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Ocean acidification and its effects upon fitness in nereidid
polychaetes

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by

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Contribution to scientific studies

Whilst data collected within this thesis is entirely my own, work carried out in Chapter 4 (Are *Platynereis dumerilii* found at a naturally occurring CO₂ vent in Ischia different to other known *P. dumerilii* populations?) is part of a larger collaboration with Piero (Plymouth University) and members of the Stazione Zoologica Anton Dohrn, Ischia (Naples, Italy). Worms were collected at the CO₂ vent of Ischia by members of the Functional Ecology Research Group, Hull (myself included) and Stazione Zoologica Anton Dohrn, Ischia. Following shipment to the UK, worms were processed by Marlene Jahnke and I to produce the phylogenetic tree for use within this thesis and a publication by Calosi et al. (2013).

Abstract

In recent years there has been increasing focus on predicting the potential effects of greenhouse gas driven global warming; this has proven to be a major challenge for science. In the last decade, there has been a major shift in research with growing scientific concern over the changing ocean carbonate chemistry as a result of ever increasing anthropogenic CO₂ emissions.

Major changes to the basic chemistry of seawater, such as the water pH, are likely to have substantial implications for marine life in the future (Hardege et al., 2011). Research to date has focused largely upon those organisms that require calcium carbonate to build protective shells or skeletons (Orr et al., 2005).

Using semelparous polychaetes, *Platynereis dumerilii* and *Alitta succinea*, it is shown that when exposed to pH levels forecasted to occur by 2100 (pH 7.8) survival, development, reproductive output and essential behaviours e.g. feeding and predator avoidance, are negatively impacted. *A. succinea* show severely reduced responses to natural chemical signals with subsequent low fertilisation and larval success.

The ubiquity of chemical communication in the aqueous environment indicates that chemoreception disruption can potentially have dramatic consequences. Data show that if ocean acidification continues as predicted, marine chemoreception will have to adapt rapidly with potentially profound consequences for marine life and animal interactions.

It is clear from this investigation that *P. dumerilii* and *A. succinea* are not capable of acclimatisation within one lifetime. Interestingly, *P. dumerilii* sampled and sequenced from a naturally occurring CO₂ vent in Ischia (Naples, Italy) are genetically different from other *P. dumerilii* populations within Europe. Individuals appear to show signs of adaptation in behavioural trials with few significant differences between pH treatments 8.2 and 7.8. Future studies are needed to ascertain how these organisms are adapted to life in low pH waters.

Chapter 1:
Introduction

1.0. Introduction

1.1. Ocean acidification: the other CO₂ problem

Since the Industrial Revolution began, there has been a sharp increase in atmospheric carbon dioxide (CO₂) as a result of human activity, primarily from the burning of finite fossil fuels. Recent figures show emissions of anthropogenic carbon dioxide to be in the region of 8Gt per year with some 25% absorbed by the oceans (IPCC, 2007). Cumulative anthropogenic emissions spanning the industrial era now amount to approximately 560 billion tons.

Increasing atmospheric CO₂ (ppm) is clearly evident in time series such as that established by Charles David Keeling in 1958 from the summit of Mauna Loa volcano in Hawaii. At the beginning of the investigation CO₂ levels were 315 parts per million (ppm) increasing today to some 387 ppm, an increase of greater than 37% since pre-industrial times. If fossil fuel consumption is to continue unchecked, this figure could double or triple before the end of this century (Tans, 2009). The rapid rise in atmospheric CO₂ is 30 times faster than natural rates in geologic history with present levels higher than at any time in the last 850,000 years (Kump et al., 2009).

As atmospheric CO₂ increases, air-sea gas transfer processes drive additional CO₂ into the surface waters of the ocean. The addition of CO₂ to seawater leads to the formation of carbonic acid (H₂CO₃) (See Figure 1.1) which readily dissociates into hydrogen ions (H⁺) and bicarbonate ions (HCO₃⁻) (Doney, 2006).

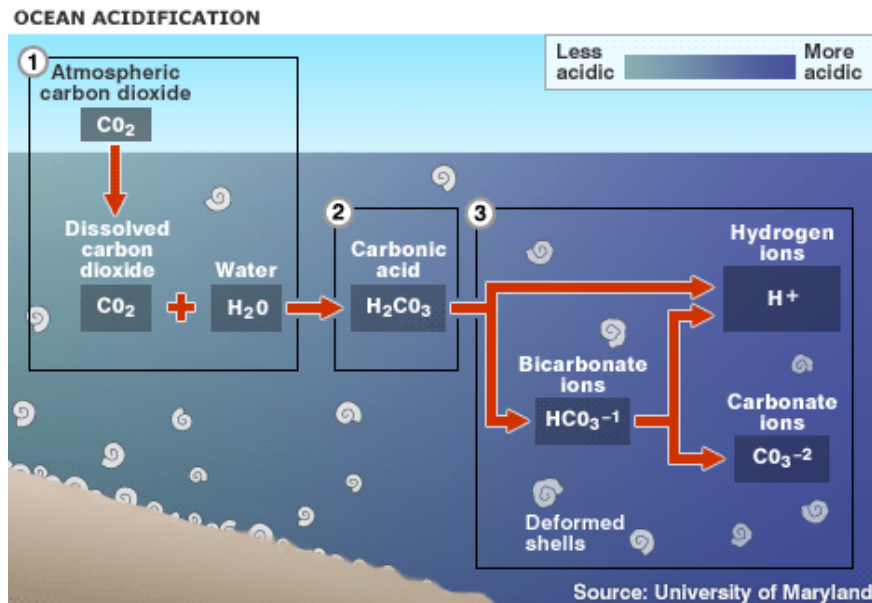


Figure 1.1. Chemistry of dissolved CO₂ in seawater (Source: University of Maryland).

Seawater is naturally maintained at a pH of approximately 8.2 by a pre-existing natural buffering system (Widdicombe and Spicer, 2008). It is estimated that surface ocean pH has dropped by slightly more than 0.1 units, a result of excess H⁺, since the beginning of the industrial revolution (Orr et al., 2005). This significantly represents an increase in acidity of 29% (Orr et al., 2005). These chemical changes are referred to as “ocean acidification”. Estimates of future trends in ocean acidification can be made for different CO₂ emission scenarios. Based on current emissions, ocean pH will drop a further 0.3 units by the end of the century as predicted by the IPCC’s IS92a scenario (Orr et al., 2005). These predictions represent a total increase in acidity of 150% (Orr et al., 2005).

Ocean acidification was first described by a number of authors in the early 1970s based on early models of CO₂ exchange and the thermodynamics of the carbon system in seawater (Broecker et al., 1971; Fairhall, 1973). Whilst these authors were in agreement that the oceans would become under saturated in aragonite and calcite, there was disagreement over when this would arise. Several studies indicated corals and coral reef systems would be severely affected (Gattuso et al., 1998; Kleypas et al., 1999) but the response of non-calcifying organisms was not speculated upon.

Additional data in the 1980s (Feely and Chen, 1982) led to the conclusion that the high-latitude regions of the ocean would become under-saturated with respect to aragonite in the twenty-first century, whilst the tropical regions would remain unaffected. Today, ship-based surveys, ocean time series and on-going field observations (Dore et al., 2009; Fabry et al., 2009) contribute to our understanding of the alteration to seawater chemistry as a result of excess CO₂ uptake from the atmosphere.

