative implications for individual adult survival or reproductive success.

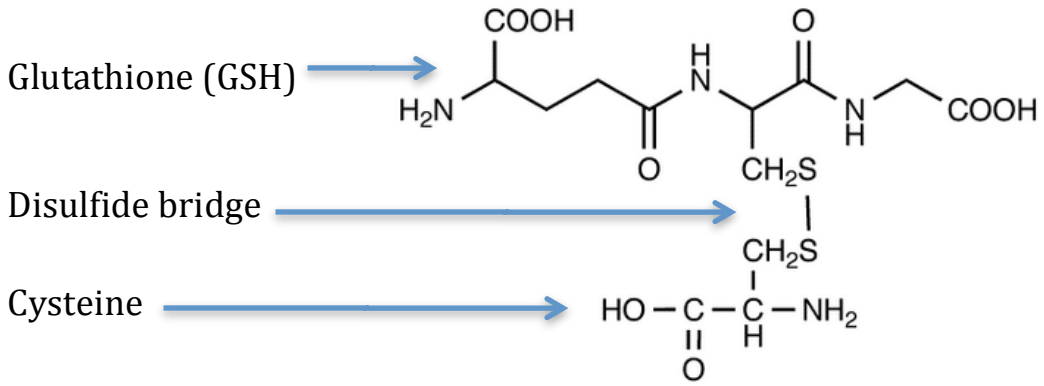

Figure 2.1: The structure of the female sex pheromone in *N. succinea*, CSSG ('Nereithione').

Assessing levels of glutathione within animal and plant tissue is a widely used technique to assess the health status of organisms (Baker, *et al.,* 1990). After tissue preparation, which involves de-proteination, several methods to quantify the total content of glutathione (reduced + oxidized forms, GSH and GSSG respectively) are common place (Pastore, *et al.,* 2003). These include the use of spectrophotometric, fluorometric and bioluminometric assays, liquid chromatography-mass spectrometry and nuclear magnetic resonance techniques (Griffith, 1980; Compagnone, *et al.,* 1993). This project will use spectrophotometric techniques to assess total levels of glutathione in both immature and mature *N. succinea* cultured in control conditions (pH 8.2) and in conditions synonymous with OA (pH 7.8) (mainly due to cost

effectiveness) to indicate whether this phenomenon may have implications on the health status of *N. succinea.*

2.3.4 Burrowing behaviour

Behaviours can be identified and quantified and are subject to change when optimum environmental conditions deteriorate (Depledge, *et al.,* 1995) allowing them to be used as biomarkers to identify stress occurrence within a species. Burrowing is a common behaviour in benthic marine invertebrates and has been used extensively in a number of studies to investigate the impact of chemical pollutants such as pesticides (Møhlenberg and Kiørboe, 1983) and heavy metals (Bonnard, et al., 2009). Such studies showed burrowing behaviours to be impaired in a variety of marine invertebrates and led to the use of similar techniques to be used in this study to assess whether pH stress may also impact burrowing efficiency.

Burrowing is particularly common in marine polychaetes, such as in the lugworm *Arenicola marina* (Newell, 1948), which spends the whole life cycle inside a U-shaped burrow on the seashore. On the other hand, *N. succinea* spends only the immature life stage in burrows (Hardege, 1990) after the initially pelagic larvae settle in benthic habitats to continue development. Prior to metamorphosis, *N. succinea* dig semi-permanent mucus lined U-shaped passages into benthic sediment to feed on detritus and evade predation (Rasmussen, 1973). As with many Nereid species, *N. succinea* is cannibalistic and concealment within sediment also helps to minimize aggressive contact with conspecifics. Durou, *et al*. (2008) used comparisons of burrowing activity and efficiency in *Nereis diversicolor* between 'clean' and chemically polluted estuarine environments as a potential biomarker of ecosystem health, showing the relevance of using burrowing activity to assess organism health when subjected to stressful conditions. *N. diversicolor* exhibits a similar life history and ecology to *N. succinea* (including burrowing behaviour) leading to digging activity being chosen as

a suitable behavioural biomarker to aid fitness assessment of *N. succinea* in this project.

2.3.5 Hypothesis

Long-term culture (4-6 months) of N. succinea in seawater enriched with CO_2 gas to pH 7.8 will negatively impact overall fitness in comparison to N. succinea cultured in control conditions (pH 8.2, 4-6months). pH 7.8 cultured individuals are expected to show the following results on removal from culture:

- Reduction in maximum size (length and weight)
- Delayed metamorphosis of immature adults into fully mature heteronereids.
- Reduced burrowing efficiency due to reduced size and poor health status.
- Reduced intracellular levels of glutathione (GSH) as a result of increased intracellular detoxification activity.

2.4 Methodology

2.4.1 Specimen origin and collection

Juvenile *N. succinea* were collected from a known population in Roath Basin, Cardiff Bay, Wales. The species exists here in amongst mussel (*Mytilus edulis*) and barnacle (*Balanus balanoides*) colonies on the harbour walls a few feet below the water line. Collection was carried out by scratching away areas of these colonies from the walls using a 7ft pole with a metal attachment and collecting basket on the end to catch the dislodged biological material. Specimens were removed and sorted into size categories on location and transported back to the laboratory at the University of Hull, England, in 1cm of damp coral sand in small boxes (max. 20 worms per box) to prevent desiccation. The boxes were placed in insulated coolers with ice to maintain temperature while travelling at 8 – 12°C. This minimized stress to the animals and maximized survival during transit by slowing down the worm's metabolic rate. Total time of translocation from field to lab culture did not exceed 36hrs. This method of collection and transportation to establish a laboratory culture of *N. succinea* has proven effective in past projects.

2.4.2 Establishment of laboratory culture

After collection, poor quality (damaged) worms were identified and discarded and 400 undamaged worms immediately transferred to laboratory culture. The preferred and widely recommended method of manipulating seawater pH and recreating OA conditions in the laboratory is by the injection of CO_2 gas (Ross, *et al*, 2011). The culture systems in this project were maintained at different pH levels by automated injection of CO_2 gas from CO_2 storage cylinders (JBL Proflora m500) each coupled with a pressure reducer (JBL Proflora m001). Water pH was detected by a permanently placed pH sensor (JBL Proflora pH sensor) and continually monitored using an electronic control panel (JBL Proflora pH control –

Software version: V. 2.4.09w31). This control panel allowed a target pH to be set and kept constant by signaling CO_2 to be administered via a submerged gas diffuser (JBL Proflora Taifun) when required. The equipment used (all from JBL Proflora range) allowed pH to vary no more than 0.05pH units from the selected value for each system. Two culture systems were set up in this way (Figure 2.2), each consisting of two large tanks (4 in total, see figure 2.3 for dimensions) holding 100specimens (200 total per culture), in a temperature-controlled room at around 16°C. Salinity was maintained at 18‰ (as in the collection environment of this intertidal species) and monitored regularly using a TMC V^2refractometer (Maidenhead Aquatics). A pump (Aquaflow 100, 3.5w) was present in each of the four tanks to maintain water flow at 200L/H. Sediment in the tanks consisted of a 15cm layer of 2mm coral sand combined with various shells. The pH of control tanks (x2) was set to pH8.2 (current oceanic pH) whereas the test system (x2 tanks) was set to the reduced level of pH7.8 (oceanic pH forecast for 2100). Natural moon cycles were mimicked using a lamp with moon light bulb, which was illuminated overnight from 17:00 – 09:00 hours for 4 days during the period of full moon. Feeding occurred every three days with ground and rinsed tropical fish flakes (Aquarian Tropical Fish Flakes, see Table 2 for nutritional information).

After 4-6months of culture heteronereids began to emerge. The systems were checked twice daily in the morning and evening by lightly sifting the sediment to reveal any mature heteronereids. These were collected immediately, sexed, weighed (mg) and had total length measured (mm) using calipers, before being individually transferred to 50ml bottles containing 20ml seawater and 10g of coral sand. All harvested specimens were then incubated at 10°C in 50ml bottles containing 20ml seawater and 10g sediment to allow them to be stored for up to 10days for further experimentation.

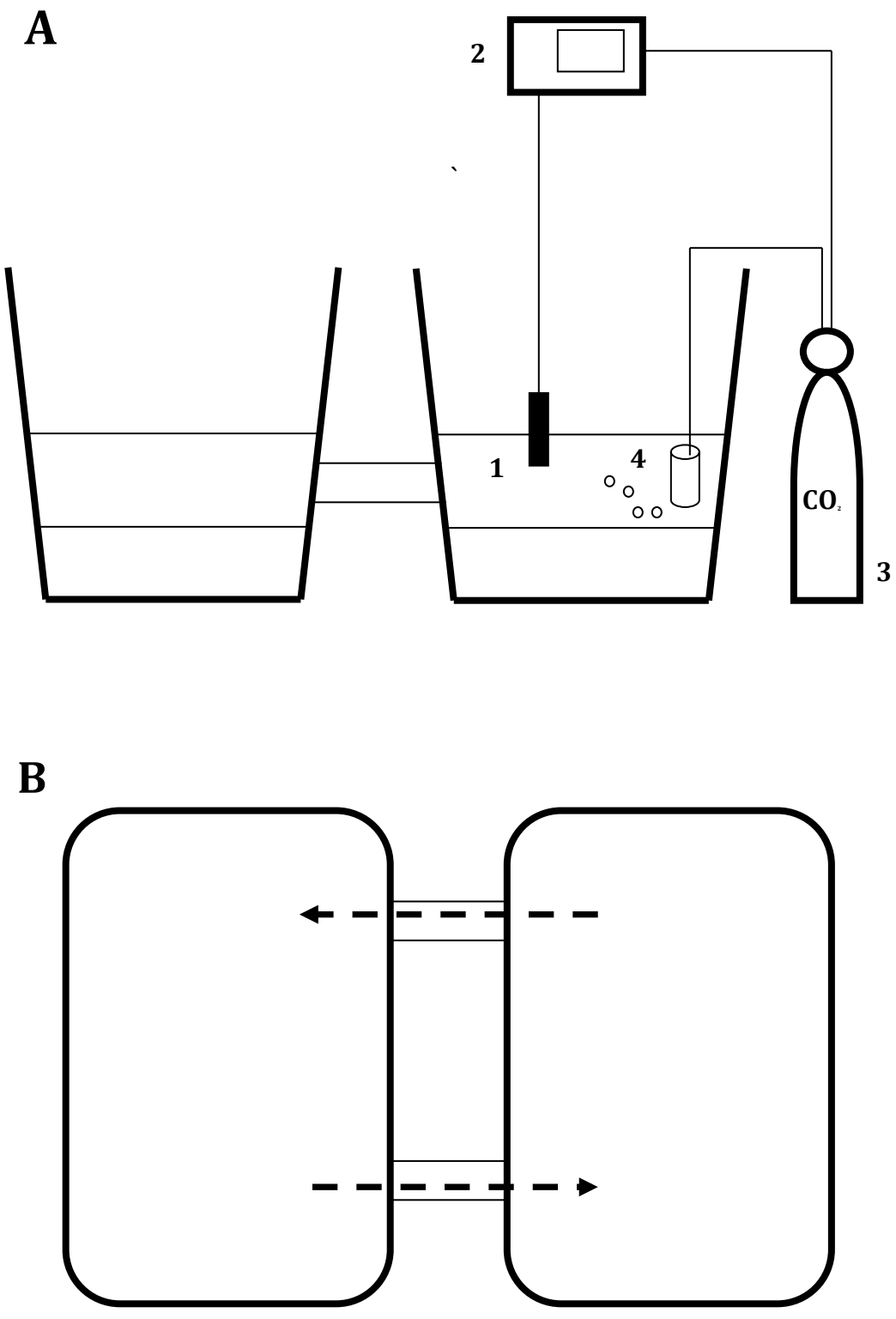

Figure 2.2: A) Equipment used to monitor and manipulate seawater pH within culture tanks. Submerged pH electrode detects seawater pH (1) and this information is relayed to the control panel (2) where a target pH can be set. When seawater pH in the tank rises above the set target release of CO_2 gas (3) is activated and bubbled into the tank via the submerged diffuser (4) resulting in a pH reduction. CO_2 gas injection ceases when target pH is reached. B) Top view of single culture system showing pipes connecting the two tanks and direction of continuous pumped water flow around the whole system.

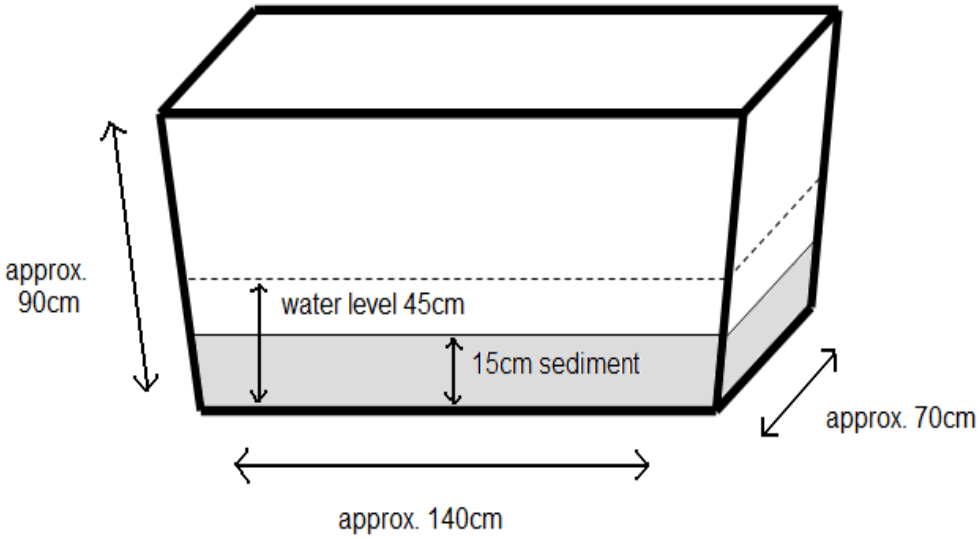

Figure 2.3: Dimensions of each individual large tank used in culture systems. 2 tanks per culture system were used with the two coupled together with large plastic pipes to allow continuous water flow between each stand-alone system.

2.4.3 Identification of heteronereids

During metamorphosis, both males and females acquire the ability to swim freely as the parapodia become modified into paddle like structures (Hardege, *et al.,* 2004). Feeding ceases, the gut becomes empty and the eyes slightly enlarged. Determining the sex of heteronereids is relatively straightforward due to distinctive colour differentiation. Males become mostly red with a white body portion behind the head where sperm is stored (Hardege, *et al.,* 2004), whereas females are green due to presence of eggs within the coelom. Figure 2.4 provides images and brief description of the main features of immature adult *N. succinea* and both male and females heteronereids are to aid identification,

A. Immature adult

- Un-modified parapodia (unsuitable for swimming)
- Food present in the gut

B. Female heteronereid

- Modified parapodia (suitable for swimming)
- Clear gut (feeding ceased)
- Enlarged eyes
- Green colouring (due to presence of eggs)

C. Male heteronereid

- Modified parapodia (suitable for swimming)
- Clear gut (feeding ceased)
- Enlarged eyes
- Red mid and posterior section
- White anterior section (due to presence of sperm

Figure 2.4: Image and description of immature and mature life stages of *N. succinea* to aid identification.

2.4.4 Recording length and weight

Length and weight of mature worms was taken at the time of harvest and used as an assessment of overall development. All juvenile worms initially added to culture measured between 8- 10mm. Total length (mm) was taken using calipers, measuring from the jaws to the tip of the tail when the worm was stretched to maximum length. Wet weight was taken so as not to damage the worms and allow them to be used for further experimentation. This would not be possible if the more conventional method of assessing dry biomass was used. Wet weight was taken by removing excess water from specimens by placing them on absorbent blotting paper for 30 seconds prior to weighing. All procedures assessing body size were carried out quickly with worms being returned to seawater immediately after to minimize any stress caused during handling.

It is normally more appropriate to acquire dry weight measurements from marine species as variation in environmental factors such as salinity and chemical stress may influence the total water content of the body (Durou, *et al.,* 2008). However, in this study it was deemed appropriate to assess 'wet weight' to allow assessed specimens not to be compromised during the weighing process and be used for subsequent behavioural experimentation. This was deemed acceptable as in other Nereid species (*Nereis diversicolor*) with similar ecology to *N. succinea* a linear regression was found between dry and wet weight (Durou, *et al.* 2008). This method of weighing has been used and recommended in other studies involving Nereids where further use of organisms was required once weighed (Durou, *et al*., 2008, Mouneyrac, *et al.,* 2010).

2.4.5 Recording time to maturation

Time to maturation (days) was calculated from the day the worms were admitted to culture to the day the individual was harvested. The mean time to maturation for each culture (control and test) was then calculated.

Individuals were harvested only when they had reached the fully mature heteronereid stage of the life cycle (Figure 2.3). Sediment in all culture tanks was sifted twice daily (morning and evening) and all heteronereids (male and female) removed and stored pending further use (See 2.4.2 for storage methodology).

2.4.6 Assessing ability to release gametes

All males harvested from all cultures were tested for their ability to release gametes successfully. This was done by stimulating each male with 5µl of 10^{-4} M CSSG in 20ml of seawater (pH equivalent to culture of origin). This dose is more highly concentrated than what would be expected to induce gamete release naturally (Hardege, *et al.,* 2004) and therefore should induce all males that are physically capable of releasing gametes to do so. Any males that were incapable of releasing gametes in this way were discarded and not used in any subsequent behavioural experiments during the study.

2.4.7 Burrowing activity

Burrowing efficiency was assessed as the total time taken for an immature adult individual to fully dig into the sediment from a position on the immediate surface. In turn, worms were placed in boxes containing 2cm of sediment (2mm coral sand) covered with 2cm of 18‰salinity seawater. Immediately after placing the worm on the sediment surface a timer was started. The timer was stopped when worms were deemed to have burrowed successfully, defined as the moment when the body became no longer visible and fully covered by sediment. In total, 15 adult worms, cultured for 3months in control tanks (pH 8.2), were used and the procedure was replicated three times with each worm in each condition explained in the following paragraph. The average time from the three replicates was used for data analysis.

Initially trials were carried out with in conditions accurate to present day ocean chemistry (pH 8.2). After this first control trial, water in each individual tank was exchanged fully for seawater enriched with CO_2 to a pH of 7.8 and the burrowing time trial repeated. From this point forward water in the tanks was monitored and maintained at pH 7.8 according to the methods outlined previously. The burrowing time trial was repeated at a further 5 intervals within a 32 day period (days 2, 4, 8, 16 and 32). Worms were kept isolated in separate small tanks through the duration of the experiment to minimize chance of cannibalization and help ensure all worms survived the length of the experiment.

2.4.8 Assessing levels of glutathione in body tissues

This procedure involved calculating total glutathione (GSH + GSSG) content of complete individual *N. succinea* specimens. Both immature adults and mature heteronereids (male and female) were assayed although sample sizes of each life stage, sex and treatment varied depending on the availability of worms from laboratory cultures. Some sample sizes were too small to attain reliable conclusions, namely females from both cultures as very few were harvested in comparison to males throughout the project. The aim was to assess whether prolonged exposure to near future OA conditions had an effect on glutathione content in the body tissues. Due to its detoxifying properties, reduced levels of GSH can be an indicator of environmental stress. Sub optimal levels of intracellular GSH may have implications for oxidative status of cells, detoxification activity and also sex pheromone production in females.

2.4.8.1 Sample collection and storage

Adult worms between 30 and 35mm in length were removed from culture (control pH 8.2 and CO_2 enriched pH 7.8) after 20weeks. They were immediately frozen in liquid nitrogen and stored in 1.5ml Eppendorf tubes

at -80°C for a further 12weeks prior to use in the assay. The same process was carried out with mature heteronereids. Prior to the glutathione quantification assay, all samples were freeze dried and then weighed to enable the correct amounts of chemicals required for the assay to be calculated. A minimum of 5mg of tissue per sample was required to carry out the bioassay, therefore, any individual samples weighing less than 5mg (14 in total) were discarded and not used for the following procedures.