CO₂-induced acidification will, potentially, threaten the fitness and survival of many species, both calcifying and non-calcifying organisms alike (Widdicombe and Spicer, 2008). An increase in acidity will result in increased solubility of calcium carbonate minerals; minerals vital in the production of protective skeleton and shell materials (Cohen and Holcomb, 2009). Resulting decreased calcification rates could impact negatively upon marine ecosystems and in turn have severe consequences for marine food resources (Cohen and Holcomb, 2009).

However, as previously stated, the effects of ocean acidification will be much more widespread than (hitherto) anticipated. Most multicellular marine organisms have evolved a regulatory system to maintain the hydrogen ion balance of their internal fluids, expending energy as they do so (Pörtner et al., 2005). An increase in H⁺ in the surrounding seawater will mean the diversion of energy from vital processes such as growth and reproduction. Moreover, changes in seawater pH will potentially affect a large, diverse number of chemicals dissolved in the marine environment. Included in these chemicals are crucial chemical signal compounds; information chemicals used by almost all marine organisms in the exchange of information and control of behaviours (Hay, 2009).

1.2. Chemical communication

The transfer of information between organisms can manifest itself in many ways. One of the oldest forms of communication is the use of chemical signals (Wilson, 1970). Chemical signals can be observed in a variety of organisms, from single cell bacteria to the more complex human (Wilson, 1970). In an aquatic environment where light is often poor, more specifically in turbid waters, such reduction in visibility can reduce the effectiveness of other communication systems (e.g. visual, mechanical and electrical). This transmission of chemical cues can therefore provide valuable information about the

surrounding environment (Watson et al., 2005). It should be noted that this, whilst being the oldest form of communication, is often the only form of communication in invertebrates and requires no special organs (Watson et al., 2005).

Pheromones are the molecules used for communication between individuals of the same species, they are a subclass of semiochemicals: a broader term for the chemicals involved in animal communication (Karlson and Lüscher, 1959). These chemical signals, detected via olfaction, consist of a single compound or specific combination of molecules in defined amounts (Wyatt, 2010) and elicit a specific behavioural or physiological response in the receiver (Wyatt, 2009). Karlson and Lüscher (1959), responsible for the creation of the term 'pheromone', originally described them as 'substances secreted to the outside by an individual and received by a second individual of the same species in which they release a specific reaction, for instance a definite behaviour [releaser pheromone] or developmental process [primer pheromone]'.

When chemicals are used between individuals from different species they are known as allelochemicals and can be further sub-divided according to the costs and benefits to both parties (Wyatt, 2003). Signals used for deceitful purposes, e.g. the bolas spider lures male moths through synthesis of the moth pheromone, are known as allomonones (Wyatt, 2003). Semiochemicals beneficial to both the signaller and the receiver, e.g. the sea anemone and the clownfish, are called synomonones.

Chemical stimuli can elicit many biological responses, e.g. feeding, reproduction and avoidance. These stimuli can be released both deliberately in communication between a signaller and a receiver or unintentionally in the aquatic equivalent of sweat, urine and faeces, etc. (Zimmer and Butman, 2000). Chemosensory behaviour is a common function for many animals in the location of food; organisms are able to switch between chemoreception and visual cues this being distance dependent (Zhou and Rebach, 1999). The American lobster uses chemical signals to locate its prey in both direction and distance (Moore et al., 1991). Chemical communication is also used to locate suitable mates and migratory routes, e.g. salmonids spend long periods of time in the ocean but return to their home stream to reproduce. Such navigation is made possible by chemical cues (Brönmark and Hansson, 2000).

Despite their ubiquity and ecological importance, the study of chemical signals is a relatively new field. The first such chemical, the silk mother pheromone bombykol, was identified by the Nobel laureate Adolph Butenandt in 1959. The field of chemical ecology has since made great progress in analytical and bioassay techniques. These developments have allowed thousands of signals, the majority of which are insects, to be successfully elucidated (see Eisner and Meinwald, 1995 for review).

The study of aquatic chemical signals is still in its infancy (Brönmark and Hansson, 2012). Few compounds have been successfully isolated and characterised (Agosta, 1992; Zeeck et al., 1996). The list of species for which there is evidence of a sex pheromone, however, in particular those belonging to the subphylum Crustacea is beginning to grow (see Hardege and Terschak, 2011 for review). Table 1.1 (pers. comm. Dr. Jörg Hardege and Dr. Thomas Breithaupt) below details chemicals in aquatic ecosystems as characterised for a range of taxonomic groups.

Sex pheromones of nereidid polychaetes are the best investigated example in marine invertebrates (Watson et al., 2003). Nevertheless, studies of the effects of ocean acidification and reduced pH upon such pheromones are scarce. In a complex environment, chemical signals are dispersed using molecular diffusion and bulk flow (Atema, 1995). Receptors must recognise specific cues against a background of chemical noise; this involves both receptor specificity and intensity recognition of the signal. Animals use spectral and temporal properties in order to recognise a 'true' signal against random formations (Atema, 1995). As to be expected, such an array of chemical compositions within the water column results in multiple sensors for the detection of different signals. These sensory systems have evolved in different species according to need (Derby and Stuellet, 2001).

Hydrodynamics can play a significant role in the sensory and behavioural mechanisms of organisms (Zimmer and Butman, 2000). Transport of molecules occurs through the physical force of the fluids they are released into. Concentration gradients form in non-moving fluids whilst in steady flow environments the interaction between the molecule and flow create what is known as an odour plume. In high turbidity, molecules are distributed in eddies and vortexes creating multiple odour patches (Vickers, 2000). The blue crab, *Callinectes sapidus* relies upon the hydrodynamic transport of metabolite attractants when searching for intact clams, predatory success is dependent upon flow

speed (Zimmer and Butman, 2000). The American lobster, *Homarus americanus*, uses the specific chemical composition of the signal and temporal odour parameters to locate food (Atema, 1995). Antennular flicking, at a maximum rate of 4 Hz, takes place in low flow conditions to increase odour access to the receptors (Atema, 1995). Search paths, walk speed and turning behaviour are additional tools to aid in the search for sustenance (Atema, 1995).

Chemical communication relies not only on the release and reception of the stimulant, but also on the hormonal status and physiology of the emitter and receiver (Zimmer and Butman, 2000). Molecular structure, concentration and temporal and spatial distribution all play important roles in the behavioural reaction of the receiver. Common stimulatory molecules occur naturally within the water column. Organisms need to distinguish chemicals from background noise; many chemoreceptors respond not to the appearance of a chemical but rather a specific change in concentration or “threshold” (Wyatt, 2003). Response to concentration change has been evidenced in the aquatic spiny lobster, *Panulirus interruptus* (Zimmer-Faust, 1991). This crustacean is able to successfully detect changes in glycine concentrations, between 2 and 8 %, above background levels (Zimmer-Faust, 1991). Such response to minute concentration changes of stimuli undoubtedly shows a well-developed olfactory system in lobsters (Zimmer-Faust, 1991). Other examples of stimulatory molecules include uric acid in *Platynereis dumerilii* and the peptide CSSG in *Alitta succinea*. When released in low concentrations these chemicals act as mate attractants whilst in high concentrations they induce the release of gametes by males (Ram et al., 1999; Hardege, 1999).

Table 1.1. Selected chemically characterised pheromones: aquatic systems (pers. comm. Dr. Jörg Hardege and Dr. Thomas Breithaupt).

Taxonomic group	Sender species and gender	Chemical category of pheromone	Physiological or behavioural response	References
Polychaetes	<i>Platynereis dumerilii</i> , both sexes, use sex specific enantiomers	5-methyl-3-heptanone	Increases swimming behaviour; induces spawning behaviour, nuptial dance	Zeeck et al., 1988
	<i>Platynereis dumerilii</i> , male	L-Ovothiol-A	Egg release	Rohl et al., 1999
	<i>Platynereis dumerilii</i> , female	Uric acid; unknown small lipophilic compound	Sperm release	Zeeck et al., 1998
	<i>Alitta succinea</i> , male	Inosine; glutamic acid	Egg release	Zeeck et al., 1998
	<i>Alitta succinea</i> , female	Nereithione, a tetrapeptide (CSSG)	Increases swimming behaviour; trail following in males; sperm release	Hardege et al., 1997; Zeeck et al., 1998; Ram et al., 1999
	<i>Arenicola marina</i> , male	Small lipophilic cues in a bouquet incl. hexanal	Increases pumping activity required by females to 'collect' sperm	Hardege et al., 1996
	Crustacea	<i>Carcinus maenas</i> , female	Nucleotides, mainly Uridine-Diphosphate	Male attraction; induces grasping behaviour during pair formation; released in urine
<i>Callinectes sapidus</i> , female		Small molecule <500D	Courtship display in males; forms precopulatory pair bond; released in urine	Kamio, 2009

	<i>Erimacrus isenbeckii</i> , female	Novel Ceramides	Male mate guarding	Asai et al., 2000
Mollusc	<i>Aplysia californica</i>	Attractin, a 58 residue peptide	Mating aggregation pheromone	Painter et al., 1998
Fish	<i>Brachyodanio rerio</i>	Steroid glucuronid	Induces ovulation in females	Van Den Hurk & Lambert, 1983
	<i>Phoxinus phoxinus</i>	PGF _{2α}	Induces courtship behaviour in males	Ostroumov, 1992
	<i>Carassius auratus</i> , female	17,20β-P together with PGF _{2α}	Male attraction; induces reproductive behaviour	Dulka et al., 1987
	<i>Carassius auratus</i> , male	Androstenedione	Female attraction; induces reproductive behaviour	Dulka et al., 1987

1.3. Effects of pH change on chemical communication in the aquatic environment

The chemical sense is predominant in the aqueous environment. A large number of behaviours have been described to be stimulated via chemical signals with examples including: location of the mating partner, the synchronised release of gametes (Brönmark and Hansson, 2012), sperm activation and attraction towards eggs (Foltz, 1995), predator-prey interaction, detection of settlement sites and induction of metamorphosis and the establishment of dominance hierarchies in male lobsters, i.e. social cue (Atema, 1995).

The effects of pH upon olfaction and chemical signalling are well documented in freshwater systems. In weakly acidic conditions, *Oncorhynchus mykiss* (the rainbow trout) and *Salmo salar* (the Atlantic Salmon) have been shown to exhibit reduced anti-predator response when exposed to their own species chemical alarm cues (Leduc et al., 2004a; Leduc et al., 2006). Further investigation in *O. mykiss*, revealed juvenile fish learned response to predator odour was also inhibited in low pH conditions (Leduc et al., 2004b). Subsequent exposure to the correct pH did not result in the ability to recognise and respond appropriately to predator odour. An increase in pH, on the other

hand, has been shown to have a positive effect upon chemical communication in the three-spined stickleback (Heuschele and Candolin, 2007) whereby females showed greater attraction to male scented water.

The orange clownfish, *Amphiprion percula*, has become the focus of a number of pH studies. Munday et al. (2009a) have shown that individuals readily swim towards stimuli they would normally, avoid when reared at low pH. Larval recognition of parents was also affected. Animals no longer responded to olfactory cues essential to finding a suitable habitat in which to settle and survive. Such changes to their behaviour may result in inbreeding, an occurrence avoided by the control group by avoidance of parents and their natal habitat. More recently it has emerged that in addition to eradicating their sense of smell ocean acidification will compromise their ability to hear (Simpson et al., 2011). This study demonstrates that ocean acidification can affect external sensory systems as well as those inside the body of the fish. The ears of fish are set deep within the head, suggesting pH stress may have a profound impact upon the entire functioning of the sensory system. Yet whilst these studies show ocean acidification to negatively affect the clownfish, another study by Munday et al. (2009b) showed there to be no negative effect upon embryonic duration, egg survival or larval hatching size. Instead, a reduction in pH was shown to positively affect the length and weight of the emergent larvae. Further investigation by Dixon et al. (2010) found larvae were unable to discriminate between predator and non-predator odour. It is clear that this is a complex story; ocean acidification, it seems, will have different effects upon different species. Further studies, utilising a wide range of taxa are needed to understand the impact that ocean acidification will have upon the marine ecosystem and chemical signals.

Although it has now been shown that pH affects the detection of predators and homing ability in some freshwater and marine species, it is not yet known how widespread this phenomenon is. Furthermore, the cues used by organisms in these studies, e.g. clownfish, have not been chemically characterized. It is not possible, therefore, to make conclusive predictions with regards to the effects of changing pH upon these chemicals, their reception and equally upon marine life's ability to adapt their communication systems to low pH.

1.4. Acclimation, acclimatisation and adaptation

It is important to clearly define the following terms for use within this thesis: acclimation; acclimatisation and adaptation. Herein, I will follow the terminology of Brown (1997) and Gates and Edmunds (1999). Acclimation refers to changes in tolerances under laboratory or other experimental conditions over a short period of time (Brown, 1997). Acclimatisation refers to phenotypic changes by an organism in response to stresses in the natural environment, for example, becoming accustomed to a change in temperature, altitude or pH (Brown, 1997). These stressors result in the readjustment of an organism's tolerance levels with no change to the gene pool of the species; these changes cannot be inherited. The ability to acclimatise is limited by an organism's genotype, which determines the boundaries beyond which acclimatisation cannot occur, i.e. their phenotypic plasticity. Finally, selective adaptation occurs only when the more stenotopic members of a population have been eliminated by the environmental stress. A change occurs in the species; the more tolerant organisms remain and are able to reproduce and recruit to available habitats (Gates and Edmunds, 1999). Change caused by the environmental pressure is permanent and inherited by the next generation (Gates and Edmunds, 1999).