2.4.8.2 Tissue preparation

Each freeze-dried sample (containing 1ml of cold homogenizing sodium phosphate buffer: 50mM sodium phosphate, pH 7.5, containing 1mM EDTA) was homogenized using a continuous 5-10sec ultrasonic pulse (using Dawe Soniprobe: Branson Sonic Power Co.), before being centrifuged at 15000 x g for 15mins at 4°C. The supernatant was then removed and stored at -80°C in 2ml Eppendorf tubes (stable for 1month) until the next step of the procedure.

2.4.8.3 Deproteination of supernatent

This step of the procedure involved using metaphosphoric acid (MPA) to remove all traces of protein from the prepared supernatant. The presence of protein may cause interference when quantifying GSH levels and so it is vital it is removed prior to the glutathione assay for accurate GSH quantification.

5g of ACS grade MPA chips (Sigma: 239275) were dissolved in 50ml of purified water (solution stable for 4hours at room temperature) and the resulting solution added in equal volume to the sample tissue supernatant. The mixture was then vortexed and left to stand for several minutes at room temperature before being centrifuged at 2000-x g for 2-3 minutes. A delicate white precipitate of protein formed and the supernatant was carefully removed and frozen in 2ml Eppendorf tubes at -80°C for later use (Stable for up to 6months).

Immediately prior to commencement of the assay of total glutathione, a 4M solution of triethanolamine (TEAM) was prepared by mixing 531µl of stock (Aldrich: T58300) with 469µl of purified water. 50µl of this solution was then added per ml of supernatant and vortexed immediately for 20seconds.

2.4.8.4 Sample preparation (Using 2-vinylpyridine to derivatize GSH)

A 1M solution of 2-vinylpyridine was prepared by mixing 108µl of stock (Aldrich: 132292) with 892µl of ethanol. 10µl of this solution was added per ml of sample, vortexed thoroughly and incubated at room temperature for 1 hour before immediate commencement of the assay of total glutathione. (Note: 2-vinylpyridine prevents colour formation to some extent therefore the preparation of the standards (Table 2.1) must be the same as the samples.

2.4.8.5 Total glutathione assay: Reagents preparation

All reagents required for the total glutathione assay were prepared in advance according to the procedures outlined in Table 2.1.

Table 2.1: Preparation procedures of reagents required for total glutathione quantification assay.

Reagent	Stock origin	Preparation procedure	Storage details.
Assay Buffer	-	100mM sodium phosphate buffer, pH 7.5 containing 1.mM EDTA.	Store at 4°C
1.5mM DTNB (5, 5'-Dithiobis (2-nitrobenzoic acid))	(Sigma: D8130)	Dissolve 1.2mg of stock DTNB in 2ml of double distilled water.	Store at -20°C
3mMβ-NADPH (β-Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate)	(Sigma: N6505)	Dissolve 2.25mg of stockβ-NADPH (in 1ml of cold assay buffer.	Store at -20°C
20U/ml GR (Glutathione reductase)	(Sigma: N6505)	Mix 44µl of GR with 956µl assay buffer.	Store at -20°C
100µMGSSG (oxidized glutathione) (for preparation of standards only)	(Sigma: g4626)	Dissolve 6.13mg of stock GSSG in 1ml assay buffer to make 10mM GSSG solution. Dilute this 10mM solution by 1:100 (10mM GSSG: assay buffer).	Store at -20°C

2.4.8.6 Total glutathione assay: Specifications for preparation of standards

The absorbance values acquired from assayed *N. succinea* samples must be compared to the absorbance values of known concentrations of GSH to determine the amount of intracellular GSH present. The quantities of 100µM GSSG and assay buffer required to prepare a set of 13 standard

concentrations of GSH is provided in Table 2.2. It is vital that 10µl 1M 2-vinylpyridine per ml of standard is added following preparation and the resulting solution vortexed and incubated at room temperature for 1 hour prior to the assay. 2-vinylpyridine may affect colour intensity and therefore the standards were treated with this solution in exactly the same way as the samples in section 2.4.7.5.

Table 2.2: Specifications for preparation of standards for total glutathione assay.

Tube	100 µM GSSG (µl)	Assay buffer (µl)	Final GSSG concentration (µM)	Equivalent GSH concentration (µM)
A	0.0	500.0	0.0	0.0
B	0.25	499.75	0.05	0.1
C	0.5	499.5	0.1	0.2
D	1.0	499.0	0.2	0.4
E	2.5	497.5	0.5	1.0
F	5.0	495.0	1.0	2.0
G	10.0	490.0	2.0	4.0
H	25.0	475.0	5.0	10.0
I	37.5	462.5	7.5	15.0
J	50.0	450.0	10.0	20.0
K	75.0	425.0	15.0	30.0
L	100.0	400.0	20.0	40.0
M	150.0	350.0	30.0	60.0

2.4.8.7 Total glutathione assay: Master mix preparation

The master mix was prepared using 20U/ml GR, 3mM β-NADPH, 1.5mM DTNB and assay buffer in the ratio of 1:1:2:12 respectively (See section 2.4.7.6 for reagents preparation). The mix had to be used quickly, within 10minutes of preparation.

2.4.8.8 Total glutathione assay: Preparing 96-well plates and reading absorbance

40µl of sample/standards and 160µl master mix (see section 2.4.7.8 for preparation details) were pipetted into the designated wells on 96-well plates, mixed thoroughly and left to stand at room temperature for 5minutes. Absorbance was then measured using a BioTek Absorbance microplate reader (model: ELx800) at 405nm for 5minutes at 1minute intervals.

2.4.9 Data handling

All data was analyzed using IBM SPSS (Version 19). Details of the exact statistical analysis used in individual experiments, including the outcome, can be found in the results section (figure legends and accompanying paragraphs). However, the types of statistical analysis used in this chapter are summarized below:

- **Continuous data** (collected from two independent samples) was analyzed using a two-sample T-test. This was able to confirm whether or not the means of the two samples differed significantly. This test was appropriate for data collected on length, weight, maturation time and intracellular GSH content.
- **Binomial data** (collected from two independent samples) was analyzed using a chi-square test for association. This test was appropriate for data collected on whether maturation was successful and male capability of gamete release.
- **Continuous repeated measures data** (collected from a single sample tested multiple times in different conditions) was analyzed using repeated measures ANOVA. Bonferroni *post hoc* analysis allowed for determination of which data sets differed significantly from the control. This method was used exclusively for data collected on burrowing activity where burrowing speed of a single cohort of control cultured adult worms was initially tested in control seawater (pH 8.2) and then in CO2 enriched seawater (pH 7.8) at several time points over a 32day period.

2.5: Results

2.5.1 Impact of ocean acidification on heteronereid length

Mean total length (mm) of both male and female heteronereids after culture for 4-6months was not affected by culture condition (control, pH 8.2 or CO_2 enriched, pH 7.8) (Figure 2.5). The two samples for mean total length were completely independent (each group consisted of different individuals subjected to different culture conditions (either pH 8.2 or pH 7.8) leading to a two sample t-test being applied to determine whether total heteronereid length was significantly different between cultures. Data for males and females was treated separately due to ecological variance in size between sexes (females are usually larger due to presence of eggs). Sample sizes for length and weight data were unequal due to different numbers of individuals reaching maturity in each culture. It was noted that a considerably higher percentage of heteronereids developed into males rather than females in both cultures (control and CO_2 enriched). Length was found to be insignificantly different between culture conditions in both males ($t=0.535$, $p> 0.05$) and females ($t= 0.058$, $p>0.05$).

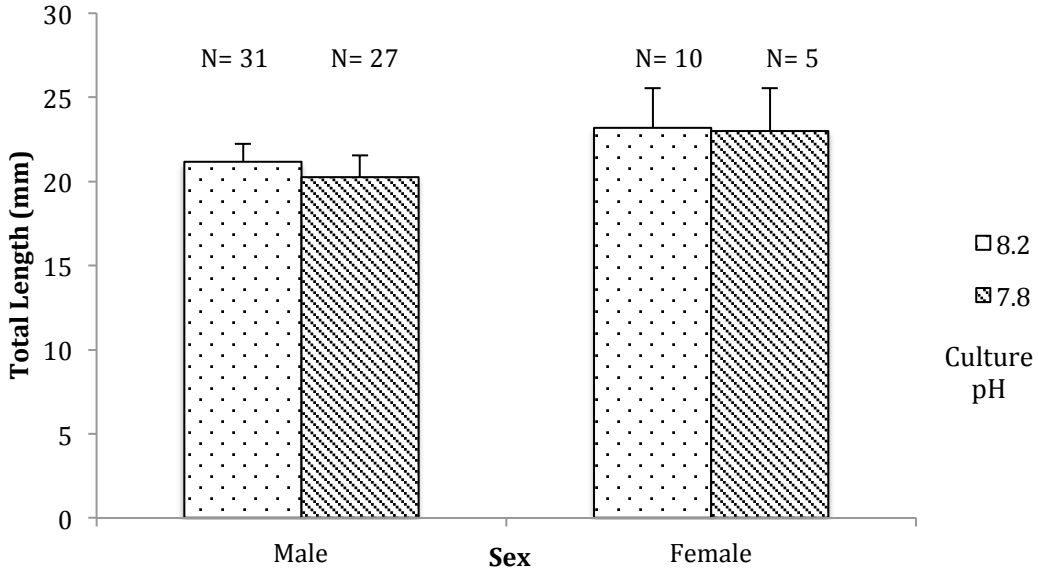

Figure 2.5: Average total length (mm) of male and female heteronereids from pH 8.2 control culture (dotted bars) and pH 7.8 CO_2 enriched culture (striped bars). Error bars show standard error. Mean length between cultures in both males and females not significantly different (two sample t-test, $p> 0.05$).

2.5.2 Impact of ocean acidification on heteronereid weight

Mean weight (mg) of male and female heteronereids after culture for 4-6months was not affected by culture condition (control, pH 8.2 or CO_2 enriched, pH 7.8) (Figure 2.6). The two samples for mean total weight were completely independent (each group consisted of different individuals subjected to different culture conditions, either pH 8.2 or pH 7.8) leading to a two-sample t-test being applied to determine whether total heteronereid length was significantly different between cultures. As with length data, weight data for males and females was treated separately due to ecological variance in size between sexes (females are usually larger due to presence of eggs). Sample sizes for length and weight data were unequal due to different numbers of individuals reaching maturity in each culture. It was noted that a considerably higher percentage of heteronereids developed into males rather than females in both cultures (control and CO_2 enriched). Weight was found to be insignificantly different between culture conditions in both males (t=0.432, p> 0.05) and females (t= -0.005, p>0.05).

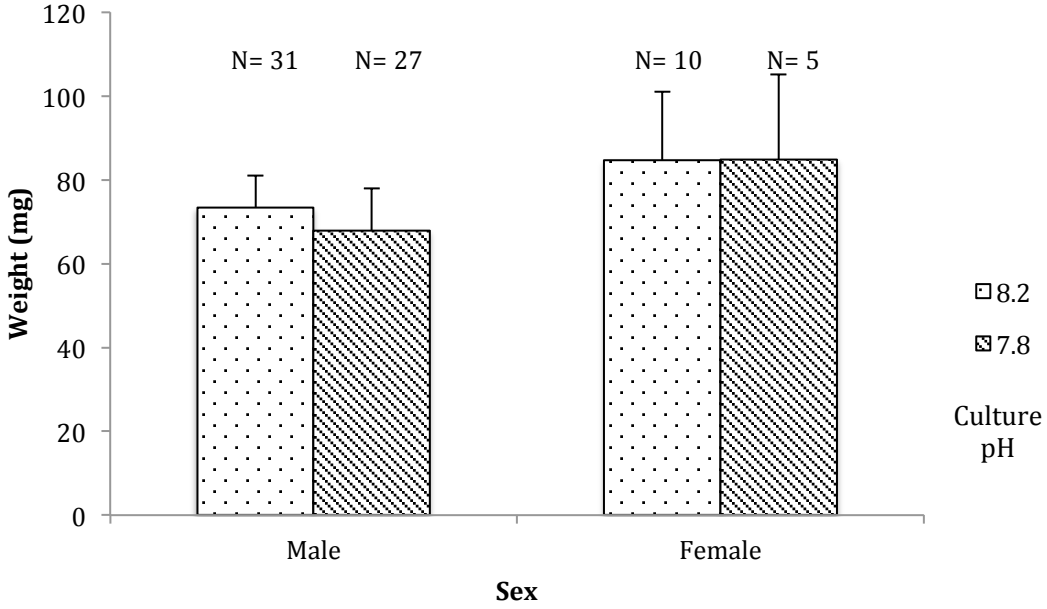

Figure 2.6: Average wet weight (mg) of male and female heteronereids from pH 8.2control culture (dotted bars) and pH 7.8 CO_2 enriched culture (striped bars). Error bars show standard error. Mean weight between cultures in both males and females not significantly different (two sample t-test, p> 0.05).

2.5.3 Impact of ocean acidification on time to metamorphosis

Data collected on the average time to maturation was calculated as the number of days for each individual to reach the fully mature heteronereid stage from initial addition to either culture (Figure 2.7). Sample sizes from each culture were not equal as less worms reached maturity in pH 7.8, CO_2 enriched culture. The samples were completely independent (each group consisted of different individuals subjected to different conditions) leading to a two sample t-test being applied to determine whether mean maturation time differed significantly between cultures. A t value of -3.786 and p value of 0.000 (<0.005) confirmed that the means of the two data sets were significantly different.

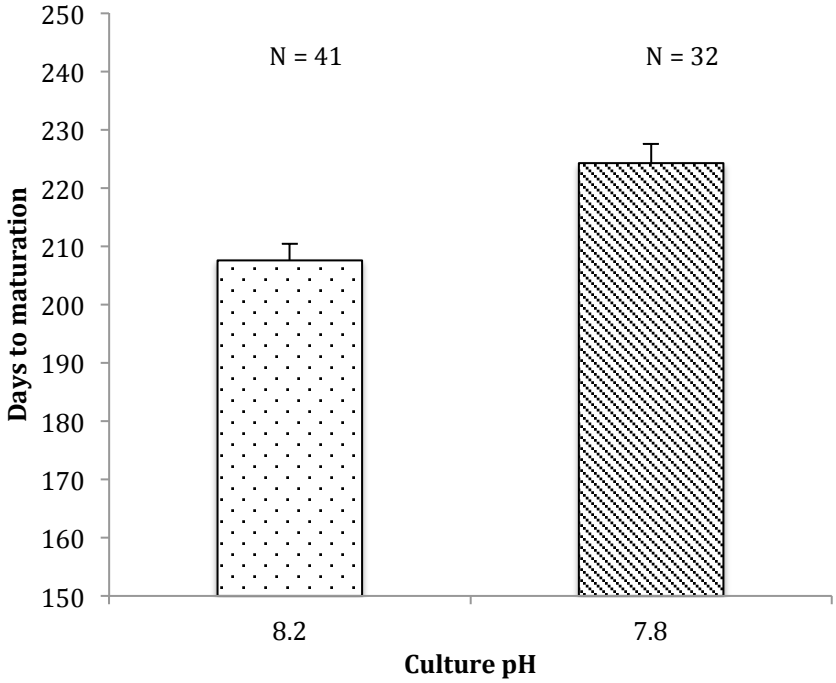

Figure 2.7: Average days taken for individuals to reach fully mature heteronereid stage after addition into culture at juvenile stage. pH 8.2 culture shown on dotted bar (N = 41), pH 7.8 CO_2 enriched culture shown on striped bar (N =32). Error bars show standard error (pH 8.2 culture = 2.88416, pH 7.8 culture = 3.33330). Two-sample t-test confirmed sample means are significantly different (t = -3.786, p = <0.005).

2.5.4: Impact of ocean acidification on successful maturation

Maturation success is displayed as the percentage of total individuals added to culture as juveniles that progressed to the fully mature heteronereid stage of the life cycle (Figure 2.8). Each culture initially consisted of 200 worms, 41 matured successfully in pH 8.2 and 32 in pH 7.8. As there was no expected outcome to these results and raw data was binominal (matured/did not mature), a chi-square test for association was carried out to identify any significant difference in maturation success between cultures. Prolonged exposure to CO_2 enriched seawater (one whole life cycle, 4-6 months at pH 7.8) was found to have no significant impact on maturation success (Pearson chi square = 1.357, p = 0.244).

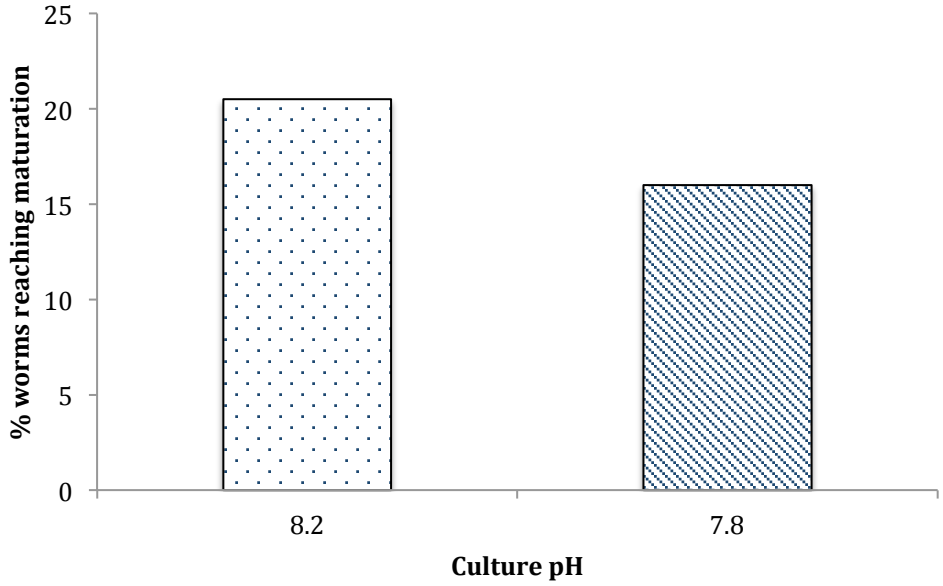

Figure 2.8: Percentage *N. succinea* that progressed successfully from juvenile to heteronereid stage during culture period (4-6months). pH 8.2 culture shown on dotted bar, pH 7.8 CO_2 enriched culture shown on striped bar. No significant difference in percentage maturation between cultures (Pearson chi square = 1.357 p = > 0.05).

2.5.5 Impact of ocean acidification on capability of gamete release by males

Fewer males from the pH 7.8 CO_2 enriched culture (70.37 %) were capable of releasing gametes when stimulated with high dose female pheromone (5µl 10^{-4} M CSSG) in comparison with males from the pH 8.2 control culture (83.87%)(Figure 2.9). Data sets were uneven (31 males harvested from pH 8.2 control culture, 27 harvested from pH 7.8 test culture), therefore the data are displayed as percentage of males capable of releasing gametes. The reduction in gamete release response by pH 7.8 cultured males was not significant in comparison to pH 8.2 cultured males when assessed using a chi-square test for association (Pearson chi square = 2.466 p = > 0.05).