1.5. Chemical communication in polychaetes

Reproductive sex pheromones are a chemical bouquet released into the environment by the emitter, to be detected by the receiver (Wyatt, 2003). This can occur over relatively small spatial scales or much larger distances. As a result, both the emitter and receptor of the receiver are acutely sensitive to specific chemical signals (Roelofs, 1995). Coordinating reproduction is very important, especially for those animals which release their gametes at the same time as their partner(s) and fertilise them externally as so called broadcast spawners. The polychaetes *P. dumerilii* and *A. succinea* also face the additional challenge that reproduction is a terminal single event making timing of gamete release important. Sperm do not live long and are also quickly diluted in the water column; the opposite sexes, therefore, need to be in close proximity. Marine invertebrates, from different phyla, have been reported to reproduce simultaneously, for example, at coral reefs (Babcock et al., 1992) and in the Irish Sea (Minchin, 1992). Many marine invertebrates, including nereidid polychaetes, use chemical signalling via sex pheromones to coordinate their reproductive behaviour and "time" the exact

moment of spawning. Synchronised spawning ultimately maximises the chances of gamete contact and thereby fertilisation. This is especially important for animals such as the nereidid worm which have only one opportunity to reproduce, as they die following the event (semelparity).

1.6. The experimental species

1.6.1. Polychaetes

The annelids are a large phylum more commonly referred to as segmented worms. Polychaetes comprise the majority of the diversity and can be found in almost every marine habitat, from intertidal algal mats to the deepest sediments and extreme hydrothermal vent communities. There are a small number of pelagic species which swim freely in the water column preying upon microscopic plant-like organisms, phytoplankton. Most polychaetes, however, are secretive creatures, living under rocks or burying themselves in the sediment. They are rarely found in fresh water and are almost absent from terrestrial habitats. Earthworms and leeches, both members of the class Clitellata, are found in these environments and it is possible that they evolved from a polychaete group (Rouse and Pleijel, 2001).

Nerididae, more commonly known as ragworms, are a widely distributed family of polychaete worms containing approximately 500 species in both marine and brackish environments (Fischer and Dorresteijn, 2004). They are commonly found at all depths of the water column; foraging in seaweeds, seeking refuge under rocks or burrowing in sand or mud. Individuals vary in colour from transparent to red-brown whilst some display additional colour and pigmentation patterns (Rouse and Pleijel, 2001). Size is equally variable, from a few millimetres long, as in *Micronereis*, for example, to over a metre long in *Nereis virens*. The larger, more mobile species often have well-developed eyes, various sensory appendages and elaborate jaws for grasping and ingesting prey. Whilst most ragworms are omnivorous there are those species which are active carnivores (Kristensen, 1988). Known predators of polychaetes include fish, birds and epibenthic invertebrates (Reidel et al., 1989).

1.6.2. *Platynereis dumerilii*

Experimental species

Phylum: Annelida

Class: Polychaeta

Family: Nereidae

Species: *Platynereis dumerilii* (Audouin and Milne-Edwards, 1834)



Figure 1.2. Heteronereid *P. dumerilii* (male specimens). The scale bar to the lower left corner represents 1 cm (Source: European Molecular Biology Laboratory).

With a full-size length of approximately 35 mm, up to 75 segments, *P. dumerilii* (Figure 1.2) is one of the smaller of the nereidid species spending the majority of its lifetime in a self-spun tube with openings at either end (Fischer and Dorresteiijn, 2004).

The prostomium, or head, is easily recognizable by two pairs of eyes, two pairs of sensory appendages, four pairs of peristomial cirri and powerful jaws. Each segment holds a pair of parapodia equipped with setae which can, in combination with a complex set of musculature, be used for crawling and slow swimming (Fischer and Dorresteiijn, 2004). These segments are similar in morphology, a condition called “homonomous segmentation”, segments and parapodia differ in many other polychaete species, for example, the “parchment worm” *Chaetopterus*, a condition called “heteronomous segmentation” (Fischer and Dorresteiijn, 2004). Whilst largely shallow sublittoral in

distribution, *P. dumerilii* have also been recorded at depths as great as 4850 m (Fischer and Dorresteijn, 2004).

In recent decades, *P. dumerilii* has been established as a marine animal model for developmental (Fischer and Dorresteijn, 2004), evolutionary (Arendt et al., 2001), ecological and toxicological research (Hutchinson et al., 1995), owing in part to the large range of environmental conditions they can tolerate (Fischer et al., 2010). Evidence indicates its evolutionary lineage has been slow-evolving with a highly conserved gene structure. As such, it is highly suitable for comparative studies. It has been successfully kept in laboratory culture since 1953 where it can be easily bred with the ability to produce more than 2000 eggs at one time. These gametes undergo embryonic and larval development in a highly conserved manner. Eggs, embryos and larvae are all transparent in appearance, eggs measuring 160 μm in diameter. This makes them easily accessible by conventional light or dissecting microscope (Fischer et al., 2010). For the purpose of this investigation they will be monitored with the use of a compound light microscope.

As previously stated, *P. dumerilii* is one of the better investigated examples of a sex pheromone in marine invertebrates owing largely to its well defined, simple organisation, well documented reproductive behaviour (Hauenschild and Fischer, 1969), accessibility and finally, the ease with which it can be continuously bred in the laboratory. Its sex pheromones, uric acid and 5-methyl-3-heptanone have been successfully identified and synthetic compounds are readily available for use in bioassays. It is these attributes which have led to its selection for the current investigation.

1.6.3. *Alitta succinea*

Experimental species

Phylum: Annelida

Class: Polychaeta

Family: Nereidae

Species: *Alitta succinea* (Leuckart, 1847)



Figure 1.3. Heteronereid *A. succinea*, male (top), female (bottom). The scale bar to the lower left corner represents 1 cm (Source: L. Davidson).

A. succinea (Figure 1.3) are distributed throughout the world in shallow, brackish waters (Marine Species Identification Portal). They are known to occupy a range of marine and estuarine intertidal to subtidal infaunal and epifaunal habitats including sand and mud bottoms, seagrass meadows, rocky benthic areas, mussel and oyster beds, and dock pilings (Craig et al., 2003). Non-swimming juveniles reside in benthic environments, to approximately 20 m depth, whilst the mature heteronereis swim freely within the water column (Hardege et al., 2004). Individuals can reach lengths of up to 190 mm with 160 segments equipped with well-developed, setose parapodia (Pettibone, 1963). It has a dark pigmented head with: four large eyes; one pair of antennae; one pair of long feeding palps, four pairs of small tentacles and a pharynx with chitinous jaws (Craig et al., 2003). The anterior region is brown in colour whilst the posterior region is green, yellow or red (Marine Species Identification Portal). Macroscopically it is very

similar to other nereidid species, particularly those belonging to the same family, for example *N. virens* and *N. diversicolor*. Microscopically it possesses a distinct pattern of paragnaths (small chitinous toothlike structures on the eversible pharynx) and parapodial lobes (European Network on Invasive Alien Species).

Unlike *P. dumerilii*, which reside in self-spun tubes, *A. succinea* are commonly found in U-shaped sediment burrows in estuaries. It should be noted, however, that its distribution is not restricted solely to estuarine salinities and can therefore be said to be more tolerant of a fluctuating environment than many other nereidid species. Successful reproduction has been reported in the Salton Sea in California at salinities as high as 45 – 50 with survival as high as 65 (Kuhl and Oglesby, 1979). In brackish water environments pH readily fluctuates, it is possible therefore that *A. succinea* are able to adapt more readily to large scale environment changes such as or caused by CO₂ induced acidification (European Network on Invasive Alien Species).