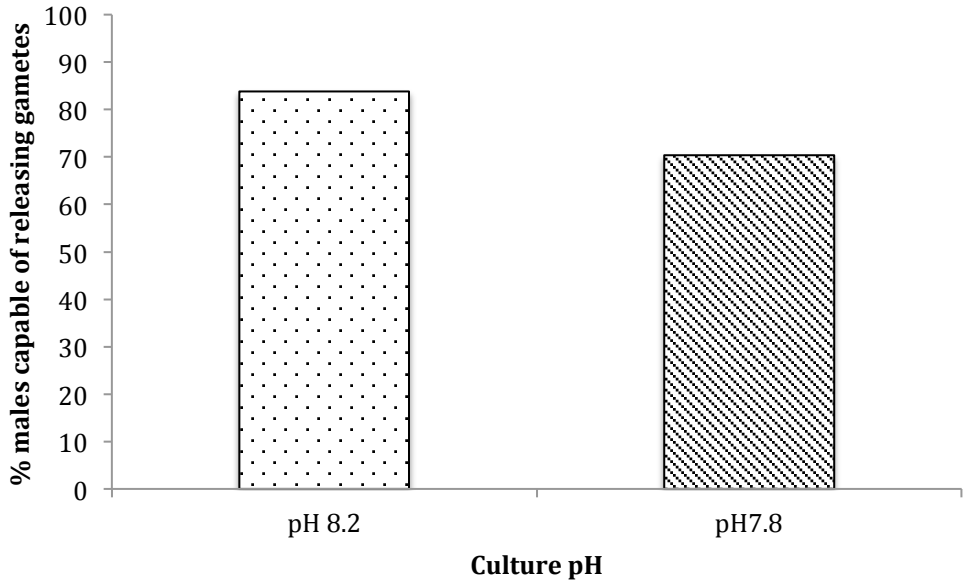

Figure 2.9: Percentage of male *N. succinea* from each culture (control pH 8.2 and CO_2 enriched pH 7.8) capable of releasing gametes when stimulated with 5µl 10^{-4} M CSSG. pH 8.2 culture shown on dotted bar, pH 7.8 test culture shown on striped bar. No significant difference between cultures in the proportion of males capable of gamete release (Pearson chi square = 2.466 p = < 0.05).

2.5.6: Impact of ocean acidification on burrowing activity

Data collected during burrowing experiments was paired; each of the 15 adult worms were tested in control conditions (pH 8.2) and then again in test conditions at pH 7.8 at several time points over a 32 day period. Repeated-measures ANOVA (with associated Bonferroni *post hoc* analysis) was used to compare mean digging time in pH 7.8 test conditions at each time point during the experiment with the pH 8.2 control. Time to dig into the sediment was significantly reduced at all time points after addition to pH 7.8 culture in comparison to the control (pH 8.2) (Repeated- measures ANOVA + Bonferroni post hoc analysis sig. < 0.05 for all time points).

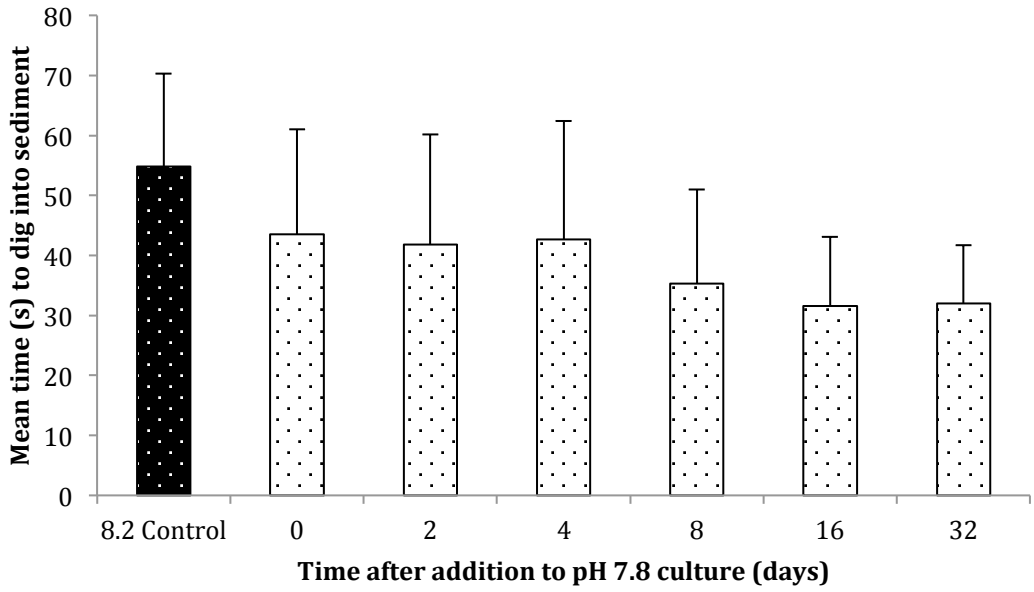

Figure 2.10: Mean time (s) for adult *N. succinea* to dig from the sediment surface until completely covered in control conditions pH (8.2) and at several time points after addition to pH 7.8 culture. N = 15. Error bars show standard deviation of means. * Indicates mean time to burrow into sediment is significantly different from pH 8.2 control (Repeated- measures ANOVA with Bonferroni *post hoc* analysis sig. < 0.05).

2.5.7: Impact of OA on total glutathione content of body tissues

GSH content of the body (mM/mg) of adult (Figure 2.11) and male (Figure 2.12) *N.succinea* was not impacted by long-term exposure to OA conditions (pH 7.8). A two- sample t test showed any difference to be insignificant ($p > 0.05$). Results for females were not analyzed as sample size was too small to perform reliable statistical analysis (only one specimen was recovered from pH 7.8 culture).

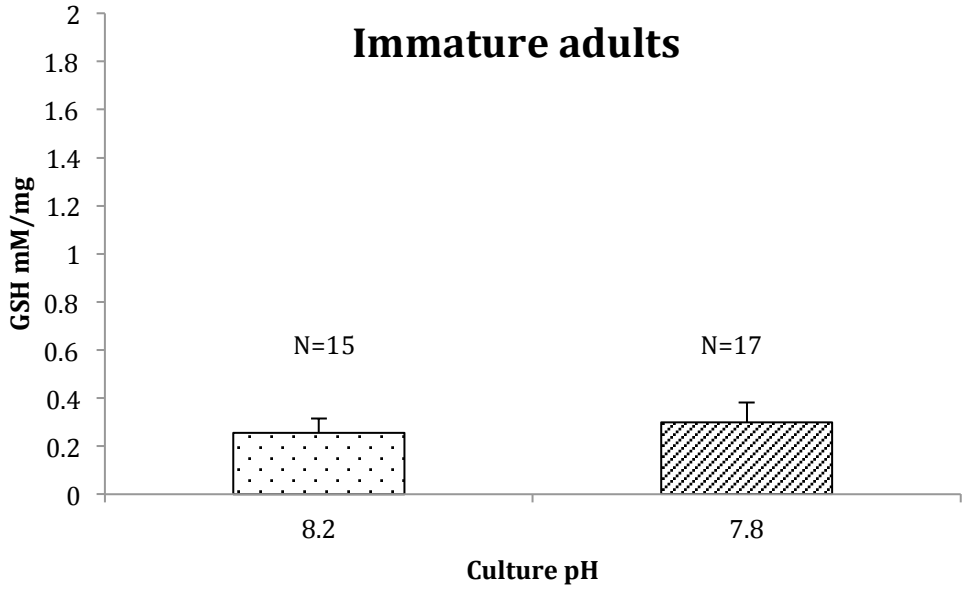

Figure 2.11: Mean amount of GSH (mM) per mg of body tissue in immature adult *N. succinea* cultured for 20weeks in either pH 8.2 control (dotted bars) or pH 7.8 CO_2 enriched (striped bars) seawater. N for each culture displayed above bars. Error bars show standard error of data. A two- sample t test showed no difference between data sets ($t = -0.440$, p 0.663).

Figure 2.12: Mean amount of GSH (mM) per mg of body tissue in mature male *N. succinea* cultured for 4-6months in either pH 8.2 control (dotted bars) or pH 7.8 CO_2 enriched (striped bars) seawater. N for each culture displayed above bars. Error bars show standard error of data. A two- sample t test showed no difference between data sets (t = -0.208, p 0.838).

2.6 Discussion

2.6.1 Physical development

Heteronereid body size as a measure of length and weight was not reduced after culture in CO_2 enriched conditions (pH 7.8) from juvenile to heteronereid stage in either males or females in comparison to the pH 8.2 control culture (Figures 2.5 and 2.6). This does not agree with previous conclusions, which saw a slight reduction in maximum size in males after prolonged culture in pH 7.8 conditions and a more prominent size reduction in females with regards to length only. The difference in conclusions could be attributed to the very low sample size of females in this study, as cultures produced a highly male oriented sex ratio and time constraints prevented replicate procedure necessary to inform a more robust conclusion. Size variation in natural *N. succinea* populations, particularly in males, is high (Ram and Hardege, 2005). The conflicting results between this and previous studies may be a reflection of this.

The affect of OA on body size is reported to be variable between species and the extent to which detrimental effects may occur is unclear. Calcifying species are assumed to suffer the most, with respect to growth due to OA driven changes in seawater chemistry reducing the amount of free carbonate available in seawater for them to synthesize hard exoskeletons (Stummp, *et al.,* 2011a). However, more recent studies have concluded that growth rate in some calcifying species is in fact increased and that acid dissolution of calcified body parts creates the illusion of reduced growth (Rodolfo Metalpa, *et al.,* 2011; Wood, *et al.,* 2007).

The tropical fish species *A. percula* also shows no difference in body size when reared in OA conditions compared to current seawater chemistry (Munday, et al., 2009b). Fish are assumed to be more robust to OA with respect to development due to them possessing more highly developed systems for internal acid-base regulation (Ishimatsu, *et al.,* 2008) in

comparison to invertebrates (Widdecombe and Spicer, 2008). In addition, Munday, *et al.,* (2009b) only made size comparisons during the larval stages of the *A. percula* life cycle which may not have been enough time for any OA driven size deficiency to become apparent.

N. succinea in this study were added to culture at the juvenile stage when specimens were around 10-15 segments long. Percentage survival to heteronereid stage (Figure 2.8) and maximum size (Figures 2.5 and 2.6) was not significantly affected throughout the 4-6 month culture to maturity. However, previous studies have shown that survival is less than 5% for larvae developing from the point of fertilization in pH 7.8 CO_2 enriched seawater. During fertilization and the pelagic larval stage of the life cycle*, N. succinea* is exposed directly to the water surface and therefore the chemical changes associated with OA. Once the benthic life stage is reached, *N. succinea* burrow into the sediment and may have potential to regulate pH and chemical conditions within their burrows (Zhu, et al., 2006). It is apparent that the fertilization process and early developmental stages of the life cycle in *N. succinea* and other marine invertebrates (Havenhand*, et al.,* 2008) are likely the most sensitive to OA.

As with most marine species, it is advantageous for *N. succinea* to attain adequate size and failure to do so may have negative implications for species persistence. Males locate sexual partners by detecting and following a pheromone trail, a cue, which increases swimming activity in the male to enable him reach the female (Hardege, *et al*., 2004). Swim speed is dependent on body size (Ram and Hardege, 2005) in *N. succinea*, with larger males capable of reaching faster swim speeds therefore providing an advantage when pursuing a partner. Body size is also important in females. In the closely related species *N. diversicolor*, female body size is directly correlated with fecundity; larger females carry more eggs (Durou, *et al*., 2008)). Producing a large amount of eggs is particularly relevant in broadcast spawning species with pelagic larvae, such as *N. succinea*, to

maximize fertilization and the amount of subsequent offspring that are highly susceptible to mortality in early life stages.

The percentage of individuals reaching maturation did not differ between cultures. *N. succinea* cultured in CO_2 enriched condition from juvenile stage took approximately 10 days longer to reach full sexual maturity than those raised in control conditions (Figure 2.7). The finding supports evidence that other marine invertebrate species also display delayed metamorphosis when reared in low pH conditions (Talmage and Gobler, 2010). The timing of the reproductive event in *N. succinea* is seasonal and highly coordinated by environmental cues including lunar phase, temperature and day length (Hardege, 1990). It is essential that the population reach maturity at the correct time to participate in mass spawning events. A delay in successful metamorphosis to the sexually mature heteronereid may have implications for correct coordination and timing of the reproductive event. However, although OA is increasing, change in ocean chemistry is still relatively gradual. Estuarine species, such as *N. succinea*, are often relatively robust to fluctuation in a number of environmental factors such as temperature, salinity and pH on a regular basis. This, coupled with a rapid generation time (~6months) may provide *N. succinea* with a relatively high potential to adapt to near future levels of OA.

2.6.2 Burrowing behaviour

N. succinea spends the majority of the life cycle burrowed into sediment, emerging only to participate in reproduction once mature. Highly cannibalistic and a source of food for other species, burrowing offers this species shelter and protection and is therefore a key survival behavior. Significantly less time was taken for control cultured, immature adult individuals to successfully burrow into sediment when tested in CO2 enriched, pH 7.8 seawater, compared to tests in control (pH 8.2) seawater (Figure 2.10). Additionally, this increased burrowing efficiency was

maintained when individuals were retested at several time points during 32-day culture.

It is suggested here that the increased affinity to burrow by *N. succinea* when in acidified conditions may be a type of avoidance behaviour. *N. succinea* has the ability to regulate pH within their burrows to some extent and therefore create optimal conditions for growth and survival (Zhu, et al., 2006). Many freshwater fish species have been shown to exhibit avoidance behaviour of areas of low pH when given the choice, including salmon (Atlund and Barlup, 1996; Ikuta, *et al.,* 1999), snapper, yellowfin bream, and Australian bass (Kroon, 2005) and the sand smelt (Davies, 1991). Similar conclusions have also been drawn from invertebrate species including freshwater prawns (Kroon, 2005) and crayfish (France, 1985). France (1985), tested the pH preference of the crayfish *Orconectes virillis* from two different lakes, one reference and one acidified, and found that crayfish from the reference lake showed stronger avoidance of low pH than those from the acidified lake. This suggests *O. virillis* shows potential to acclimatize to conditions of reduced pH.

Studies investigating whether aquatic species show avoidance for a particular environmental condition, such as reduced pH, use fluvarium equipment whereby a continuous flow of water may be modified to hold particular properties at either side of the flume. The area where the test species spends most time is assumed to be the preferred condition. Future studies should apply such a technique to adult *N. succinea* to help determine whether increased burrowing efficiency may be a result of low pH avoidance.

2.6.3 Glutathione content in the body

Intracellular glutathione (GSH) is a key component for detoxification and maintenance of cell oxidative status (Kirlin, *et al.,* 1999; Pastore, *et al.,* 2003). In *N. succinea*, GSH plays an additional role in acting as the precursor to the synthesis of the female sex pheromone cysteine glutathione di-sulphide (CSSG) (Hardege, *et al.,* 2004), presenting a potential trade off between maintaining cell oxidative status and pheromone production.

Levels of GSH per mg body tissue in adult (Figure 2.11) and mature male (Figure 2.12) *N. succinea* were found not to be affected by prolonged culture (one lifetime) in CO_2 enriched, pH 7.8 conditions in comparison to pH 8.2 control culture suggesting that OA does not impact GSH production. Solid conclusions about GSH levels in females in response to prolonged pH stress could not be made due to low sample size. Assessment of females is arguably most relevant as these are the ones facing the trade of between detoxification and pheromone production and so the need for further experimental clarification on GSH content of female body tissues is required.

Stress by heavy metals such as zinc, has been shown to significantly reduce intracellular levels of GSH in *N. succinea* (Shafi, M., personal communication). It is suggested that although reduced pH is a potential stressor, it may not be this in isolation that presents the most negative biochemical consequences. Stress may accumulate in response to multiples factors such as the combination of OA with rise in heavy metal pollution and temperature (Hardege, *et al.,* 2011). Initial examination of this hypothesis with *N. succinea,* showed worms subjected to zinc stressed culture with the additional stress of pH reduction to pH 7.8 by CO_2 injection did not show any further reduction in intracellular levels of GSH than when subjected to zinc stress alone.

A more accurate assessment of GSH levels and activity in pH stressed *N. succinea* would require assessing multiple parameters involved in GSH

production and activity, rather than the GSH content of cells in isolation. It is possible to measure levels of both GSH synthetase, the enzyme required for GSH synthesis and Glutathione-S-transferase (GST), the enzyme associated with the detoxifying activity of GSH. Such techniques have been used by Ayoola, *et al.,* (2010), who present evidence of increased levels of GST in *N. succinea* after exposure to the endocrine disruptor nonylphenol. This indicated that GSH was actively being used for detoxification in response to a known stressor. Quantifying levels of GSH in combination with the enzymes associated with its production and biological activity would give a more comprehensive assessment of whether OA is responsible for causing oxidative stress with respect to GSH.

Chapter 3:
The effect of acute exposure to near future ocean acidification on chemical communication

3.1 Introduction

Poor light transmission and varying levels of turbidity (Bronmark, *et al.,* 2000), have helped lead to chemical strategies becoming the dominant method of communication in the ocean (Wisenden, 2000). All chemical cues and signals must be produced, released, transmitted, received and correctly interpreted by the receiver to be a successful means of communication (Wyatt, 2003) (Figure 3.1). The term 'signal disruption' is used to describe interference with any of these steps, a process that may lead to reduced efficiency of information transfer between organisms and potentially impact survival.

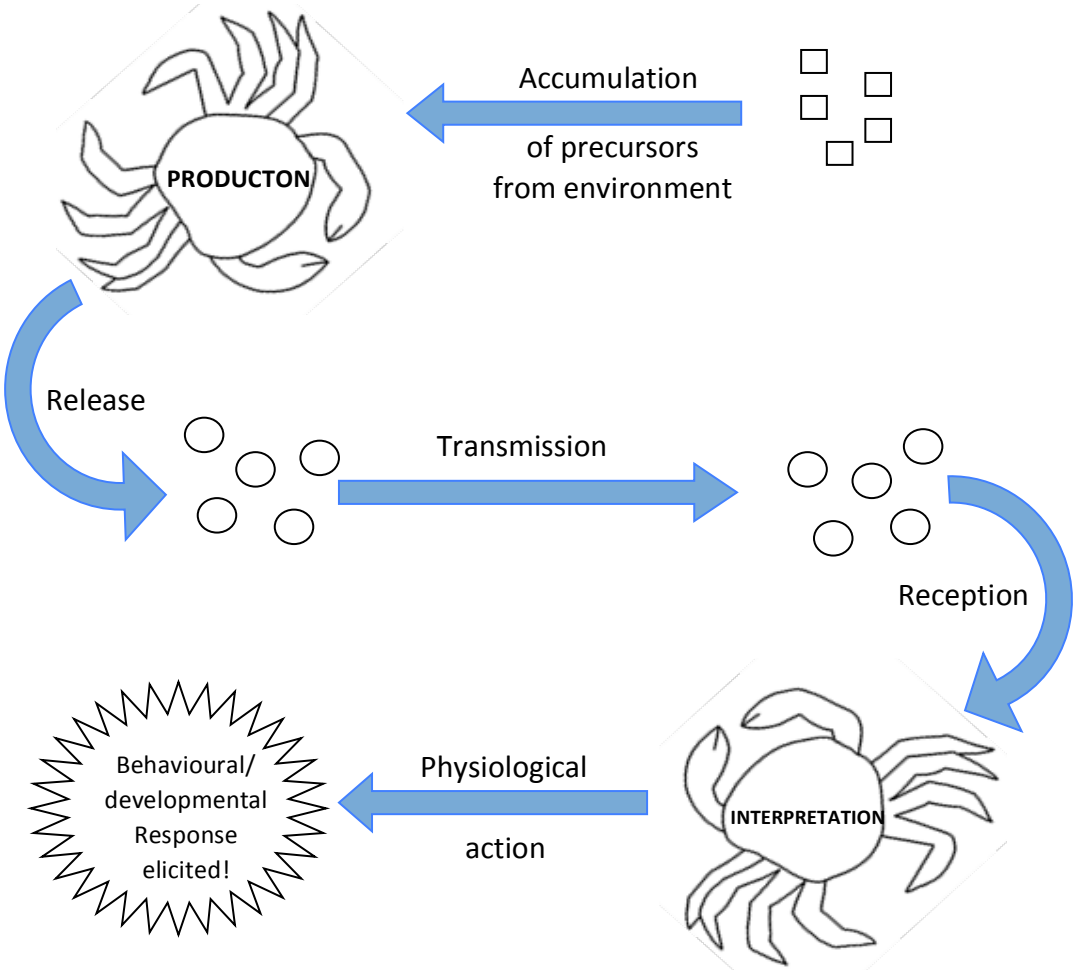

Figure 3.1: Original illustration of steps involved in chemical signaling in the marine environment. Squares indicate precursors for pheromone production within the environment and circles indicate pheromone molecules.