In the natural environment, as with *P. dumerilii*, *A. succinea* spawn in synchrony with lunar cycles and other environmental cues (Hardege, 1999). They are easily accessible and are well suited to laboratory work. They can be easily manipulated to achieve year round spawning. In addition to their ease of culture in the laboratory and short generation time, they possess a clearly defined pheromone coordinated reproductive behaviour; both male and female reproductive chemical signals have been identified (see Hardege et al., 2004 for review). Unfortunately, it has proven difficult to sustain larval development (due to problems with larval feeding) and therefore establish F1 and subsequent generations (pers. comm. Dr Jörg Hardege).

A. succinea have been selected for investigation in conjunction with *P. dumerilii* to allow for comparisons between closely related semelparous polychaetes and further examination of the effects of ocean acidification with a number of species that live in different habitats. Where experiments have not been possible with *P. dumerilii*, for example, fertilisation and larval success due to asynchronous maturation of males and females, *A. succinea* have been used for “proof of principle” owing to their similar life cycle and reproductive strategies.

1.7. Geographical distribution

1.7.1. *Platynereis dumerilii*

P. dumerilii is a widely distributed species with a geographic distribution extending from the tropics to cold temperate latitudes in both hemispheres, more commonly along the Mediterranean and North Atlantic coast of Europe and Africa (Fischer and Dorresteijn, 2004). It should be noted that this species have also been found to the East in West Thailand, Sri Lanka, Java, the Philippines, North Australia and the South China Sea (Natural History Museum). *P. dumerilii* inhabit shallow hard ocean floors and are particularly abundant at depths of 3 m or less (Natural History Museum).

1.7.2. *Alitta succinea*

A. succinea are distributed throughout the world and are said to be cosmopolitan in distribution, being common in both temperate and tropical marine habitats (Hardege et al., 1990). Whilst considered native to the Atlantic coast of the Americas, the species can now be found as an introduced species along the coast of Europe and Africa, in the Black Sea, Caspian and Aral Sea, and southern Australia (Pardo and Dauer, 2003). *A. succinea* also occur along the US Pacific coast as an introduced, non-indigenous species (Pardo and Dauer, 2003). Introduction pathways include natural dispersal in addition to ship ballast water and hull fouling as a result of maritime transport and shipping activities (ISSG, 2007).

1.8. Life stages and growth

1.8.1. *Platynereis dumerilii*

P. dumerilii have a relatively short life cycle (Figure 1.4) changing their habitat twice in a lifetime. Developing eggs drift in the water column kept afloat by a thick gelatinous layer from which they hatch as planktonic trochophore larvae within the first 18 hours of development. Polychaete larval development traditionally comprises three major stages equally applicable to *P. dumerilii*: the trochophore, the metatrochophore and the nectochaete. The trochophore is a spherical larva with an equatorial ciliated belt (the prototroch), and an apical organ with a ciliary tuft (Hauenschild and Fischer, 1969). The

metatrochophore is slightly elongated in comparison to that of the trochophore, comprising a now segmented trunk (Fischer et al., 2010). The subsequent and final stage is that of the nectochaete, larvae bearing parapodial appendages used in swimming and crawling, closely resembling the adult form in key traits. These three-segmented young worms eventually settle in the benthic environment. Additional segments are proliferated by a subterminal zone of proliferation throughout their life as a benthic “atoke”. Upon reaching sexually maturity the “epitoke” briefly returns to the water column in search of a mating partner, dying some hours later.

For reproduction, many nereidid polychaetes undergo “epitokous” metamorphosis during which time the worms transform into swimmers better known as epitokes, or “Heteronereis” (Hardege et al., 2004). These heteronereis are easily distinguishable, mature males possess a white (sperm filled) anterior and red (muscle rich) posterior whilst females are yellow in colour due to the large number of yellow oocytes filling the body cavity (see Figure 1.2). This transformation is, however, at the expense of many somatic cells of the immature worm (Fischer and Dorresteijn, 2004), individuals die after reproduction.

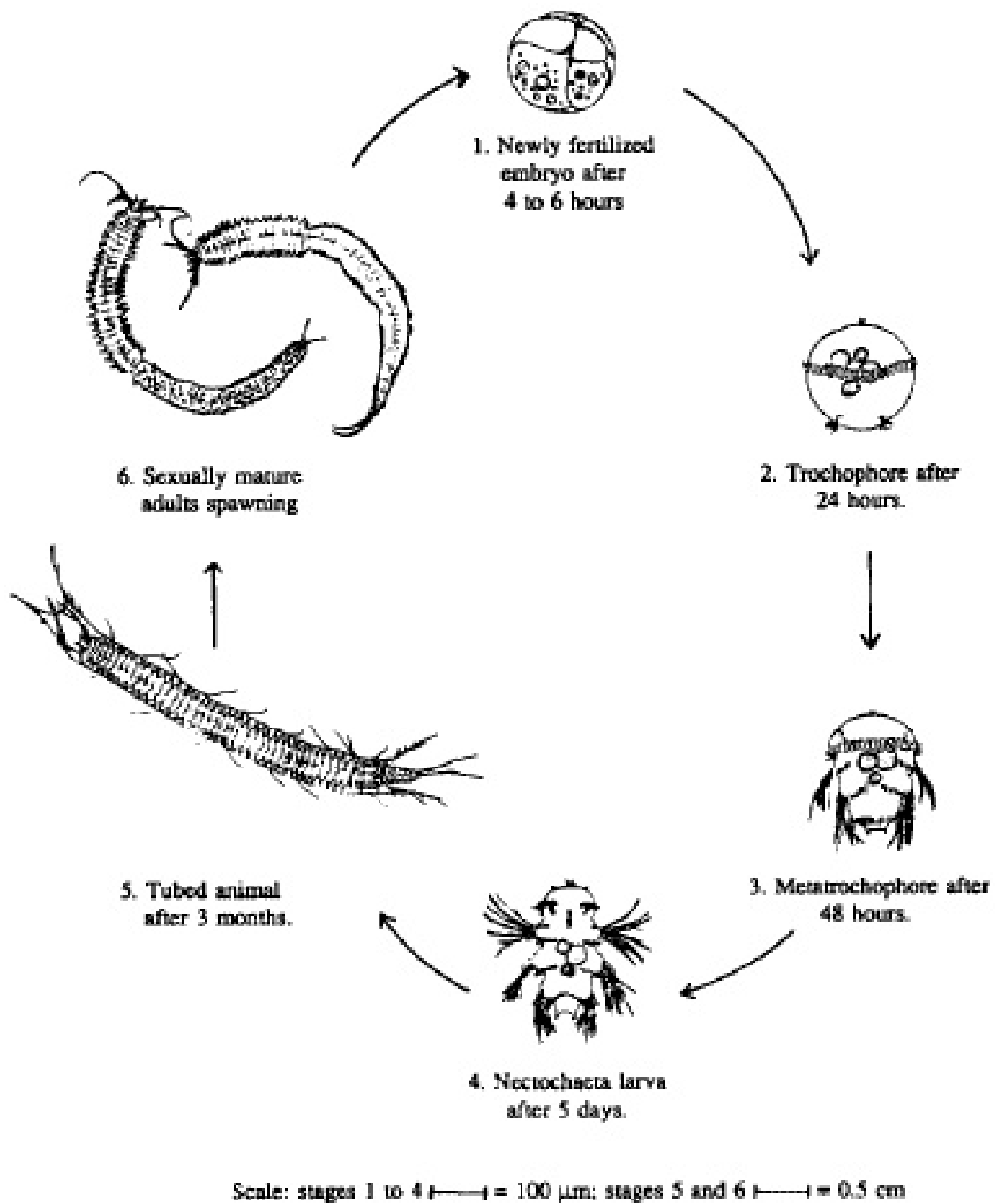


Figure 1.4. Schematic diagram of the life cycle of *P. dumerilii* at 19 ± 1 °C (Hutchinson et al., 1995).

The eyes of heteronereids enlarge and a new type of muscle fibre, rich in mitochondria, forms. The posterior section is modified; starting at segment no. 16 in males and at no. 22 in females (Fischer and Dorresteijn, 2004). The hook and needle-shaped setae are shed being replaced by new paddle-like setae (Fischer et al, 2010). Together with newly formed muscle these modified setae propel the worm through the water at great speed. The setae and parapodia of the anterior section remain the same thus replacing the previously homonomous segmentation of the trunk with a sex-specific, rigid subdivision into two sub dissimilar regions (Fischer et al., 2010).

1.8.2. *Alitta succinea*

As previously stated *A. succinea* possess a relatively short generation time, a minimum of 1 year (Hardege et al, 1990) with a very similar life cycle to that of *P. dumerilii*, as detailed below. Within 36 hours of fertilisation, eggs develop into small setose, two-segmented planktonic larvae. These larvae float freely within the water column until they reach at least 9 segments in length, at this point they settle in the benthos where they remain throughout the non-swimming juvenile stage (Tiffany et al., 2002).

Upon reaching sexual maturity individuals undergo “epitokous” metamorphosis (Detwiler et al., 2002). At this stage the body is divided into three regions, only the middle of which is used for swimming. Males possess a white anterior and red posterior, as with *P. dumerilii*, whilst females are greenish brown in colour. Heteronereids develop modified parapodia which allow them to swim freely within the water column. These parapodia also hold receptors responsible for detecting essential sex pheromones (Hardege, 1999). Being a semelparous polychaete, such changes are at great cost and animals die following a once in a lifetime reproduction.

1.9. Feeding

The feeding behaviour of many aquatic organisms relies heavily upon a complex mixture of chemicals to stimulate sensory organs (Mackie et al., 1980). All nereidids have eversible jaws which in some instances are adorned with small auxiliary jaw pieces called paragnaths (Fauchald and Jumars, 1979). Many nereidid species, including those used within this investigation, form mucous tubes and feed preferentially from the mouth of such ‘structures’ (Fauchald and Jumars, 1979). When required to do so,

organisms can leave the refuge of these tubes in order to forage elsewhere when conditions become unacceptable.

1.9.1. *Platynereis dumerilii*

P. dumerilii regularly and rapidly produce mucous tubes within which they reside, both in the field and in laboratory experiments (Fauchald and Jumars, 1979). As with *A. succinea* and many nereidid species, *P. dumerilii* are generally herbivorous and feed from the safety of their tubes on a diet consisting largely algae and diatoms (Fauchald and Jumars, 1979). Unlike *A. succinea*, *P. dumerilii* show little tendency toward cannibalism and carnivory. It should be noted, however, that in high densities some cannibalism will take place. Faecal pellet analysis, food-choice experiments and photosynthetic pigment analyses of Mediterranean *P. dumerilii* indicate preferential consumption of erect micro- and macroalgae (Gambi et al., 2000). *P. dumerilii* are maintained in the laboratory upon a diet of spinach and commercial fish food.

1.9.2. *Alitta succinea*

A. succinea is an opportunistic feeder (NIMPIS, 2006). Spending most of the day in a mucous-lined tube they are an active forager primarily at night (NIMPIS, 2006). Whilst the jawed, eversible proboscis is used primarily for the ingestion of sediment deposits, it is also used in the grazing of plant material and facultative capture of small invertebrates (NIMPIS, 2006). They are cannibalistic in nature with small amphipods and other polychaete species reported in gut content analysis (NIMPIS, 2006). Fong (1987) found feeding to be largely non-selective with a wide range of consumption from 20 – 300 µm. A study by Cammen (1980) estimated almost one-fourth of the organic carbon requirement of North Carolina *A. succinea* to be microbial in origin. Additional sources of carbon were believed to be derived from the uptake of plant substratum, ingestion of meiofauna and possible uptake of dissolved organic matter.

Whilst the importance of chemoreception in reproduction has been discussed above it is important to remember that chemical signals are responsible for a great number of behaviours both within and between organisms. To survive, organisms, including nereidid worms, must be adequately equipped to successfully detect food and predators within the marine ecosystem, seeking out and avoiding them where necessary. The

feeding behaviour of many aquatic animals relies upon a mixture of chemicals to stimulate sensory organs (Mackie et al., 1980). Feeding behaviour can be classified into those that affect at distance (attractants), and those that require physical contact (stimulants) (Mackie et al., 1980).

Whilst this topic is, again, in its infancy with regards to the effects of ocean acidification a recent study by Cripps et al (2011) indicated that future oceanic conditions may prevent fish adapting rapidly to fluctuating food availability. When exposed to both current-day CO₂ levels and those predicted to occur by 2100, the brown dottyback (*Pseudochromis fuscus*) exhibited reduced feeding and increased activity levels in the latter treatment. Whilst a reduction in feeding may suggest a reduction in olfactory ability, an increase in activity may be indicative of compensation via visual detection, i.e. they have to spend a lot more time in search of food. It is important to consider both parties of the predator- prey interaction as each will be affected in different ways ultimately altering the fate of each.

1.10. Predation

Predation impacts heavily upon the ecology of an ecosystem with important effects for both the predator and prey (Barta et al., 2004). Chemically induced defence responses are particularly common in complex aquatic ecosystems (Griffiths and Richardson, 2006). Chemicals released by the predator or alarm pheromones from a conspecific may alert prey to threat of predation (Wisenden, 1999). Equally so, anti-predator behaviour may be induced through the release of injury cues from conspecifics and heterospecifics alike (Wisenden, 1999). Whilst the predator-prey relationship is a complex one, it is often controlled by external factors, both physical and chemical. Water condition, movement, substratum complexity and flow rate can affect the predator's ability to locate prey species and similarly the prey species detection of a predator (Powers and Kittinger, 2002). It could be argued therefore, that acidified waters may substantially alter the basic chemistry of seawater and prevent the detection of both predator and prey. Such implications could dramatically alter aquatic ecosystems.