3.1.1 Sex pheromones in *Nereis succinea*

Nereis succinea exhibits a complex series of reproductive behaviours closely regulated and coordinated by chemical and environmental cues during the lead up to the ultimate event of gamete release (Hardege, 1999). This process was introduced and covered in detail in Chapter 1 (section 1.5.1) but is summarized briefly here, as it is key to the experiments in this chapter.

As a broadcast spawning species, *N. succinea* must ensure maximum participation of all individuals within a population to maximize the possibility of contact between male and female gametes. This synchronization is achieved by environmental factors such as temperature and the lunar cycle to achieve the correctly timed mass-spawning event (Hardege, *et al.,* 1998). Both male and female pheromones are then used to more finely tune the probability that gametes will meet (Figure 1.3) (Zeeck, *et al.,* 1998a, Zeeck, *et al.,* 1998b). The sexual behaviours induced by these pheromones ensure gametes from each sex are released both in quick succession and only when male and female partners are in close proximity to one another (Hardege, *et al.,* 1998). Disruption of these pheromonal cues and subsequent sexual behaviours may therefore prevent gamete release from occurring at the correct time (or not at all) potentially reducing the chances of fertilization and overall fecundity within a population.

The female sex pheromone in *N. succinea* (CSSG) (Figure 2.1) is responsible for inducing nuptial behaviours (increased swimming activity and the 'nuptial dance') and ultimately gamete release in males (Zeeck, *et al.,* 1998a). The behaviours elicited are fully dependent on the concentration of CSSG detected by males (Hardege, *et al.,* 2004). CSSG concentration acts as an indicator of how close the male is to the female to ensure gamete release only occurs when sexual partners are in the closest possible proximity to one another (Hardege, *et al.,* 2004). It is important that all 3 ritualized behaviours are conserved and carried out correctly to maximize

reproductive output. This chapter will assess to what extent long-term exposure to near future conditions of OA may affect the behaviours associated with reception of CSSG.

My own previous research concluded that increased acidity of seawater has a negative effect on swim speed in male *N. succinea*. These earlier investigations showed male response to low dose CSSG (10^{-6} M, required to increase swimming activity) was significantly reduced when exposed to seawater treatments <pH 8.0 for short periods, suggesting that OA is likely to affect the ability of a male to efficiently pursue a female. This present study used a revised version of the original bioassay to assess *all three* major stages of male behavior during reproduction: increased swimming activity, swimming in tight circles around the female ('nuptial dance') and sperm release, and to what extent reduced pH may affect them. This aims to give a clearer picture of to what extent pheromone mediated sexual behaviours and subsequently reproductive success may be influenced by reduced pH in the marine environment.

3.1.2 Feeding cues in the marine environment

Arguably, the most important chemically mediated behavior in the marine environment is the detection and location of food sources. Many marine organisms, vertebrates and invertebrates, locate moving prey and stationary food sources through olfaction and chemical reception (Hayden, *et al.,* 2007). This chemical sense is also important for discriminating between food choices, for example between distasteful or harmful substances and nutritious ones (Croll, 1983). Behaving appropriately to detect or choose food items is essential to optimize health and individual survival. Unlike pheromonal cues which consist of either a single or small bouquet of specific molecules (Wyatt, 2010) and similarly are received by specific receptors, feeding cues are usually more general and often of multiple chemical components. If a few components of the bouquet are affected by OA, those

that remain may still be sufficient to trigger a response. For this reason, it is hypothesized that the ability of organisms to detect food will be impacted to a lesser extent by OA than the detection of highly specific, single component cues such as the sex pheromone, CSSG in *N. succinea*.

Amino acids are well-known effective chemical stimuli for many species (Tierney and Atema, 1987) and a common technique in researching feeding response is to use single amino acids as feeding stimulants. Hara (2006) examined the behavioural and physiological responses of a variety of freshwater fish to numerous amino acids to assess feeding interest. This project will use a similar technique combined with examining complete food source odour (fish food) to see whether *N. succinea* are still capable of responding to food sources when exposed temporarily to OA conditions.

3.1.3 Hypothesis

Acute exposure of control cultured (pH 8.2) *N. succinea* to CO_2 enriched seawater (pH 7.8) will negatively impact reception of chemical feeding cues (taurine, glycine and fish food extract) and sex pheromones (CSSG). Typical physical behaviours indicating recognition will either be less pronounced or not exhibited at all.

3.2 Methodology

3.2.1 Assessing impact of acute exposure to ocean acidification on behavioural response to feeding cues in *Nereis succinea*

Feeding experiments were used to assess the impact of low pH on the ability of *N. succinea* to detect chemical cues. Both single component (glycine and taurine) and multicomponent (tropical fish flakes) feeding cues were used. Single component cues taurine (Sigma) and glycine (Sigma) at 10^{-3}M concentration were administered separately in the amount of 50µl per each feeding trial. The multicomponent fish food cue was less straight forward, both to prepare and administer. 500mg of fish flakes (Aquarian Tropical Fish Flakes, nutritional information in Table 3.1) were ground to a fine powder using a pestle and mortar and then combined with 50ml of water. Dissolving fish food in water causes a dramatic drop in pH of the surrounding water. This must be taken into account to avoid addition of another variable and to prevent any interference associated with this during behavioural experiments. The pH of the fish food mixture was therefore adjusted to the correct pH (that of the surrounding water used in each particular trial) with the addition of 0.1M NaOH.

For each cue (taurine, glycine, fish flakes), 15 control cultured (See section 2.4.2 for culture methods) immature adults (45 in total) were subjected to feeding trials in control pH seawater (pH 8.2) to assess response in conditions appropriate to present day ocean chemistry. Details of the amount of cue administered per trial can be seen in Table 3. Exposure to each cue was repeated three times with an overall positive response to the cue being recorded if a positive response was recorded in two or more of the three repeats. This approach was used to help minimize error in attributing a positive result by mistake by ensuring the positive result was maintained and behaviour did not occur by chance. This technique has also

been used in feeding experiments with the polychaete *Platynereis dumerilii* in previous studies. Individuals were deemed to be responding positively to a cue if they displayed certain pre-defined behaviours. These included: change in direction and actively moving toward cue, flaring of palps and exposure of internal jaws. Negative responses included: no movement or moving away from the cue. These behaviours were identified following preliminary observation of the species during feeding (Dr. Jörg Hardege, personal communication)

Table 3.1: Preparation concentrations and quantity of feeding cues added per feeding trial.

Feeding cue	Nature of cue	Concentration	Amount per exposure
Seawater control	No feeding cue present	-	50ul
Taurine	Single component, amino acid	10^{-3}M	50ul
Glycine	Single component amino acid	10^{-3}M	50ul
Tropical fish flakes *	Multicomponent	500mg in 50ml water	10ul

* Brand: Aquarian Tropical Fish Flakes. Ingredients Fish and Fish Derivatives, Cereals, Derivatives Of Vegetable Origin, Oils and Fats, Algae, Vegetables, Minerals, Egg and Egg Derivatives, Yeasts, Mollusks and Crustaceans. Typical Analysis: Moisture <5%, Protein 36.5%, Oils and Fats 12.5%, Fibre 3%, Ash 14%.

Prior to experimentation, all worms starved for 2 days and were allowed to feed again on completion of the trials. This was to standardize hunger levels to minimize any influence this may have on level of behavioural response during feeding trials. Before exposure to a cue, worms were separated and placed individually in 67mm diameter crystalizing dishes containing 20ml of 18‰ salinity filtered seawater (to eradicate any chemical 'noise' present before filtration). Prior to addition of any stimuli, 10 minutes settling time was allowed to make any change in behaviour on addition of a cue to be

more easily identified. Feeding cues were added using a P200 micropipette approximately 4cm away from the head region of the worm and behaviour was observed for 1 minute. Preliminary testing in control conditions (control cultured worms in pH 8.2 seawater) showed that positive responses to feeding cues were exhibited within one minute following exposure. Worms were transferred to clean dishes containing clean water and allowed to settle for 10 minutes between exposures, deemed an appropriate amount of time for the worm to desensitize to the previous cue (pers. Comm. Dr. Jörg Hardege). After 2 days of starvation, the above procedure was repeated in CO_2 enriched, pH 7.8 seawater. During all feeding trials, visual stimulation was kept to a minimum by blocking the experimenter from view during cue administration using cardboard sheeting. The influence of visual stimulation on behaviour caused by the procedure was also controlled for by carrying out the experiment as described, but with the addition of 50µl seawater only as a negative control.

3.2.2 Assessing impact of acute exposure to ocean acidification on behavioural response of male *Nereis succinea* to female sex pheromone

Behavioural responses by male *N. succinea* to the female pheromone, CSSG, were recorded according to a numerical grading system whereby 4 possible specific responses were identified (Table 3.2). In the cases of responses 0-2, these were awarded if the behavior was maintained for 3+ seconds following addition of CSSG. Response 3 was awarded if ejaculation of sperm occurred within 5 seconds of the addition of CSSG (See section 1.5.1 for further details of *N. succinea* sexual behaviour).

Table 3.2: Definitions of grades used to describe behavioural response of male *N. succinea* on addition of 10^{-6} M CSSG (female sex pheromone).

Grade	Behavioural response description
0	No measurable response – swimming activity remains unaffected/ no gamete release
1	Notable increase in swimming activity – swim speed is increased by more than 40%/swimming path remains regular/ no gamete release
2	Nuptial dance –Immediately follows behaviour 1. Swim pattern of small tight circles/no gamete release.
3	Ejaculation – Immediately follows behaviour 1 and 2. Sperm is released, visible as surrounding water becomes cloudy.

All males used in this experiment were removed from incubation (10-12°C) and allowed to acclimatize to room temperature (for 1 hour) in 20ml of 18‰salinity seawater in a 67mm diameter crystalizing dish prior to experimentation. Males were then tested for the ability to release gametes via stimulation with high dose (10^{-4} M) CSSG and only males that had been deemed fit and capable of releasing gametes were used for experimentation (Figure 3.2).

Data was collected using males cultured in pH 8.2 seawater (control group), (see section 2.4.2 for culture technique). 15 individuals were used. Defined amounts of 10^{-6} M CSSG were pipetted into 20ml 18‰ salinity, filtered seawater in 67mm diameter glass crystalizing dishes containing 1 male worm.

As per the above procedure, each male was exposed once to 1µl, 5µl, 10µl, 20µl, 30µl, 40µl, and 50µl 10^{-6} M CSSG in both pH 8.2 seawater and pH 7.8 seawater, meaning each male was stimulated a total of 14 times. Each addition of pheromone was carried out in fresh filtered seawater. All males were transferred to clean seawater after stimulation and allowed to resettle for 10mins before the next stimulation (Figure 3.3). No order was followed when administering pheromone cues (both concerning seawater pH and amount of cue) to control for any improvement/degradation of response that may be caused by multiple stimulations. This technique of multiple pheromonal stimulation was justified as male *N. succinea* are capable of being stimulated by and responding to CSSG repeatedly without a reduction in performance (Ram and Hardege, 2005). The amount of sperm released does become reduced after multiple stimulations (Ram and Hardege, 2005), however this experiment is only testing the capability of males to release sperm and not the amount ejected.

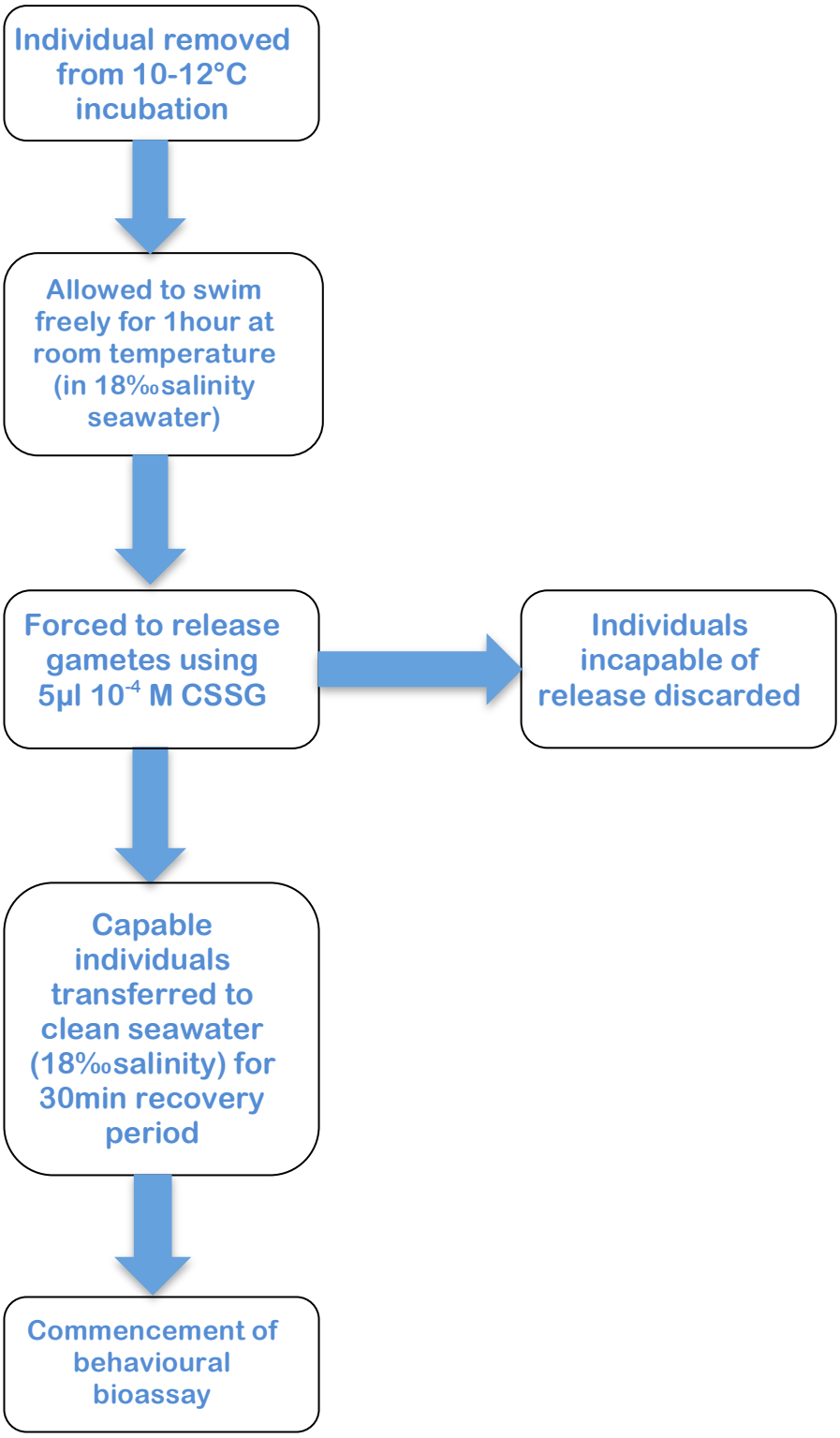

Figure 3.2: Preparation steps prior to behavioral bioassays to ensure competence of all male *N. succinea* used.

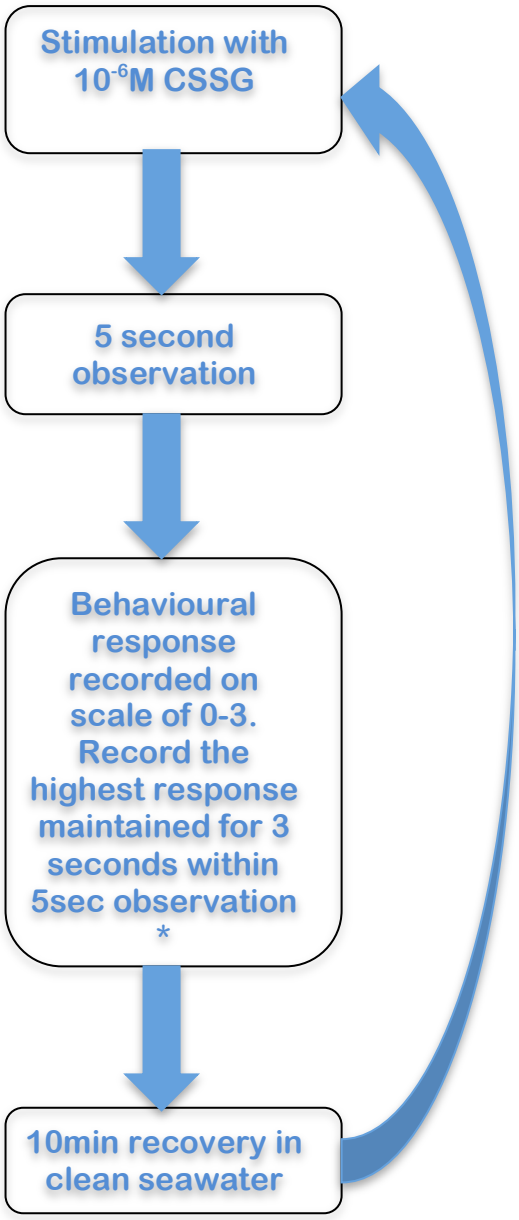

Figure 3.3: Behavioural bioassay to assess male response to CSSG. With each individual the process was repeated with the amounts of 10^{-6}M CSSG specified in the method and in pH 8.2 and pH 7.8 seawater. *Gamete release does not need to be maintained for 3 seconds to be graded.

3.2.3 Data handling

All data was analyzed using IBM SPSS (Version 19). Details of the exact statistical analysis used in individual experiments, including the outcome, can be found in the results section (figure legends and accompanying paragraphs). All data in this chapter was analyzed using the McNemars statistical test. This is a variation of Chi-square suitable for matched pair, binomial data and shows whether the outcome of such paired data sets differ significantly. Experiments in this chapter involved ranking behaviour of control cultured worms as occurring positively (1) or negatively (0) after stimulation with a chemical cue. The same experiment was repeated with the same sample in CO_2 enriched water to provide a paired data set. McNemars test was therefore used to assess whether behaviour changed significantly as a result of pH change of test conditions.

3.3 Results

3.3.1 Impact of acute exposure to ocean acidification on behavioural response of male *Nereis succinea* to female sex pheromone

Male *N. succinea* from the control culture (pH 8.2) only were stimulated with 10µl of 10^{-6}M CSSG in both control (pH 8.2) and CO_2 enriched (pH 7.8) seawater. As the data were binomial and paired (each male was subjected to testing in both pH conditions), McNemars test (variation of chi-square suitable for repeated measures data) was used to test for differences in behavioural response according to treatment. Each behavioural response (increased swimming, 'nuptial dance' and gamete release) was tested separately. For all 3 behavioural responses, no significant difference in level of response between treatments was observed ($p > 0.05$) (Figure 3.4).