1.10.1. Nereidid predators

Due to the nature of the environment *P. dumerilii* and *A. succinea* inhabit, they are at risk from both terrestrial and aquatic predators. During low tide when polychaetes are readily exposed in the intertidal zone, they are at risk from avian predators; whilst in the aquatic environment they are preyed upon by both fish and crustaceans including *Rhithropanopeus harrisii* (as witnessed at sample sites, in particular, Cardiff) and *Carcinus maenas* (Fischer et al, 2010). It is crucial that polychaetes are able to detect predators in order to survive through avoidance and/or escape. Such interactions are also controlled via chemical cues, for example, Copeland and Wieman (1924) found that when exposed to crushed periwinkles and other animal food *N. virens* would emerge from its tube. Exploiting this feeding behaviour, Watson et al. (2005) exposed *N. virens* to whole-body extracts of conspecifics, polychaete prey species and flatfish muscle (predator). Feeding and foraging activity were significantly reduced when *N. virens* were exposed to whole-body extracts of conspecifics; these extracts were deemed to act as an alarm signal. Extracts of flatfish muscle reduced the number of worms feeding but did not impact upon activity outside the burrow (Watson et al., 2005).

1.11. Reproductive strategies of polychaetes

The different reproductive strategies of polychaetes have been extensively studied (Watson et al., 2003). *P. dumerilii*, specifically, is one such species and its culture in the laboratory is described in detail by Fischer and Dorresteyn (2004) as adapted from Hauenschild and Fischer (1969) with its development described in detail by Fischer et al., 2010.

Whilst the reproductive strategies of polychaetes vary in their form, from broadcast spawning to vivipary, they generally exhibit one of two reproductive patterns: iteroparous and semelparous. Iteroparous species, such as *Nephtys hombergi* (Olive et al., 1997) take part in an annual reproductive event whereas semelparous species are characterized by a single reproductive event before death. *P. dumerilii* are a well-studied example of semelparity (Zeeck et al., 1998). The reproductive strategies adopted by a number of polychaete species are summarised below (Table 1.2)

Table 1.2. Examples of the reproductive strategies of polychaetes (pers. comm. Dr. Jörg Hardege).

Species	Reproductive Strategy	References
<i>Platynereis dumerilii</i> <i>Alitta succinea</i>	Broadcast spawner, contact, heteronereis	Zeeck et al., 1988
<i>Platynereis megalops</i>	Female and male spawn, female bites male, internal fertilisation	Just, 1914
<i>Nereis diversicolor</i> <i>Nereis virens</i>	Males swim, no real heteronereis, female brood care in <i>N. diversicolor</i>	Olive et al., 1997
<i>Nereis acuminata</i>	Pair formation, male paternal care	Storey et al., 2013
<i>Nereis limnicola</i>	Viviparous hermaphrodite, low salinity	Baskin & Golding, 1970
<i>Arenicola marina</i>	Gametes broadcast, stay in burrow, female collects sperm	Hardege et al., 1996

Semelparous marine invertebrates, such as *P. dumerilii*, die after a once in a lifetime reproductive event. *P. dumerilii*, as seen from the table above, is a broadcast spawning polychaete. It is important, therefore, that spawning be carefully coordinated (Hardege, 1999). This feature has prompted the evolution of mechanisms to reduce the potential for gamete loss if there are any individuals which are asynchronous in gamete release with other adults of a given population.

Photoperiod, temperature, lunar periodicity and tidal cycles all act to synchronize maturation in broadcast spawning nereidids (Olive et al, 1997). Spawning hormones and pheromonal communication are responsible for the transduction of this information (Bentley and Pacey, 1992) ultimately resulting in the mass spawning events described for nereidid species.

1.11.1. *Platynereis dumerilii*

Specialized heteronereis assemble at the water surface where they exhibit a characteristic reproductive behaviour called the nuptial dance (Boilly-Marer, 1974). Upon meeting, the sexual partners swim in circles of decreasing size until the male releases a small amount of sperm. This stimulates the female to release eggs followed by a second release of sperm by the male, this time in large quantities to fertilize the eggs. This “nuptial dance”, in addition to the release of gametes into the seawater, is controlled largely by sex pheromones (Boilly-Marer, 1974) located in the coelomic fluid of gravid specimens (Townsend, 1939). Exposure of ripe heteronereids to such body fluid (containing the sex pheromones) induces the release of gametes with evidence for heterospecificity between many nereidid species (Boilly-Marer and Lasalle, 1980). It is this behaviour that has formed the basis of simple, unambiguous behavioural assays. Ablation experiments undertaken by Boilly-Marer and Lasalle (1980) indicated ripe individuals detect these chemical cues via chemoreceptors located at the parapodial cirri (Boilly-Marer, 1974). See Figure 1.5.

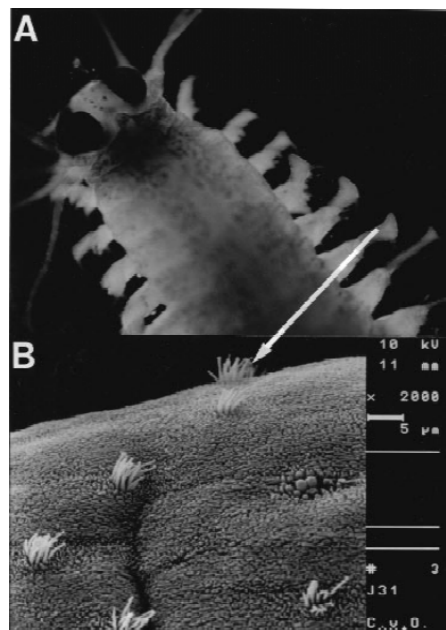


Figure 1.5. (A) Head and first segments of mature female *P. dumerilii* with modified anterior parapodia. (B) Scanning electron microscope image of the parapodial cirri of a modified female of *P. dumerilii*. (Hardege, 1999).

The reproductive behaviour of the free spawning nereidid polychaete *P. dumerilii* can be divided into four simple steps as shown in Figure 1.6:

1. Males swim over large distances at the water surface in search of slow swimming females. Occasionally females will vary their swimming patterns, moving in circles of smaller size for a couple of seconds. At this time they emit small amounts of the sex pheromone 5-methyl-3-heptanone and begin to swim in tight circles of decreasing size (Zeeck et al., 1988).
2. Ripe males passing a swimming female detect the pheromone in the water column and immediately change their swimming characteristic. They return to the origin of the 'smell' circling the female whilst emitting a small amount of the coelomic fluid, a small 'sperm cloud' (Hardege, 1999). The sperm cloud contains the egg release pheromone (ERP), L – Ovoidiol A (Röhl et al., 1999) (Figure 1.6).
3. The ERP then stimulates the female to swim with high velocity in narrow circles, the 'nuptial dance'. After a few seconds the female discharges the eggs (Hardege, 1999). The female body fluid contains the sperm release pheromone (SRP), uric acid (Zeeck et al., 1998) (Figure 1.6).
4. Upon detection of this substance the male swims with increased speed emitting large amounts of sperm easily detectable in the water column. The female dies within hours of reproduction whilst the male swims away in search of another female (see Hardege et al., 2004 for review).