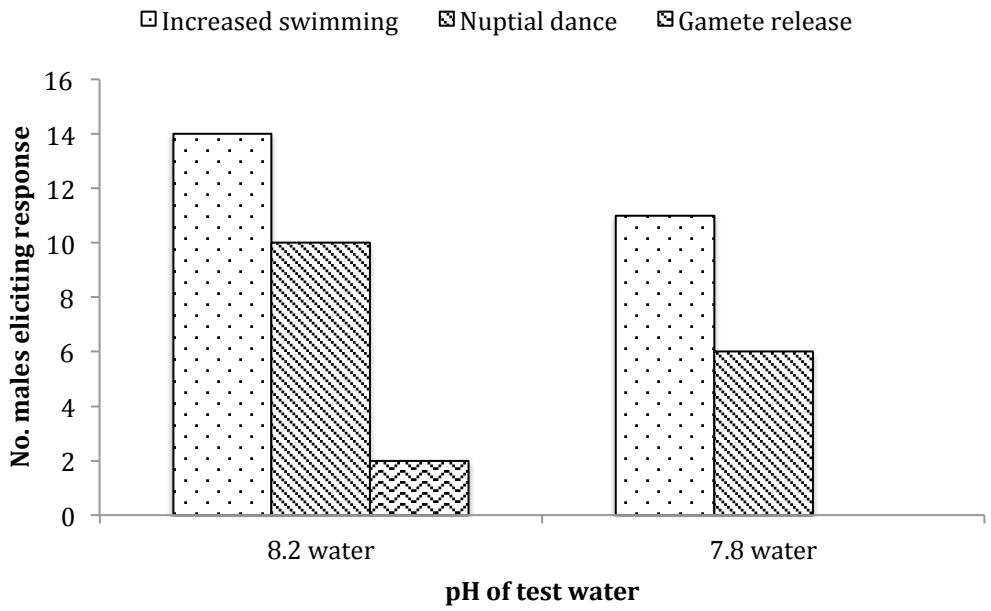

Figure 3.4: Behavioural response of control cultured (pH 8.2) male *N. succinea* after stimulation with 10µl of 10^{-6}M CSSG in control (pH 8.2) and CO2 enriched (pH 7.8) conditions. All three characteristic behaviours (increased swimming, the nuptial dance and gamete release) were examined. N= 15. Paired data. No significant reduction in no. males eliciting a positive response in acidified conditions for all three behaviours (McNemars test, $p>0.05$).

3.3.2 Impact of acute exposure to ocean acidification on pheromone dependent gamete release threshold in male *Nereis succinea*

The amount of 10^{-6}M CSSG (female pheromone) required to induce gamete release in male *N. succinea* was greater in CO_2 enriched treatment (pH 7.8) compared to control treatment (pH 8.2) (Figure 3.4). Response was consistently higher in pH 8.2 water compared with acidified pH 7.8 water, however this difference was not significant for all quantities of pheromone tested (McNemars test, $p > 0.05$). Data was binomial and paired as the same worms were tested in both conditions to see if individual response improved/deteriorated according to test condition, justifying the use of the McNemars statistical test (variation of chi-square suitable for repeated measures data).

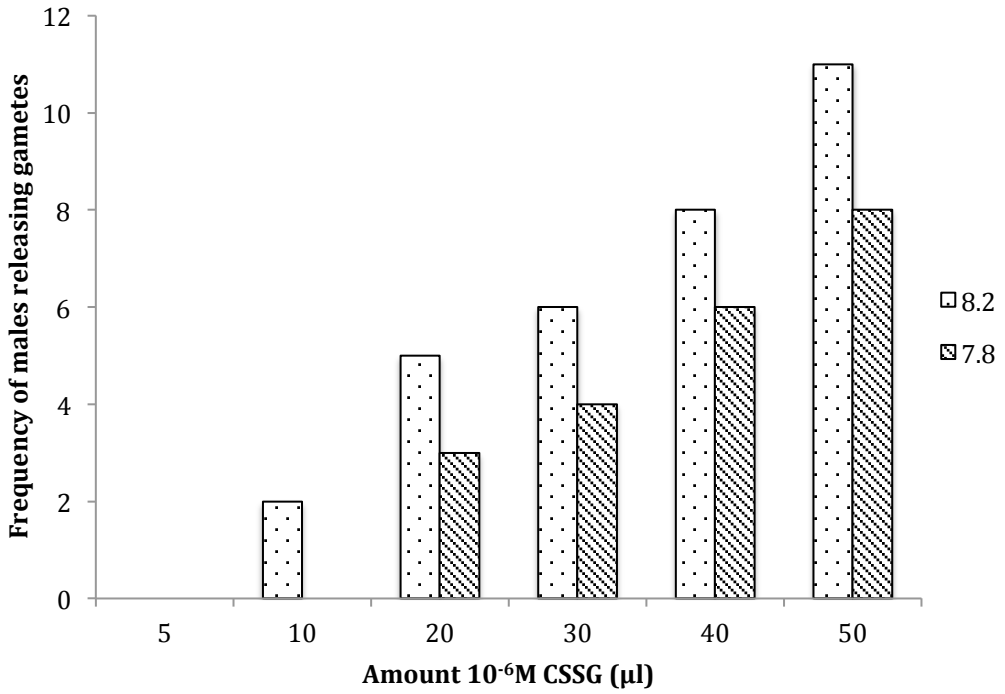

Figure 3.5: Amount of 10^{-6} M CSSG required to induce gamete release in control (pH 8.2) cultured *N. succinea* when stimulated in control (pH 8.2, dotted bars) and CO_2 enriched (pH 7.8, striped bars) seawater conditions. Paired data. N = 15. No significant difference in number of males capable of gamete release between seawater treatments at each 10^{-6} M CSSG quantity (McNemars test, $p > 0.05$).

3.3.3 Impact of acute exposure to ocean acidification on ability of immature adult *Nereis succinea* to respond to feeding cues

Response of control cultured (pH 8.2) adult *N. succinea* to 3 different feeding cues (glycine, taurine and fish food) was reduced in CO_2 enriched (pH 7.8) seawater in comparison to control (pH 8.2) (Figure 3.6). Data was binomial and paired as the same worms were tested in both conditions to see if individual response improved/deteriorated according to test condition. McNemars test was used to test whether a significant proportion of individuals changed their response from positive to negative when tested in acidified conditions (pH 7.8). The result showed a significant amount of individuals changed their response from positive (in pH 8.2) to negative (in pH 7.8) (McNemars test, $p < 0.05$) for all 3 feeding cues.

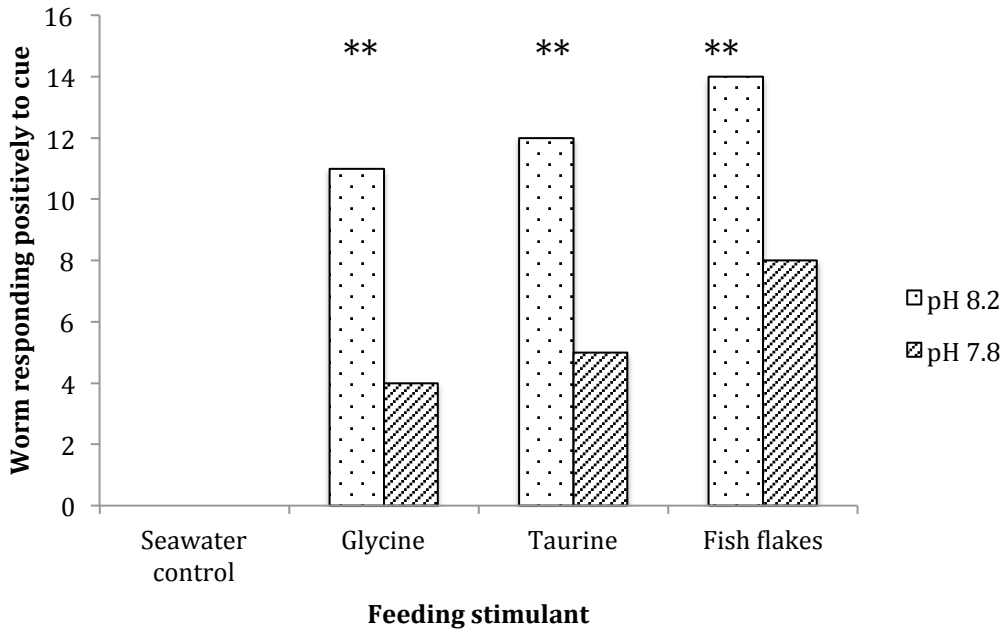

Figure 3.6: Number of adult *N. succinea* positively displaying detection behaviours when presented with feeding stimulant in pH 8.2 seawater (dotted bars) and in pH 7.8 seawater (striped bars). Paired data. N = 15 per cue (total 45). ** Indicates significant proportion of individuals changed their behaviour from a positive (in pH 8.2 treatment) to negative (in pH 7.8 treatment) response (McNemars test, $p < 0.05$).

3.4 Discussion

3.4.1 Impact of acute exposure to ocean acidification on chemical communication

Responses to both single and multicomponent feeding stimuli were depressed when control cultured (pH 8.2) *N. succinea* were presented with feeding stimuli in CO_2 enriched (pH 7.8) seawater (Figure 3.6). Feeding experiments with the related polychaete species, *Platynereis dumerilli,* drew similar conclusions showing reduced feeding response to both single amino acids and food extract in seawater enriched with CO_2 (pH 7.8). Furthermore, a recent study on the impact of pH on the feeding response of intertidal hermit crabs (*Pagurus bernhardus*) concluded similar findings (de la Haye, *et al.,* 2012). *P. bernhardus* presented with fish extract as a feeding stimulant displayed reduced levels of feeding response (reduced antennule flicking, increased time to locate food source and less contact time with food source) in acidified seawater (pH 6.8) than when food was presented in control seawater (pH 8.2). However, acidification in the de la Haye, *et al.,* (2012) study was very extreme in comparison to the conditions used in this study, which are appropriate to OA conditions expected in the next 100 years.

Several aspects of behavioural response by males to the female sex pheromone, CSSG, were reduced but these reductions were not found to be significant (Figure 3.4). Acute exposure to CO2 enriched conditions (pH 7.8) did not significantly affect the incidence of males increasing swim speed (by 50% or more) in comparison to control (pH 8.2) conditions (Figure 18) However, previous experiment where an alternative experimental design was used, showed that the actual swim speed (mm/s) of control males stimulated with 3ul 10^{-6} M CSSG in pH 7.8 seawater was significantly less than when the same males were stimulated with the same cue in pH 8.2 seawater.

The amount of 10^{-6} M CSSG required to induce gamete release in males was greater when the cue was presented in pH 7.8 seawater in comparison with

pH 8.2 control seawater (Figure 3.5), however, this difference was also found to be insignificant. Nuptial behaviours, including increased swimming activity and the 'nuptial dance' induced by low dose ($10^{-6/7}$M) CSSG are key to ensure sexual partners become close to one another during the reproductive event (Ram, *et al.,* 2008). Similarly, adequate gamete release, induced by higher doses ($10^{-4/5}$ M) of the same pheromone (Hardege, *et al.,* 2004), is critical to the success of the reproductive event. From the findings of this study and others before, it seems unclear to what extent the reception of sex pheromones is disrupted by acute exposure to OA conditions in this species. These conflicting conclusions may infer variability in the way individuals respond to pH stress with regards to chemical reception and any such plasticity may suggest adaptation potential. Further study is imperative to clarify this.

3.4.2 Mechanisms of disruption to chemical signaling

It has been identified that to some extent, the behavioural response to both sex pheromones and more so feeding stimulants (Figure 3.5) in *N. succinea* is disrupted by acute exposure to reduced pH (7.8), CO_2 enriched seawater predicted to occur in our oceans in the next 100 years. The next logical step in assessment of chemical communication in this species is to ask why such disruption occurs.

Chemical communication can be disrupted at many stages in the signaling process: signal production, transmission, reception, transduction and even physical ability to perform to appropriate response. De la Haye, *et al*. (2012), propose 4 main mechanisms by which chemical reception in the marine environment may be disrupted by acidification:

1. pH driven change in ionic state of odour molecules to disrupt receptor-ligand interactions,

2. Alteration in charge distribution of the odour receptors to disrupt receptor-ligand interactions,
3. Physical damage to sensory organs
4. Reduced motivation as a result of increased metabolic load associated with maintaining acid-base balance in low pH conditions

Points 1 and 2 are most appropriate to the results presented in this chapter as all experiments involved acute exposure (1 minute maximum) to low pH only. It is therefore unlikely that exposure was long enough to physically damage the individuals used or induce major challenges to acid-base regulation over such a short period.

All chemical cues, both pheromones and environmental odour (such as feeding cues) must be transmitted from the releaser and bind successfully to chemical receptors within the receiver. Such receptors are structurally specific to receive and bind with the appropriate signal molecule (Hardege, *et al.,* 2011) and alteration in the structure of either the receptor or signal molecule may therefore prevent successful receptor-ligand binding (de la Haye, *et al.,* 2012). As a consequence, appropriate behavioural responses may be altered or not be activated at all.

The chemoreceptors in male *N. succinea* responsible for detecting the female sex pheromone, are positioned on cirri on the modified parapodia to the front and rear of the body (Hardege, 1999) and are present in adult worms only after metamorphosis occurs. The exact receptor structure and method of transduction in marine polychaetes is currently poorly understood (Lindsay, 2009). Chemoreceptors typically consist of a membrane bound receptor proteins which, when bound to signal molecules, initiate a series of internal events leading to either a developmental or, in this case, a behavioural response (Wyatt, 2003). Sufficient changes in the pH of the liquid medium in which chemical molecules exist, may affect the charge distribution of such molecules causing alterations in overall charge and hydrogen content according to the acid dissociation constant (pKa) of the

molecule affected (Hardege, *et al.,* 2011). Such isoelectric changes can ultimately affect the shape and structure of molecules, including those, which act as sensory cues. Alteration in shape or partial charge of a peptide pheromone molecule may prevent the successful binding of the signal molecule to the corresponding receptor, ultimately leading to interference with the associated behavioural response.

Similarly, pH change may modify charge distribution and structure in the protein receptors responsible for detecting signal molecules. Even if the integrity of the signal molecule itself is conserved, structural changes in the receptor protein, particularly around the active binding site may also interfere with reception. The extent to which the efficiency of such receptors may be altered is largely dependent on the pKa of ionizable groups around the area of the active site of the receptor protein (Tierney and Atema, 1988). If such groups are susceptible to ionization within the pH change presented (here 8.2 – 7.8), changes in charge at the binding site may repel the cue ligand or structural change may prevent it from binding successfully (Xu, *et al.,* 2010). Such alteration in protein receptor structure as a result of pH manipulation was shown experimentally by Xu, *et al.,* (2010) who examined the effect of pH on the structure of a pheromone binding protein (AtraPBP1) positioned on the antennae of the navel orange worm moth (*Amyebis transitella*). The study concluded that at reduced pH (pH 4.5) a C-Terminal helix forms within the receptor structure and effectively blocks the binding site where the pheromone attaches. Subsequently, this prevents the appropriate behavioural response from being activated.

CSSG is a peptide pheromone (Figure 1.4) with an acid dissociation constant (pKa) of 8.6. When in solution of pH lower than this value, the molecule becomes subject to isoelectric changes and subsequently conformational change. These changes in molecular shape have been determined and modeled using NMR techniques, illustrating how CSSG changes shape dramatically when at pH 7.8 compared with current oceanic pH of 8.2 (Figure 3.7). Reduction in pH facilitates the formation of hydrogen bonds

within CSSG, transforming the molecule from the regular 'T' shape to a more ball shaped one. As discussed, such changes may pose difficulties for the molecule to successfully bind to receptors and induce the sexual behaviours associated with CSSG in male *N. succinea*.

Interference by conformational change of either receptor or cue ligand is not exclusive to peptide pheromones such as CSSG. The amino acids used (glycine and taurine) in feeding experiments also have pKa values in a region sensitive to pH reduction of seawater caused by near future OA (9.60 and 9.06 respectively). It is suggested that the majority of info chemicals in the marine environment that have been identified to date are potentially susceptible to pH dependent structural change (Hardege, *et al.,* 2011). Such chemicals are responsible for a vast range of survival behaviours associated with sensing the surrounding environment such as predator detection and evasion, homing and settlement, food detection and discrimination, mate choice and reproductive behaviours. This highlights the importance of research in this area to assess the extent to which communication via chemical means in marine systems may be impacted by near future OA.

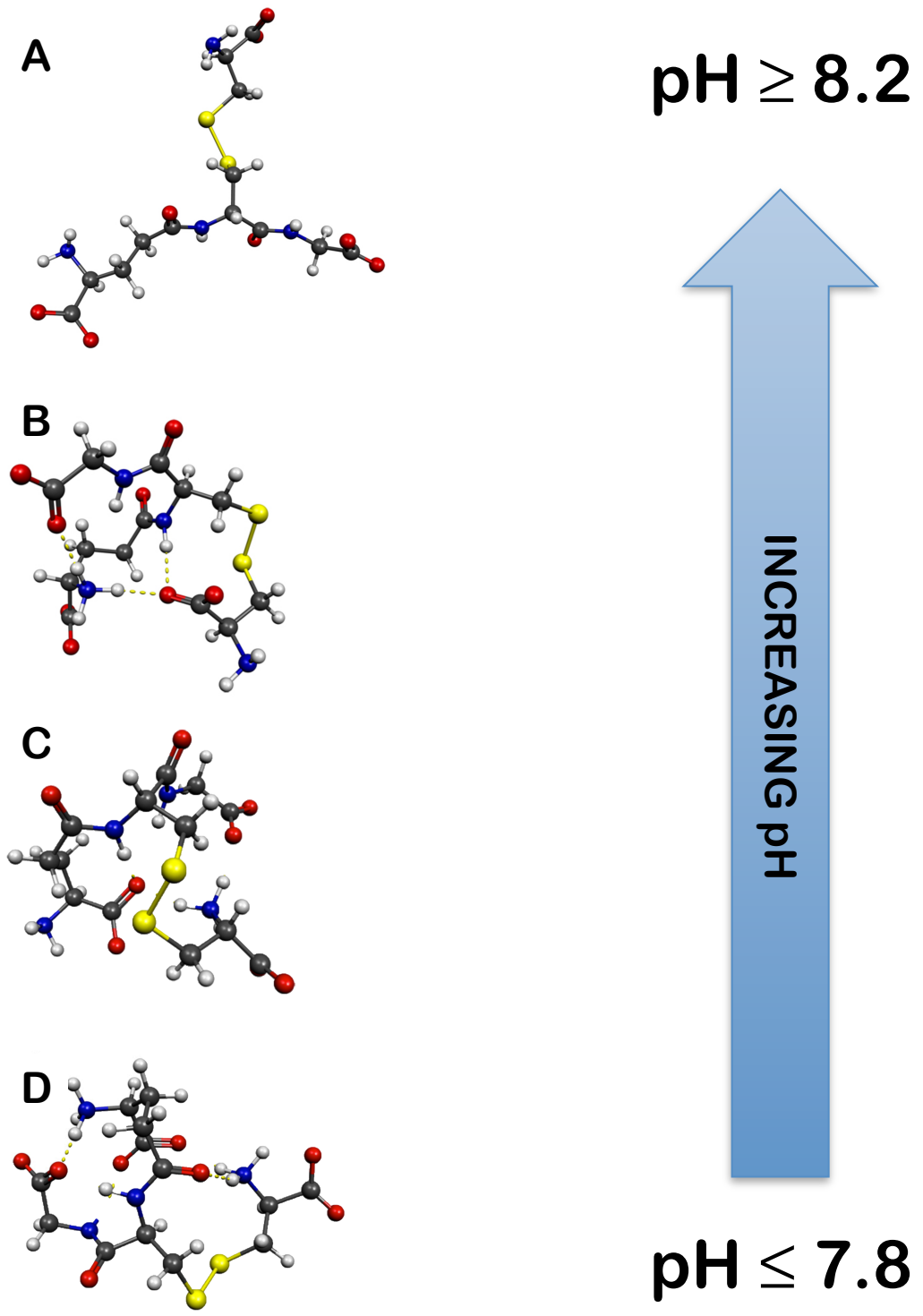

Figure 3.7: Ball-and-stick diagrams of the four protonation states of the CSSG peptide, optimised at the B3LYP-D/6-31+G(d,p) level of theory. Molecule A neither groups are protonated, in molecule B only the glu–NH2 is pro-tonated, in molecule C only the cys–NH2 group is protonated and in molecule D is fully-protonated. Arrow indicates how the protonation state and subsequent structure of the molecule (CSSG) changes according to increasing pH. (Molecular modeling diagrams courtesy of Dr. David Benoit).

It has recently been proposed that olfactory impairment in marine organisms as a result of OA may be due to interference with neurotransmitter function (Nilsson, *et al.,* 2012). Rearing clownfish (*Amphiprion percula*) in CO_2 enriched seawater during larval stages impacts olfactory discriminatory ability (Munday, *et al*., 2009b) causing association with predator odour when given a choice. Nilsson, *et al*. (2012), showed that this uncharacteristic olfactory association behaviour could be reversed by exposing *A. percula* reared in OA conditions to gabazine for 30minutes prior to choice tests. Gabazine is a highly specific antagonist of GABA-A receptors in neural tissues. These receptors are responsible for maintaining correct ion gradients over neural membranes to ensure correct neural activity. Reversed behaviour in larval fish in response to exposure to OA conditions is thought to be a result of high levels of CO_2 interfering with GABA-A receptor function to cause shifts in sensory preferences (Nilsson, *et al.,* 2012). Correction of olfactory impairment following exposure to the GABA-A receptor antagonist, gabazine, indicates that altered GABA-A receptor activity is responsible for the effects of high levels of CO_2 on neural function.

Chapter 4:
Examination of the acclimatization potential of chemical signaling in *Nereis succinea* to ocean acidification

4.1: Introduction

Initial studies on the topic of OA involved exposing organisms to reduced pH conditions only for relatively short periods (Dupont*, et al.,* 2010). More recently, such methods have seen criticism owing to the fact that acute exposure is not realistic to the challenges these organisms would face in natural ecosystems affected by OA, leading to new, more long term experimental techniques to be recommended (Fabricus*, et al.,* 2011). The main issues raised relate to experimental method causing 'pH shock' in test organisms, ignorance of the effects of long-term exposure to OA and overlooking whether organisms may have the capacity to acclimatize or adapt. Although relatively few past studies have used methodology that incorporates long-term exposure to future conditions of OA (Dupont*, et al.,* 2010), many recognize and state its importance in gaining realistic and reliable conclusions on the subject.

The most recent and developed technique of studying the impact of OA on marine ecosystems is the use of naturally occurring areas of CO_2 enriched waters (Riebesell, 2008, Hall-Spencer, *et al*., 2008, Kerrison, *et al.,* 2011) across the globe. Depending on the proximity to vents, a range of 'natural laboratories' (Kerrison, *et al.,* 2011) with varying levels of pCO_2 have been identified and are beginning to be put to use in investigating long term affects of OA *in situ*. Such sites have already been useful in the study of community structure in habitats that have been exposed to reduced pH and carbonate chemistry associated with OA for often thousands of years. Extensive use of areas of submarine volcanic activity around the Italian island of Ischia has also raised new insights into the study of OA (Hall-Spencer*, et al*., 2008). Typically, volcanically acidified sites in this area show a reduction in calcifying species such as coralline algae, sea urchins and stony corals (Reibesell, 2008) however, further examination of settlement in non calcifying benthic organisms is conflicted. Cigliano, *et al.,* (2010) concluded that recruitment of several taxa including serpulid polychaetes,

gastropods, was reduced in CO_2 enriched areas, whereas more recently Kroeker, *et al.,* (2011) found some polychaete species were more abundant in low pH zones. There are also technical constraints of conducting experiments *in situ* calling for the development of laboratory technique to assess acclimation and adaptation potential in marine species. These issues are addressed as best as possible in this project by culturing *N. succinea* from juvenile to mature heteronereid stage (one complete life cycle) in both control and CO_2 enriched conditions to examine the impact such long term exposure to conditions associated with OA may have on chemical communication in the species. Shorter, but nevertheless extended in comparison to much published literature, exposure to OA experiments were also conducted on the ability of immature adults to detect various food sources and dig successfully after prolonged (32day) exposure to CO_2 enriched, reduced pH conditions.

It should be noted that the current study examines only the ability of *N. succinea* to acclimatize to conditions associated with OA and not the genetic adaptation potential. These are two separate and distinguished terms where acclimatization refers to the usually reversible adjustment of physiology to changing environmental factors (Hofmann, *et al.,* 2010), whereas true adaption results in a genetic change in the population due to natural selection which leads to improved function with respect to some aspect of the environment (Hofmann, *et al.,* 2010).

Increased pCO_2 and reduced pH may affect *N. succinea* in a number of ways. Physiology and acid-base regulation may be impacted leading to reduced physical fitness and reallocation of effort to different processes, as seen in brittle stars which display upregulation of calcification and metabolic rate in response to OA, but suffer muscle wastage as a consequence (Wood, *et al.,* 2008). With regards to chemical communication, both signal production and reception may be impacted. Here, only reception will be examined both of environmental cues (feeding stimulants) and *N. succinea's* own sex pheromones.

4.1.1 Hypothesis

Long-term (4-6month) exposure of *N. succinea* to CO_2 enriched seawater (pH 7.8) will negatively impact reception sex pheromones (CSSG). Typical physical behaviours indicating recognition of CSSG (including increased swimming, nuptial dance and gamete release) will be less pronounced or not exhibited at all.

Medium-term exposure (32 days) of *N. succinea* to CO_2 enriched seawater (pH 7.8) will negatively impact reception of chemical feeding cues (taurine, glycine and fish food extract). Typical physical behaviours indicating recognition of feeding cues (orientation toward food source, flaring of palps) will either be less pronounced or not exhibited at all.

4.2: Methodology

4.2.1 Effect of prolonged exposure to ocean acidification conditions on chemical detection of feeding cues

In Chapter 2 the ability of *N. succinea* to retain the ability to detect a variety of feeding signals when acutely exposed to conditions of near future OA was addressed (Figure 3.6). In this chapter, these experiments are taken further by examining whether the ability to detect food is improved after longer term exposure to CO_2 enriched, reduced pH conditions.

The methods in this experiment followed exactly the procedure outlined in section 3.2.1, which assessed impact of acute exposure to OA conditions on behavioural response to a variety of feeding stimulants. To assess the possible impacts associated with longer-term exposure to OA conditions, this experiment involved adult *N. succinea* being cultured for a longer period in pH 7.8, CO_2 enriched seawater and retested at time intervals over 32 days. The same 3 feeding cues were used (glycine, taurine and fish food) in the same concentrations as previously (Table 3.1). The worms used were the same as those in section 3.2.1, as this experiment is a continuation to assess whether initial impacts on food detection caused by acute exposure to OA conditions improve or deteriorate over time.

The first feeding trial carried out in pH 7.8 seawater (section 3.2.1) was allocated as T0, as this was the first time worms were introduced to low pH conditions. From that point forward, worms were cultured in their individual tanks in seawater maintained constantly at pH 7.8 (pH checked daily, water changed fully twice weekly) by addition of CO_2. All worms were retested 2 days later and then subsequently on days 4, 8, 16 and 32 after addition to culture. Worms were fed twice weekly with ground and rinsed Aquarian Tropical Fish Flakes during the 32 day experimental period but, as previously (section 3.2.1), were always starved for 2days prior to the commencement of any feeding trials to standardize hunger levels and

therefore minimize any influence this may have on level of behavioural response during feeding trials. Please refer back to section 3.2.1 for full details of experimental procedure with regards to feeding stimulant administration and behavioural observation.

4.2.2 Effect of long-term exposure to ocean acidification conditions on pheromone reception

The effect of acute exposure to conditions associated with OA on the ability of mature male *N. succinea* to detect and respond to the female sex pheromone CSSG was assessed in section 3.2.2. This experiment continues on this theme with the aim to determine whether males exposed to OA conditions from the juvenile stage up until maturation (4-6months) are able to perform as well as counterparts cultured in control conditions (pH 8.2) with respect to sex pheromone reception. The results aim to clarify whether poor reception when acutely exposed to OA may be a result of pH 'shock' and give an indication of whether *N. succinea* may potentially be able to acclimatize to OA after long term exposure.

Unless otherwise stated, the methods in this experiment follow exactly the procedure outlined in section 3.2.2, which assessed impacts of acute exposure to OA conditions on behavioural response of males to the female sex pheromone, CSSG. Here, 15 males cultured for 4-6months in pH 7.8, CO_2 enriched conditions were tested for their level of behavioural response to CSSG according to the procedure outlined in 3.2.2. All pH 7.8 males were stimulated with pheromone in seawater of equal chemistry to the culture of origin (CO_2 enriched to pH 7.8). Culture was from juvenile stage to maturation (4-6months, see section 2.4.2 for details). As control cultured males were tested in control conditions (stimulation with pheromone carried out in pH 8.2 seawater) the previous chapter (section 3.2.2) and this experiment follows those methods exactly, the results from pH 7.8 cultured males assessed in this experiment were compared with those of control male (pH 8.2) from section 3.2.2.

4.2.3 Data handling

All data was analyzed using IBM SPSS (Version 19). Details of the exact statistical analysis used in individual experiments, including the outcome, can be found in the results section (figure legends and accompanying paragraphs).

Experiments investigating response to sex pheromone produced unmatched, binomial data (collected from two independent samples: pH 8.2 control cultured males and pH 7.8 cultured males) and was therefore analyzed using a chi-square test for association.

Experiments investigating response to feeding cues (matched pair data) were analysed using the McNemars test. This is a variation of Chi-square suitable for matched pair experimental design and shows whether the outcome of such paired data sets differ significantly. Feeding experiments in this chapter involved ranking behaviour of control cultured worms as occurring positively or negatively after stimulation with a feeding cue. The same experiment was the repeated with the same sample in CO_2 enriched water and at several time points to provide a paired data set. McNemars test was therefore used to assess whether behaviour changed significantly over time following addition of sample individuals to pH 7.8 conditions.

4.3 Results

4.3.1 Impact of long-term exposure to ocean acidification conditions on ability of male *Nereis succinea* to detect and respond to female sex pheromone

Male *N. succinea* from the control culture (pH 8.2) and CO_2 enriched culture (pH 7.8) were stimulated with 10µl of 10^{-6}M CSSG in seawater of pH equivalent to the culture of origin. Data was not paired (15 males from each culture were used, 30 in total) therefore a Chi-square test for association was used to test for differences in behavioural response according to culture origin. Each behavioural response (increased swimming, 'nuptial dance' and gamete release) was statistically tested separately. For all 3 behavioural responses, no significant difference in level of response between treatments was observed ($p > 0.05$) (Figure 4.1).

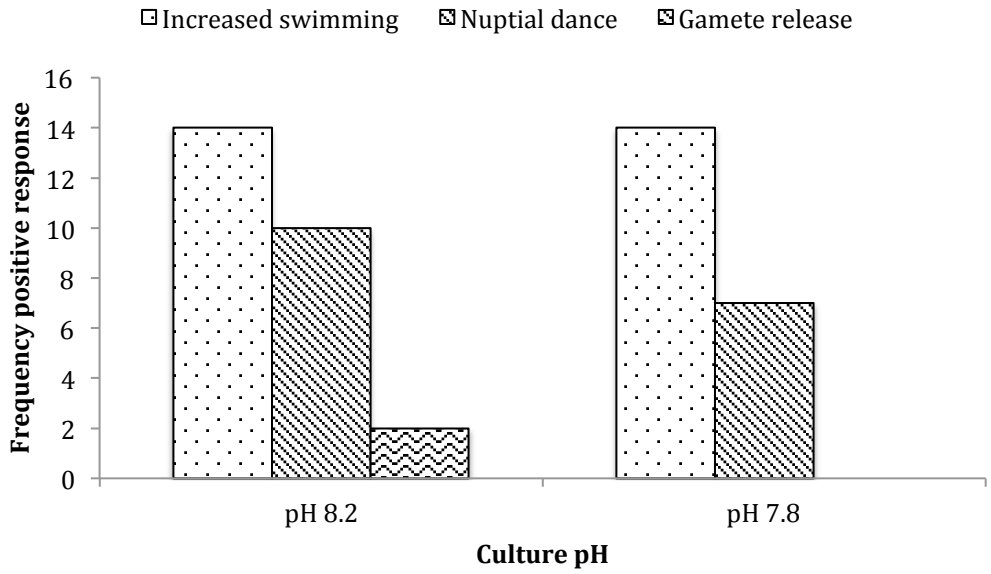

Figure 4.1: Degree of behavioural response exhibited by mature male *N. succinea* when stimulated with 10µl 10^{-6} CSSG (in 20ml seawater). Males were cultured from juvenile stage to maturity (4-6months) in either control conditions (pH 8.2) or CO_2 enriched test conditions (pH 7.8) prior to behavioural studies. N = 15 per culture. Chi squared test for association showed no statistical difference in behavioural response between cultures for all 3 behaviours ($p > 0.05$).

4.3.2 Impact of long-term exposure to ocean acidification conditions on gamete release success of male *Nereis succinea* when exposed to varying quantities of female sex pheromone

The amount of 10^{-6} M CSSG (female pheromone) required to induce gamete release in control cultured (pH 8.2) male *N. succinea* was less that that required to elicit gamete release males from inCO_2 enriched (pH 7.8) culture (Figure 4.2). Data was not paired (15 males from each culture were used, 30 in total) therefore a Chi-square test for association was used to test for differences in behavioural response according to culture origin. Response was consistently higher for pH 8.2 cultured males compared with pH 7.8 cultured males for all quantities of pheromone tested, however this difference was only significant for when stimulated with 30µl and 50µl of 10^{-6} M CSSG (Pearson Chi-square test, $p < 0.05$).

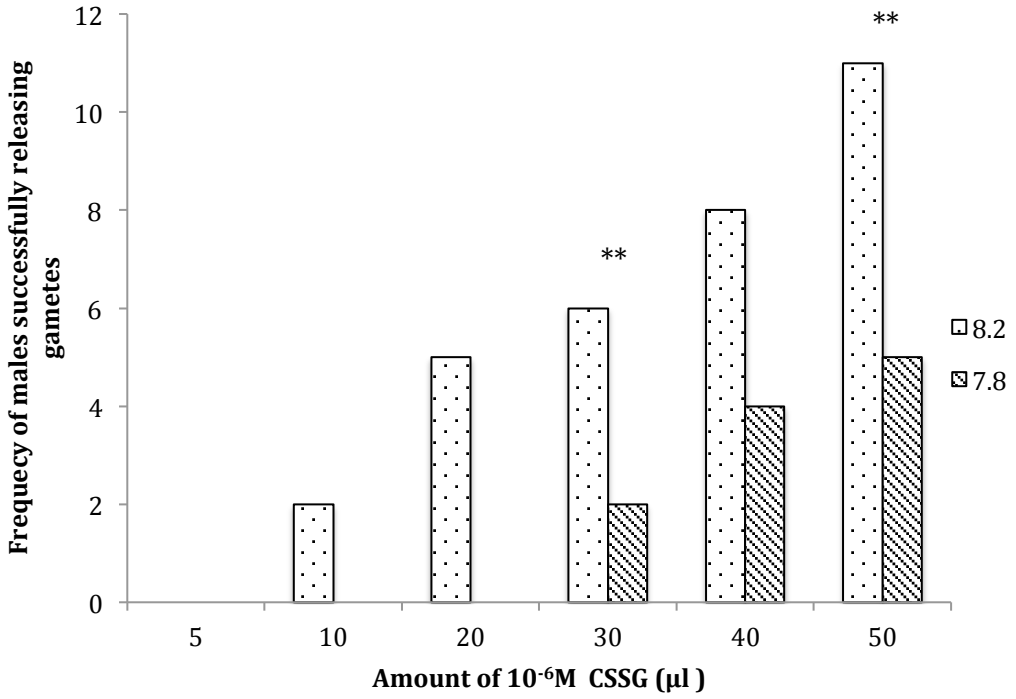

Figure 4.2: Frequency of male N. succinea successfully releasing gametes stimulated with varied amounts of 10^{-6} M CSSG. Dotted bars indicate males cultured from juvenile stage to maturity (4-6months) in control conditions (pH8.2) while striped bars indicate males cultured from juvenile stage to maturity in CO_2 enriched conditions (pH7.8). N=15 per culture. ** Indicates a significant difference in response between cultures (Chi square test for associations, sig. <0.05).

4.3.3 Impact of prolonged exposure to ocean acidification conditions on ability to detect feeding cues in *Nereis succinea*

Depressed feeding response to three different feeding cues (fish food, glycine, taurine) by control cultured adult *N. succinea* was maintained over a 32 day period after addition to CO_2enriched (pH 7.8) culture. Data was paired and binomial, therefore the McNemars test was used to test whether a significant proportion of individuals changed their response from positive to negative at 6 time points over a 32 day period after addition to pH 7.8 culture in comparison to when tested in control conditions (pH 8.2). Response was significantly less than in control conditions at all the time points tested during the experiment for all three cue types: fish food (Figure 4.3), glycine (Figure 4.4) and taurine (Figure 4.5).

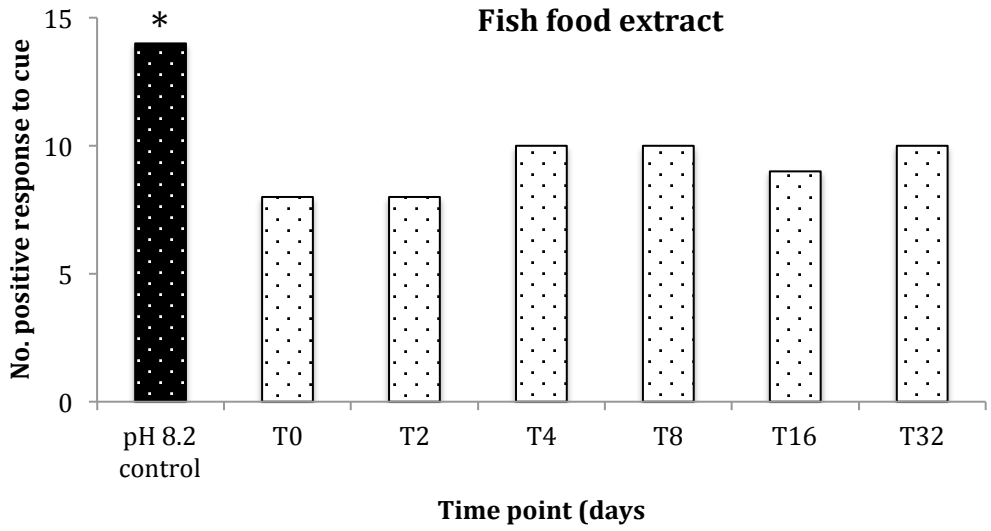

Figure 4.3: Number of adult *N. succinea* (N= 15) responding positively when presented with fish food feeding cue at several time points after addition to CO_2 enriched culture (pH 7.8). T0 indicates trial conducted immediately after addition to culture (internal control). McNemars test showed no significant improvement in response at any point across the data series ($p > 0.05$, response in internal pH 8.2 control was significantly greater than response at every time point after addition to pH 7.8 culture, indicated by *).

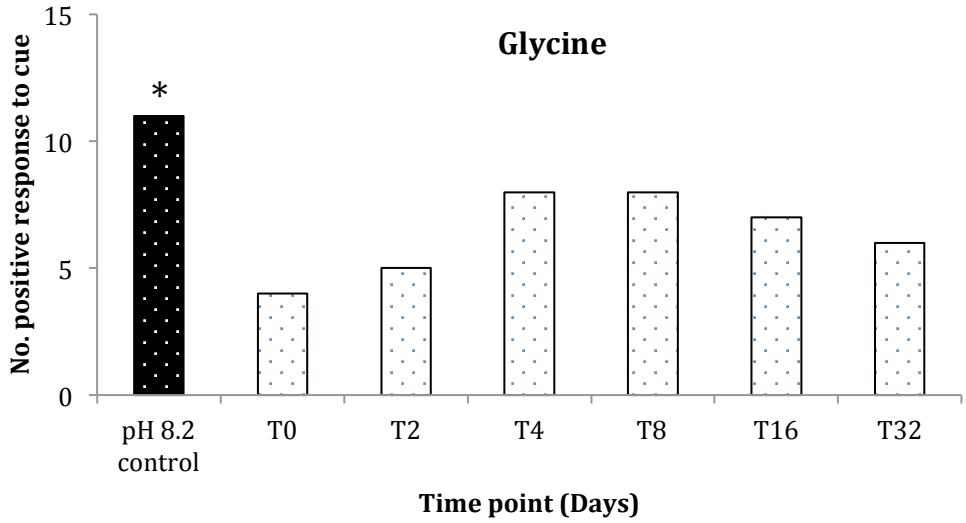

Figure 4.4: Number of adult *N. succinea* (N= 15) responding positively when presented with glycine feeding cue (50µl, 10^{-3}M) at several time points after addition to CO_2 enriched culture (pH 7.8). T0 indicates trial conducted immediately after addition to culture (internal control). McNemars test showed no significant improvement in response at any point across the data series ($p > 0.05$, response in internal pH 8.2 control was significantly greater than response at every time point after addition to pH 7.8 culture, indicated by *).

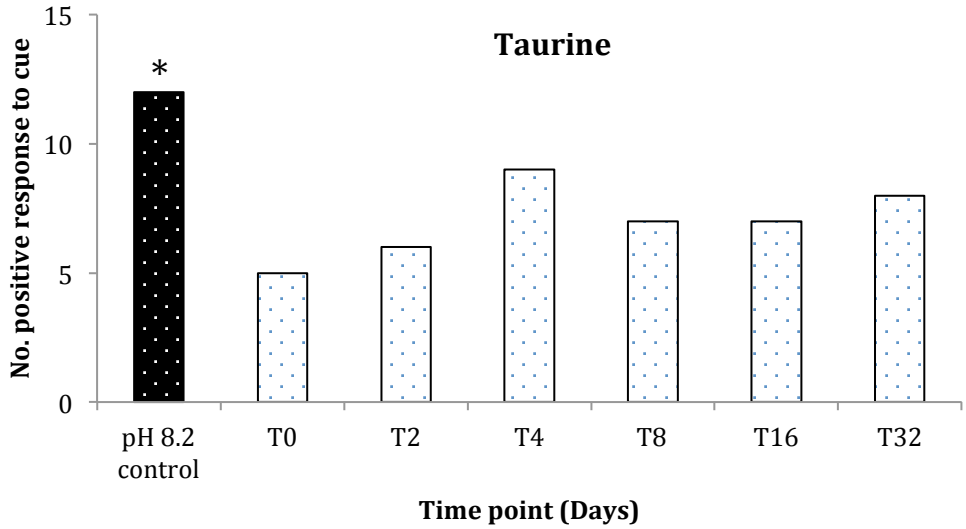

Figure 4.5: Number of adult *N. succinea* (N= 15) responding positively when presented with taurine feeding cue (50µl, 10^{-3}M) at several time points after addition to CO_2 enriched culture (pH 7.8). T0 indicates trial conducted immediately after addition to culture (internal control). McNemars test showed no significant improvement in response at any point across the data series ($p > 0.05$, response in internal pH 8.2 control was significantly greater than response at every time point after addition to pH 7.8 culture, indicated by *).

4.4 Discussion

4.4.1 Effect of prolonged exposure to ocean acidification on response to feeding cues in *Nereis succinea*

Reduced feeding response in low pH has been observed in both freshwater fish (Lemly and Smith, 1986) and intertidal invertebrates (de la Haye, *et al.,* 2012). In chapter 3, it was determined that acute exposure to pH 7.8 CO_2 enriched seawater caused a significant disruption to the ability of *N. succinea* to detect a selection of food sources (Figure 3.6). It was deemed important to assess whether this depressed response to feeding cues is maintained over prolonged exposure to low pH to determine whether organisms may have the capacity to acclimatize with regards to food detection. The experiments in this chapter confirmed that no marked improvement in response was shown to any of the feeding cues, either to single amino acids (glycine and taurine) or food extract (fish food) where feeding response of *N. succinea* was assessed at intervals over a 32 day culture in CO_2 enriched, pH 7.8 conditions (Figures 4.3-4.5).

The sustained depressed response to feeding cues over a 32 day period by *N. succinea* makes sense as reduced response to the feeding cues used is likely due to pH driven structural change in the amino molecules (see section 3.5.2: Mechanisms of disruption to chemical signaling). The cues used (glycine and taurine) have an acid dissociation constant (pKa) in a range that is susceptible to pH change from pH8.2- 7.8, therefore potentially preventing food odour from correctly binding to receptors to elicit a feeding response. If this is the case, even with time for the animals to acclimatize to low pH conditions, conformational change in feeding stimuli molecule will be sustained and therefore the detection of chemical feeding stimuli will still be disrupted.

Most marine organisms are likely to be more susceptible to pH fluctuation in comparison to freshwater organisms due to the high buffering capacity of

seawater providing a relatively unchanged environment for many years (Feely, *et al.,* 2009). However, pH driven reduction in feeding response in freshwater fish has also been observed (Lemly and Smith, 1986). Such findings illustrate how sensitive reception of chemical cues may be in response to pH fluctuations, even in a variable medium with respect to pH where organisms would be expected to be more robust to variable pH. There is no published experimental evidence of the effect of long-term exposure to near future levels of OA on the ability of marine organisms to detect feeding stimulants, making it difficult to assess to what extent marine organisms are impacted and whether they have any capacity to acclimatize. Further study is required in this area to make more informed conclusions.

4.4.2 Effect of long-term exposure to ocean acidification on response to sex pheromones in *Nereis succinea*

Male *N. succinea* display very specific sexual behaviours in response to the concentration of female sex pheromone they are exposed to (Hardege*, et al.,* 2004). These behaviours are distinctive and easy to observe and therefore could reliably be utilized to determine to what degree males responded to CSSG and whether long-term culture in a CO_2 enriched environment impacted this response in any way. When stimulated with 10µl of 10^{-6} M CSSG in 20ml of seawater, no significant differences in behavioural response were observed between control and test cultures, indicating long-term culture in conditions simulating 2100 OA levels had no effect on sex pheromone reception (Figure 4.1).

The amount of CSSG required to induce increased swimming speed and nuptial behavior in natural systems is relatively low and varies between individuals (Hardege, 1990). Gamete release is triggered only when a threshold of higher concentration CSSG ($10^{-4/5}$ M) is reached. It is vital that male and female are in very close proximity to one another before gametes are released into the surrounding water to maximize chances of fertilization.

Males will therefore only release gametes when the concentration of female sex pheromone is high enough to indicate she is very close by (Ram, *et al.*, 2008).

When examining the amount of pheromone required to elicit successful gamete release there were significant differences in response between control and test cultures (Figure 4.2). Consistently, a greater proportion of males cultured in control conditions released gametes when presented with increasing amounts of 10^{-6} M CSSG (Figure 4.2). However, a chi square test for association showed the frequency of control males releasing gametes to be significantly greater than their OA cultured counterparts only when stimulated with 30µl and 50µl 10^{-6} M CSSG. This result indicates that long-term exposure (equivalent to one life span) may cause problems with response to pheromones (based either transmission or reception of CSSG). This may potentially disrupt the coordination of the reproductive event by increasing the amount of female sex pheromone required to elicit gamete release in males. The finding also demonstrates that acclimatization to CO_2 enriched seawater (pH 7.8) over a single life span does not occur.

Reduction in the level of responses to sex pheromone exposure following long-term culture in CO_2 enriched conditions may, however, also be due to poor fitness. It is possible that male *N. succinea* are able to receive the signal, but cannot act upon on it behaviourally due to impaired physical ability resulting from prolonged exposure to low pH. Preliminary studies, found that male *N. succinea* cultured for 12 weeks in pH 7.8 seawater displayed significantly lower swimming speed (mm/s) when stimulated with low dose CSSG in pH 7.8 seawater, in comparison to pH 8.2 cultured counterparts tested in pH 8.2 seawater. Furthermore, response in pH 7.8 cultured males did not improve when stimulation was carried out in pH 8.2 seawater where the structural integrity of the CSSG molecule would be maintained and therefore the cue would be expected to be detected and responded to. This finding suggests that reduced physical fitness may be in part responsible for poor behavioural response to sex pheromones in *N. succinea* exposed OA

conditions for long periods. By assessing the swimming activity prior to and after pheromonal stimulation, previous studies have confirmed males cultured in pH 7.8 could detect CSSG to some extent. Although not as pronounced as their pH8.2 counterparts, the results showed pH 7.8 cultured males significantly increased swimming speed in response to pheromonal stimulation, confirming the cue was perceived.

Although low pH affected the threshold amount of pheromone required to induce ejaculation of gametes (Figure 4.2), all males used in this study had the ability to release gametes in response to sex pheromone regardless of culture of origin. This was verified by using very high dose (5µl 10^{-4} M) CSSG to induce ejaculation to ensure failure to release gametes was indeed due to failure to respond to CSSG and not due to physical incapability (Figure 3.1). Even if low pH does impair reception of chemical cues, using a high enough dose of stimulant may still elicit the desired response despite the signal molecule not being in equilibrium with regards to ionization at low pH. Molecular charge distribution alters on a sliding scale as pH decreases (or increases) meaning although most molecules will change structurally as a result of pH driven redefinition in charge distribution, a small percentage of molecules will still possess the correct conformation to bind successfully to receptors. Such a high dose of pheromone would not be produced by females in natural systems therefore, although theoretically males can respond to concentrated pheromone, if they cannot behave correctly in response to naturally occurring doses of CSSG reproductive success may still be compromised. This is demonstrated by pH 7.8 cultured males requiring 2 ½ times the amount of CSSG than pH 8.2 counterparts to give the same level of response with respect to gamete release (Figure 4.2).

4.4.3 Potential for chemical communication systems to adapt to changing ocean chemistry

A main concern associated with future OA forecasts is to what extent marine species may be capable of adapting quickly enough to relatively rapid OA driven changes in seawater chemistry, including reduced pH. Chemical signals, whether specific pheromones or general olfactory cues, display a mechanism whereby the chemical cue within the environment makes contact and binds to receptors on the receiver. The degree of complexity of such receptor-ligand interactions differs between taxa and organism complexity, but the basic method remains the same: chemical cues must bind to olfactory receptors both successfully and in adequate quantity to illicit the correct behavioural or developmental response in the receiver.

A lock and key principle, similar to those exhibited in enzymatic mechanisms (Hardege, *et al.,* 2011), means that the structure and integrity of both receptor and ligand must be conserved to optimize the efficiency of binding to illicit the appropriate response. Many signal molecules such as amino acids and proteins associated with feeding, peptide pheromones and alarm cues, are all subject to structural change depending on the pH of the solvent in which they exist. Different molecules may be affected to varying degrees according to their own acid dissociation constant (pKa) and those with a pKa between pH 7.5- 9.5 are most likely to be affected by the projected change in ocean pH in the next 100 years.

This chapter assessed whether *N. succinea* exhibited the capacity to acclimatize to reduced pH with respect to responding behaviourally to info-chemicals following prolonged exposure to such conditions. True genetic adaptation to such changes is a much more complex matter to assess and would require culture of a species in low pH over several generations to examine. Adaptation of chemical communication mechanisms, particularly in single molecule specific pheromone cues (such as the female sex pheromone in *N. succinea*) is extremely rare as modification of the system

would have to involve synchronized evolution of both receptor and the pheromone molecule via change in the biosynthetic pathway involved in forming it. Instead, most known studies on the evolution of chemical communication systems under selective pressure have concluded they often involve changes in the ratio of pheromone bouquets (Löfstedt, 1993) or drive speciation via pre-mating isolation (Sutton, *et al.,* 2005), rather than structurally changing the cue itself, however such change is theoretically possible.

Evolutionary speciation by pre-mating isolation is seen in the marine polychaete *Nereis accuminata* (Sutton, *et al.* 2005). Geographically different populations attain isolation by showing aggression between them due to the individual population's characteristic 'smell', thought to be caused by influence of the environment they inhabit. Although the actual sex pheromones responsible for gamete release remain the same between these populations (pers. comm. Dr Jörg Hardege), 'smell' induced aggression prevents sexual partners from differing populations pairing to reach this stage. This highlights how evolutionary change in sex pheromones is uncommon as it is not necessarily required for speciation. This also shows how similar pheromones may be used in genetically divergent different species.

Evolution of female pheromones in the turnip moth, *Agrostis segetum* has occurred by alterations in the ratio of 3 compounds that make up a pheromone bouquet (Löfstedt, 1993). Spatial differentiation in this ratio in populations across the native European range is caused by directional selection by slight differing preference for bouquet composition by males from different geographical areas. This evolutionary mechanism involves only minor changes in pheromone receptors, as it is the amount of each compound that changes not the actual pheromone molecules themselves. In *N. succinea* only a single sex pheromone (CSSG) is used so this method of signaling evolution would be unsuitable.

More recent studies on moth sex pheromones draw interesting conclusions on evolution of sex pheromones via synthesis of novel pheromone molecules (Roelofs, *et al.,* 2002) and genetic mutations that alter receptor specificity (Leary, *et al*., 2012), rather than simple changes in the ratio of components within the pheromone blends. The closely related *Ostrina* species, the Asian and European corn borers (ACB and ECB), reproductive isolation is achieved according to the slight difference in sex pheromone cues each uses to attract a mate. ECB, along with most other species of *Ostrina*, use (Z)- and (E)- 11- tetradecenyl acetate as female pheromone components (Roelofs, *et al.,* 2002). Other *Ostrina* species are reproductively isolated by the different ratios of theses components used. However, it was found that ACB use a slightly different variation of these components, (Z)- and (E)- 12- tetradecenyl acetate, which differs in that the double bond is in a different position in the chain and an alternate biosynthetic pathway for production is required (Roelofs, *et al.,* 2002). It was concluded that the production of this new pheromone blend was a result of activation of a previously un-transcribed desaturase, which is present but not functional in ECB (Roelofs, *et al.,* 2002). Further studies with these species showed that mutation within receptor genes were responsible for providing ACB with a higher affinity for the new pheromone blend (Leary, *et al.,* 2012). Substitution of a single amino acid (alanine to threonine) caused the odour receptor, OR3, to be more specific to Z/E- 12 – tetradecenyl acetate over Z/E –11– tertradecenyl acetate (Leary, *et al.,* 2012).

Another rare example of structural change in the pheromone molecule used under evolutionary pressure has been recorded in bark beetles (*Ips pini*) (Raffa and Klepzig, 1989), although the change is minor. This species emits ipsdienol as an aggregation pheromone of which it's predators uses to locate them (Raffa and Klepzig, 1989). The predators however, only respond to a specific chiral enantiomer of this compound causing evolution of chemical communication in populations of *I. pini* under this selection pressure. By switching to using the opposite enantiomer of ipsdienol to which the predator can detect, *I. pini* is able to escape undetected. This evolutionary

change in the pheromone is minor, with the molecule remaining essentially the same but in a slightly different orientation. Such minor changes in the sex pheromone of *N. succinea* are unlikely to reduce the effect of pH on reception, as the molecule will still be a peptide susceptible to structural manipulation by pH, as will the pheromone receptor.

Although not yet shown to occur with respect to chemical communication, genetic adaptation to OA has recently been suggested to exist in the economically relevant Sydney rock oyster, *Saccostrea glomerata* (Parker, *et al*., 2011). The authors suggest this species shows 'adaptive capacity' to OA as selectively bred populations were more robust with respect to shell growth than wild populations when reared in CO_2 enriched conditions. However, it should be noted that ALL populations examined were still subject to reduced shell growth when reared in OA conditions, just to a lesser extent in selectively bred populations.

The examples of evolutionary change in pheromonal communication presented in moths are associated with speciation. Although the divergent species may still interbreed, differences in sex pheromones cause reproductive isolation between species. Populations of the marine polychaete *Platynereis dumerillii* found in acidified areas near regions of volcanic activity have been shown to be genetically divergent to populations existing in non-acidified areas. This finding is promising, showing that persistence within acidified areas may be possible, but it also poses implications for the conservation of species we see today. With regards to the evolution of sex pheromones in *N. succinea*, it is unlikely the mechanisms presented here will be applicable for a number of reasons. The female sex pheromone, CSSG, is a single peptide cue susceptible to conformational change in acidified conditions. Adaptation via change in molecule orientation, such as in *Ips pini* (Raffa and Klepzig, 1989), would be unsuitable as chiral variations of the molecule would still be susceptible to isoelectric modification in low pH. As a single cue molecule, changes in blend ratio as a means of evolutionary change, such as in moths and butterflies

(Löfstedt, 1993), are also not applicable. Modification of sex pheromone communication in *N. succinea* would therefore need to encapsulate dramatic change in pheromone molecule and/or receptor, an unlikely occurrence due to the genetic and biosynthetic challenges posed at the rapid rate at which OA is occurring. Further research is required to accurately assess whether marine systems have the potential to cope with the changing chemistry of our oceans and to help inform future policy on management of OA consequences.

Chapter 5:
General Discussion

5.1: Project conclusions summary

1. Culture in OA conditions from juvenile stage to heteronereid stage did not affect maximum length and weight of male or female *N. succinea*

2. Although culture in OA conditions did not reduce maturation success it did significantly increase the time taken to reach the mature heteronereid stage of the life cycle

3.The physical ability of males to release gametes was not impaired after culture in OA conditions.

4. Time to burrow into the sediment was decreased in response to acute exposure to OA conditions. This behavioural change was maintained after 32days of culture in OA conditions.

5.Glutathione content of the cells was not reduced in adults or mature males after culture in OA conditions. Data for females was insufficient to form a conclusion.

6. Both acute and long-term exposure to OA impaired adult ability to detect a variety of feeding stimulants.

7. Male response to female sex pheromone was only significantly reduced after long-term exposure to OA.

8. Disruption to detection of sex pheromone and feeding cues is likely due to pH driven conformational change in the signal molecule.

5.2 Implications for species persistence in response to ocean acidification in *Nereis succinea*

Conditions associated with near future OA have been found to negatively impact *N. succinea* at a number of points in the life cycle (Figure 5.1). Previous research by myself has shown fertilization and larval success to be significantly reduced in seawater enriched with CO_2 (pH 7.8). Additionally, gametes fertilized from pH 7.8 cultured heteronereids also showed reduced fertilization and larval success even if actual fertilization was carried out in un-acidified seawater (pH 8.2). This study found that immature adult worms showed reduced response to a variety of feeding cues following acute and prolonged exposure to OA and also showed evidence of pH avoidance by

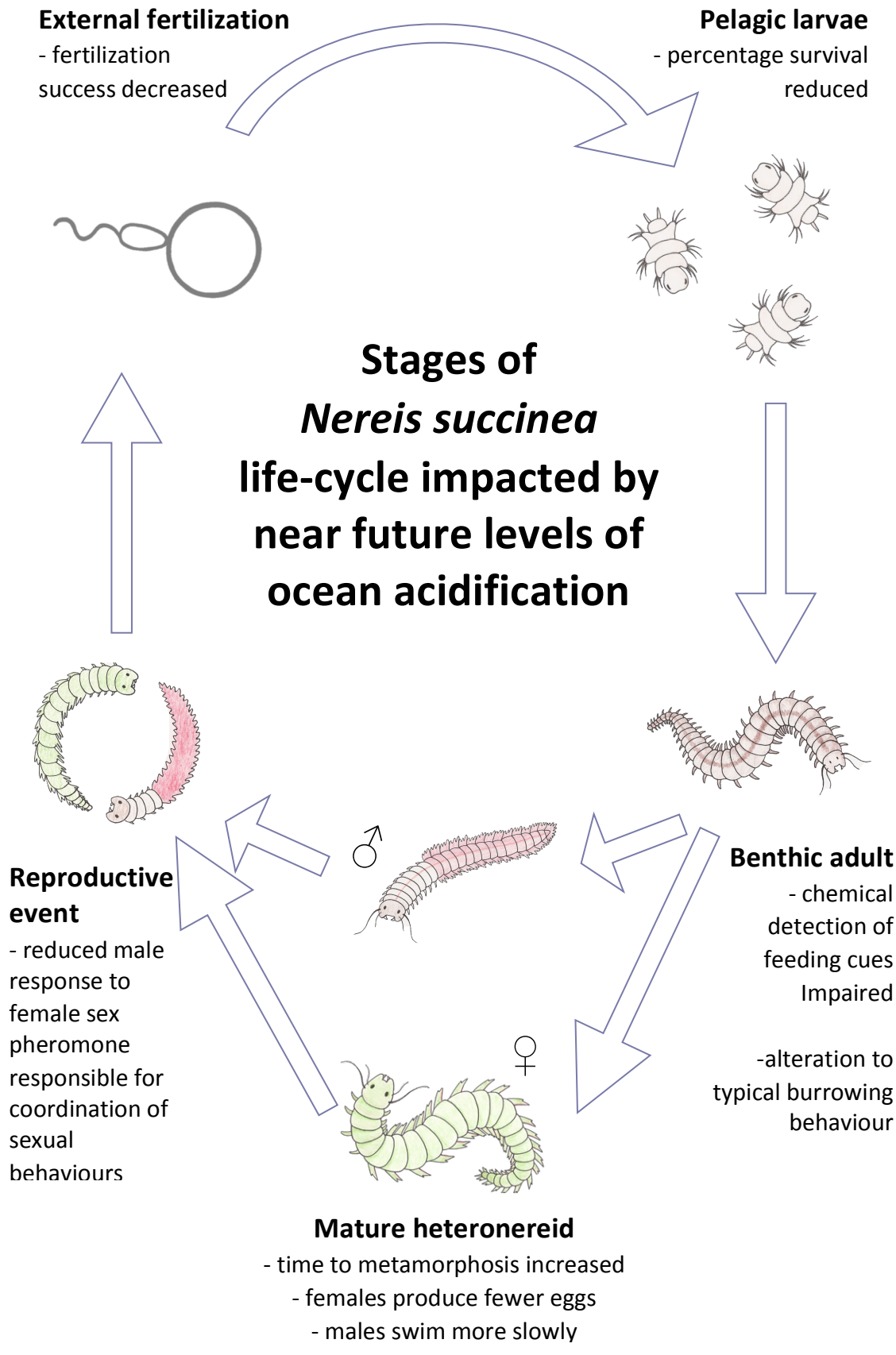

Figure 5.1: Stages in the life cycle of *N. succinea* that have been identified to be affected by OA in the current study and also in previous research by myself.

burrowing more efficiently. Maturation is delayed following culture in CO_2 enriched (pH 7.8) seawater (4-6months). Additionally, previous research by myself found that mature females cultured in pH 7.8 seawater produced fewer eggs, while males cultured in pH 7.8 reached smaller maximum size and swimming ability was impaired, a conclusion which differs from the current data. Finally, although further clarification is required, initial evidence has shown potential for disruption to reception of key sex pheromones, critical for coordinating the reproductive event. OA clearly negatively impacts *N. succinea* at several points during the life cycle. While some impacts may have relatively minor consequences regarding survival, others are likely to have more serious implications for species persistence, particularly disruption to chemical reception, low egg production and hugely reduced fertilization and larval success. The key point is that all of these impacts, whether small or large, are accumulative and should not be considered in isolation. *N. succinea* faces OA related challenges to some extent at every single developmental stage in the life history (Figure 5.1) and it is this accumulation of such challenges that will likely have severe consequences for species persistence.

It is imperative to assess further to what extent *N. succinea* may be capable of acclimatizing or adapting to our changing oceans. Little evidence was apparent to show that *N. succinea* could acclimatize and improve communication performance after longer-term exposure to CO_2 enriched conditions, supporting growing concern for the health of marine systems as a result of OA. *N. succinea* is a brackish water species, inhabiting estuarine environments where fluctuation in environmental factors such as temperature, salinity and pH is common. This may suggest a certain level of tolerance to reduced pH exists within current populations making adaptation to near future OA potentially possible. Fully marine species are not subjected to as much variance in seawater pH as estuarine species and so may be less well equipped to adapt to pH reductions associated with OA. With regards to chemical communication, disruption has been suggested to be a result of acidification causing conformational change in signal

molecules as the charge of the molecule becomes redistributed. Adaptation to such disruption is questionable as evolution of signaling systems is rare and complex.

5.3 Impacts of ocean acidification on the wider marine community

Some organisms appear more robust than others, with respect to our changing oceans, highlighting the complexity of predicting to what extent marine flora and fauna will be impacted. After examination of an Antarctic species of krill, Kawaguchi, *et al.* (2011), describe how both geographical location and migrations (both localized and over long distance) must be taken into consideration when assessing species. Furthermore, OA is not expected to occur uniformly across the globe (Doney, *et al.,* 2009). Certain areas are predicted to be more susceptible and experience more rapid change in chemistry than others, for example cold waters of the southern ocean where solubility of CO_2 and $CaCO_3$ is increased (Sabine, *et al.,* 2004). Exposure to acid conditions may also change during the life cycle of an organism due to emergence from benthic to pelagic life stage (as in *N. succinea*) or vertical migration within the water column (as in Antarctic krill) (Kawaguchi, *et al.,* 2011). It is vital that such variables are considered when assessing any impacts of OA on both established test and novel organisms in future studies to inform future policy as accurately as possible.

Marine organisms are also affected to different extents according to life strategy and physiology. Calcifying organisms face challenges associated with producing and maintaining hard exoskeletons, although even this is variable across species (Ries, *et al.,* 2009). For example, calcifying species with a layer of soft tissue covering the calcified exoskeleton are assumed to be more robust to acidic dissolution (Ries, 2011) as the carbonate exoskeleton is not in direct contact with acidified seawater. Reproductive strategy is also a factor in the extent a species may be impacted. Broadcast spawning species, such as *N. succinea*, are assumed to be more susceptible

to impacts on fertilization and larval development as gametes are released directly into the water column where acidification is occurring (Havenhand, *et al.,* 2008). Previous research on *N. succinea* has confirmed that this is the case, fertilization and larval success are significantly impacted when induced to occur in CO_2 enriched seawater conditions similar to those expected to occur in the near future. Higher organisms, such as fish, are expected to be most robust to our changing oceans due to more efficient mechanisms for acid-base regulation (Franke and Clemmensen, 2011) however, it is likely that even more developed organisms may suffer with respect to chemical communication as the basic transmission and reception mechanisms are conserved across taxa. If signal molecules are structurally affected, as has been seen here with the sex pheromone CSSG, it is likely that response will be similarly affected regardless of organism complexity. OA has been shown to affect behavioural response to chemical cues in a number of marine organisms at a number off different life stages (homing, settlement, feeding, predator-prey interactions) that may potentially impact survival of a variety of marine species.

5.4 Future research considerations

5.4.1. Assessing impact of ocean acidification on whole marine communities

This study involved culturing a single species, *N. succinea*, in isolation in laboratory conditions. Although there are benefits to this technique, there are also limitations with respect to making predictions about how the results will apply in natural systems. Comprehensive assessment of OA impact on marine communities is essential for understanding how biodiversity and ecosystem function may be affected to create well informed management and policy to minimize any negative impacts (Kroeker, *et al.,* 2011). In situ, single species form part of a larger community, interacting

with other species to for complex webs. Negative impacts observed in one species may have implications not only for the survival of that species but also for the community as a whole. *N. succinea*, along with other benthic invertebrates, is an important food source for many predatory species in our oceans, such as fish and birds. Reduction in the success of this species could therefore also impact species at higher trophic levels. Similarly, reduced success could also lead to increases of more robust benthic invertebrates able to fill the niche, reducing biodiversity and creating less complex marine communities. Such a shift in organism diversity has been observed at acidified areas of volcanic activity (Ischia, Italy), where species richness is reduced and the types of species present altered (Hall-Spencer, *et al.,* 2008)). OA studies which utilize these naturally occurring areas of acidified marine habitats, focus mainly on comparing habitat structure with areas nearby that are not subjected to CO_2 influx (Hall-Spencer, *et al.,* 2008; Porzio, *et al.,* 2011) to show how diversity and structural complexity of macro-algal and reef habitats is reduced as a result of OA. Many report on reduced diversity and structural complexity of reef habitat. The promotion of seagrass and macro algal growth has also been found to be associated with areas around submarine CO_2 vents in Papua New Guinea, where pH ~ 7.8 – 8.0, but can drop below pH 7.7 (Fabricus, *et al.,* 2011).

5.4.2 Using areas of volcanic activity to study long-term effects of ocean acidification on marine organisms and ecosystems

Several techniques within the field of OA that address the above issues are beginning to be used. In the past few years, both the mesocosm approach and the use of natural volcanic vents have increased in popularity. The emerging technique of using natural areas of volcanic activity to study OA have grown quickly in popularity with a large number of studies being published in the past few years (Rodolfo-Metalpa, *et al.,* 2011)). It enables the researcher to examine communities which have been subjected to

acidified conditions of varying intensities over generations, often for hundreds of years, a feat which would be not only difficult, but a slow process in the laboratory. With established forecasts stating that ocean pH is likely to drop by 0.3-0.4pH units in less than 100years (Feely, *et al.,* 2004), techniques such as this are invaluable to acquire information quickly to inform future policy.

These sites have also been used as ready made natural laboratories, where test species are transplanted to acidified areas and monitored over a period of time (Rodolfo-Metalpa*, et al.,* 2010, Rodolfo-Metalpa, *et al.,* 2011). Rodolfo-Metalpa, et al., used such a technique to examine calcification ability of bryozoan, mollusk and coral species by transplanting them to areas of high CO_2 around the Italian island of Ischia for extended periods (several months). Though very informative, this approach would require careful consideration to ensure the transplanted species remained localized and may be more suited to the assessment of more sessile organisms. It does, however, allow the test species to be subjected to completely natural environmental fluctuations such as temperature, diel cycles, predation and herbivory and competition with other species, a feat hard to replicate accurately in the laboratory.

Currently such *in-situ* techniques have not been used in the field of chemical communication assessment. Integrating the use of field environments that have been subject to long-term CO_2 enrichment with behavioural studies examining response to chemical signals, such as those in this study, will help to give insight into how species may cope with the pressure of OA over a long period. The presence of the polychaete *Platynereis dumerillii,* which has a similar life history to *N. succinea* including the use of pheromones to coordinate reproduction, in acidified areas around Ischia may give the opportunity to study the impact of OA in marine polychaetes *in-situ.*

5.4.3 The use of mesocosms to study impacts of ocean acidification on whole communities

All species in natural systems exist alongside a myriad of others and form complex interactions with the species present in their localized environment. It is therefore important that when assessing environmental change, species are not examined in isolation, as is often the case (Hoffman, *et al.,* 2010). For example, cultures of *N. succinea* used in this study originated from Cardiff Bay, Wales, where they inhabit established mussel beds on harbor walls. These mussel colonies form important habitat for *N. succinea* and decaying mussels provide a source of food. Several studies have reported OA may negatively impact *Mytilus* species with regards to growth (Berge, *et al.,* 2006), metabolic rate (Michaelidis, *et al.,* 2005), protein degradation and immune response (Bibby, *et al.,* 2008). Such impacts on organisms that play a crucial role in providing habitat and food would increase the pressure and challenges already identified in *N. succinea* in response to near future OA. This highlights how important it is to examine communities as a whole.

Information collected on single species, though useful, may not reflect the whole picture and lead to misinformed conclusions on the impacts of OA. The use of mesocosms as an experimental tool are a good compromise to address this problem, replicating a part of a whole community while still allowing for high levels of control and manipulation with regards to experimentation. Mesocosm experiments bridge the gap between highly controlled laboratory experiments and low control field observations. Kuffner*, et al.* (2007) successfully used outdoor mesocosms to study the impact of OA on coral reef s around Hawaii, concluding that recruitment and growth rates in crustose coralline algae were negatively affected after 7 weeks of exposure to acidified seawater. Seawater in the mesocosms was sourced from the nearby reef and the outdoor approach meant all external variables (diurnal fluctuation in solar radiation, temperature, sweater

chemistry) could be kept completely natural, with the exception of pH manipulation for the purpose of the experiment (Kuffner, *et al.,* 2007).

The source population of *N. succinea* used in this study (Cardiff Bay, Wales) exists within a relatively simple ecosystem of a handful of invertebrate species including mussels (*Mytilus edulis*), barnacles (*Balanus balanoides*) and crabs (*Rhithropanopeus harrisii*). This could easily be replicated within a laboratory mesocosm to assess how OA may impact *N. succinea* in the context of the whole ecosystem.

5.4.4 Clarifying the mechanisms involved in disruption to chemical signals

The main proposed mechanisms responsible for causing disruption to reception of chemical cues and signals are pH driven conformational change of either the signal molecule or receptor protein (de la Haye, *et al.,* 2012), pH driven disruption to neural function (Nilsson, *et al.,* 2012) and inability to respond correctly as a result of physical damage to sensory organs and increased metabolic load associated with maintaining internal acid-base balance (de la Haye, *et al.,* 2012). However, research in this area is still in its infancy. It is likely that a single disruption mechanism will not be uniformly responsible for impacting chemical communication in all species. Similarly, different species may be affected to different extents and some not all. Chemical molecules are subject to varying degrees of pH driven conformational change according to the molecule pKa. Some chemicals may remain biologically active at pH7.8, especially if their pKa is outside the critical values of 7-9. Similarly, receptor function may be maintained if pH driven structural change in the receptor protein molecule is not around the area of the active binding site (Tierny and Atema, 1988), so as not to interfere with receptor-ligand interactions.

Other proposed mechanisms involved in the disruption of chemical reception include the suggestion that reduced pH may interfere with neural activity (Nilsson*, et al.,* 2012). Interference in this way will likely be dependent on an organism's ability to regulate internal pH, as neural activity occurs within the body tissues. This is a challenge for many invertebrates (Widdecombe and Spicer, 2008), but less of a problem for fish species with more highly developed mechanisms for acid-base regulation (Ishimatsu*, et al.,* 2008).

The points discussed illustrate the need for further research into how acidified conditions may interfere with chemical communication is urgently required. Identifying the biological reasons for pH driven disruption will provide insight into the potential of organisms to overcome such challenges (if any) and help construct forecasts of how the health and biodiversity of marine systems may be impacted in the near future. Several known experimental tools may be used to investigate this further, such as the use of electrophysiology techniques to quantify biological response to chemical signals without relying on behavioural observations only. Such techniques measure the electrical activity in neurons following stimulation and so may be a useful tool to experimentally test whether response to chemical signals is depressed following exposure to acidification at the neural level. Molecular dyeing and radiolabelling techniques may also be useful to investigate receptor-ligand binding activity by using labeled signal molecules to visualize to what extent receptor-ligand binding may occur in acidified conditions.

5.5 Future research directions summary

1. Further investigation into the biological mechanisms responsible for disruption reception of chemical signals in response to OA. Techniques such as electrophysiology, receptor isolation and cloning, fluorescent dying and radiolabeling of signal molecules are suggested.

2. Investigation into potential for genetic adaptation to OA by breeding *N. succinea* over multiple generations.

3. Use of mesocosms to replicate field conditions and assess impacts of OA in context of whole communities rather than singe species in isolation.

4. Examination of structural change of feeding cues (amino acids) using NMR technology (as was used for CSSG)

5. Verification of presented results by use of larger sample sizes and increased replicates.

5.6: Conclusion

This study has identified that the marine polychaete, *N. succinea,* is impacted by near future levels of OA with regards to delayed metamorphosis and reception of chemical cues. Additionally, this species showed a limited ability to acclimatize to OA after long-term CO_2 enriched culture (pH 7.8). Further research is essential to assess the extent to which marine organisms may be impacted, as current conclusions show there to be a degree of variability in the ability of different species to cope with changing seawater chemistry, with respect to both chemical reception and the physical fitness of organism. It is now becoming clear that near future levels of OA have negative implications for the survival of many marine organisms. Even species with the ability to tolerate the changing acidity of our oceans may be indirectly affected by negative impacts on other species within the same marine communities. Physical development in early life stages of marine organisms is highly susceptible to increasing CO_2 content ocean habitats, causing additional pressure on a life stage already impacted by high mortality rates associated with predation.

The efficiency of chemical communication in the marine environment is also impacted by OA (though this may be variable between organisms), with the potential to decrease the efficiency of a number of key survival processes such as feeding, predator detection, reproduction and settlement. This is particularly problematic in ocean environments as, unlike in terrestrial habitats, the chemical sense is dominant. Attention should be paid to investigating the biological and chemical explanations for impaired chemoreception ability, as current research in this area is relatively scarce. Further development of study in this area will provide useful insight into the potential for chemical reception to either remain robust or deteriorate in response to near future levels of OA.

It is also imperative that future assessments on the health of marine systems in response to OA must consider both the consequences of long-term exposure to CO_2 enriched seawater and of communities as a whole rather than single species in isolation. Experimental techniques to address these issues, such as mesocosm and in-situ approaches, should be therefore be prioritized.

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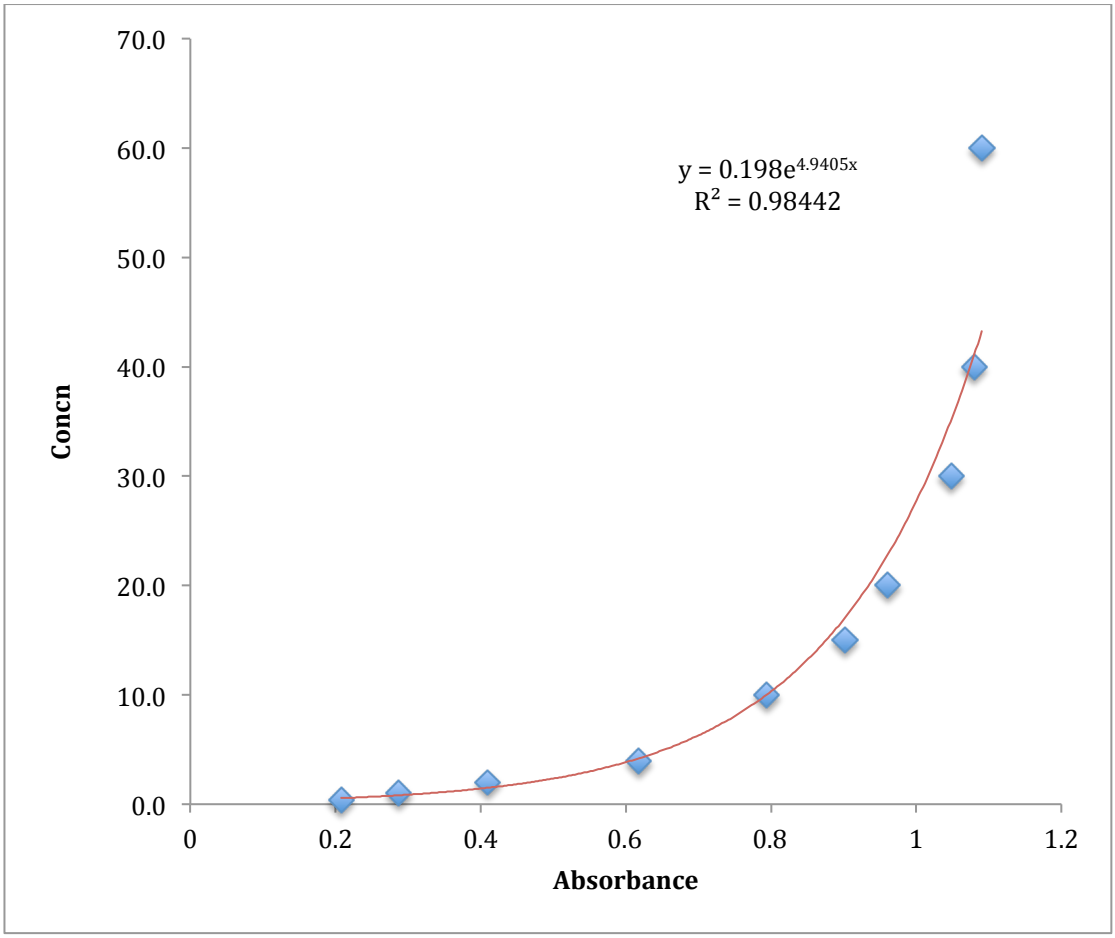
Appendices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10.0
0.0
Concn
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0.4
0.6
0.8
1
1.2
Absorbance
y = 0.198e4.9405x
R2 = 0.98442