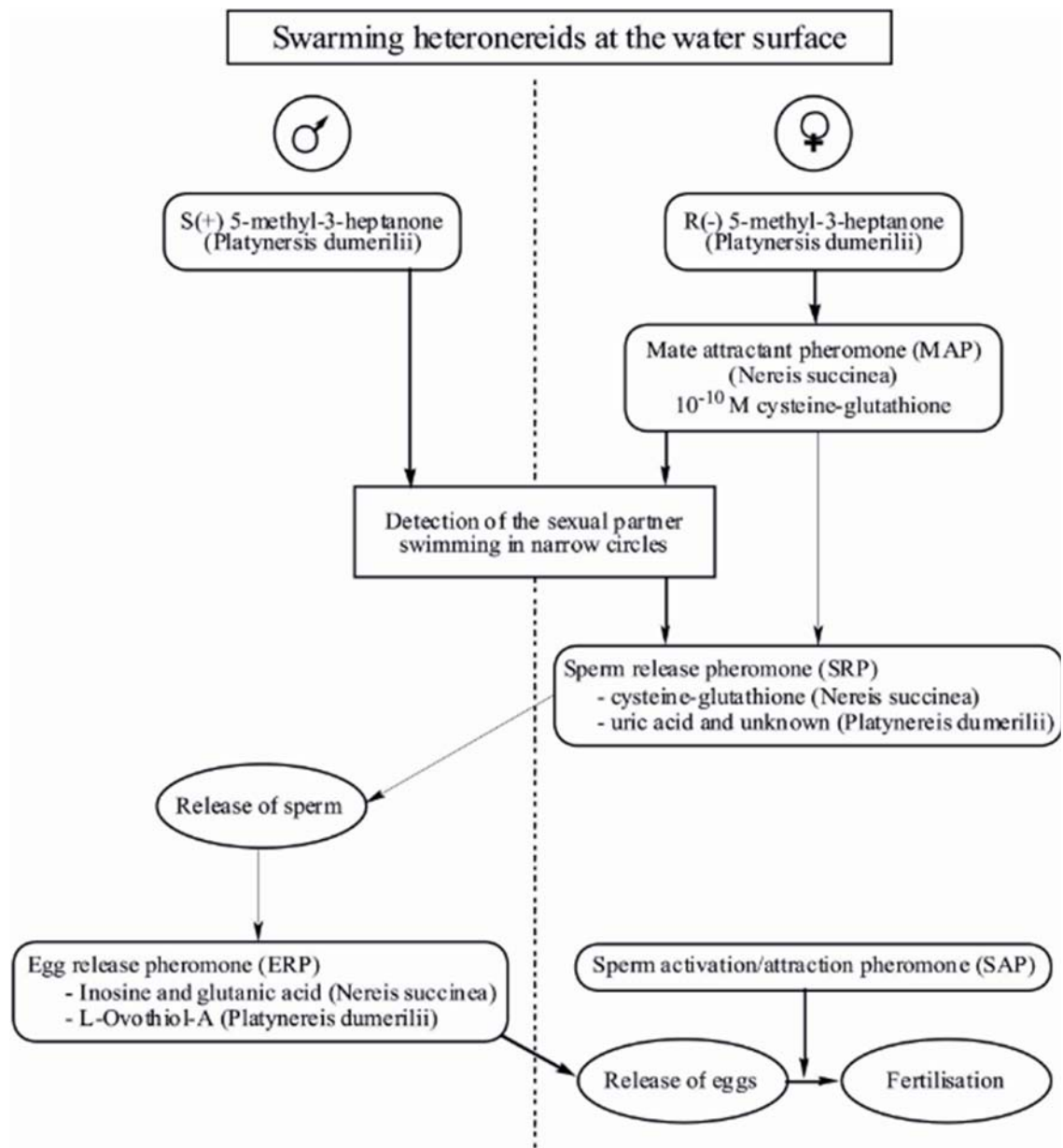


Figure 1.6. Schematic diagram of pheromone coordinated reproduction in *P. dumerilii* and *A. succinea* (adapted from Hardege and Terschak, 2011).

1.11.2. *Alitta succinea*

As with *P. dumerilii*, spawning animals swim to the water surface in response to what is thought to be a complex set of exogenous cues including temperature, salinity, photoperiod and lunar period (Hardege et al., 1990).

A. succinea mass spawning events generally occur June to September the evening of a new or full moon at temperatures above 16 °C. This is, however, dependent upon the location population (Ram et al., 1999). Females release cysteine-glutathione disulphide (CSSG) as they swim. This pheromone, first isolated by Zeeck et al. in 1998, acts as a mate attractant at low concentrations. Male swimming activity increases (Ram et al., 2008) thereby increasing the likelihood of encountering a sexually mature female (Fei et al., 2008). Upon meeting, both genders swim in tight circles of decreasing size. Males release a small amount of sperm in addition to the egg release pheromone (ERP) (Zeeck et al. 1996, 1998). This ERP consists largely of inosine with glutamic acid and glutamine and induces the female to release both eggs and large quantities of the sperm release pheromone (SRP), CSSG, this time at higher concentrations (Hardege et al., 2004). Subsequently, males release large amounts of sperm allowing fertilisation to occur.

The reproductive behaviour of the free spawning nereidid polychaete *A. succinea* can also be divided into simple steps as shown in Figure 1.6.

1.12. Chemical nature of nereidid sex pheromones

1.12.1. *Platynereis dumerilii*

1. 5-methyl-3-heptanone

The reproductive ‘nuptial dance’ of *P. dumerilii* is triggered by the release of the volatile ketone 5-methyl-3-heptanone (Figure 1.7), the first water borne sex pheromone to be identified in a marine invertebrate (Zeeck et al., 1988). It is released in ng quantities with a biological detection threshold of $3.5 \pm 0.5 \times 10^{-12}$. As indicated in Figure 1.7, the pheromone possesses optical isomers; the S(+) produced by males which acts upon females and the R(-) produced by females which acts upon males (Zeeck et

al., 1992). 5-methyl-3-heptanone has been shown to be present in the coelomic fluid of other polychaetes such as *N. virens* and *A. succinea* (Zeeck and Hardege, 1990).

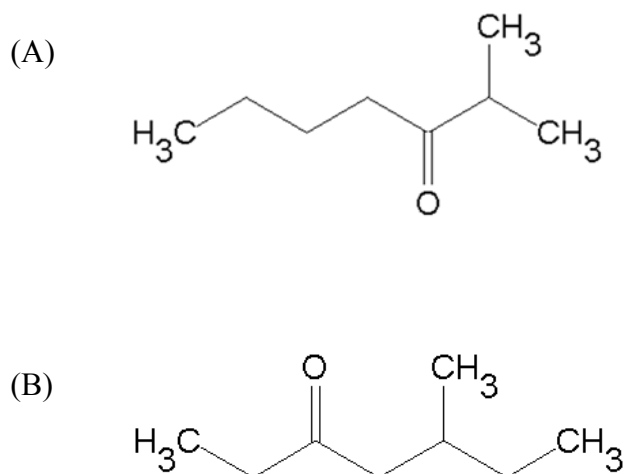


Figure 1.7. Structures of the sex pheromone in *P. dumerilii*, 5-methyl-3-heptanone: (A) S(+) isomer; (B) R(-) isomer (Source: Guidechem).

2. Uric acid

Upon meeting of the sexual partners, ripe heteronereids swim in narrow circles (the characteristic ‘nuptial dance’) around each other at the water surface. Upon exposure to coelomic fluid of the opposite sex individuals release their gametes into the water column (Zeeck et al., 1988). Uric acid (Figure 1.8), a female produced pheromone induces the release of gametes by male *P. dumerilii* at relatively high concentrations (Hardege, 1999).

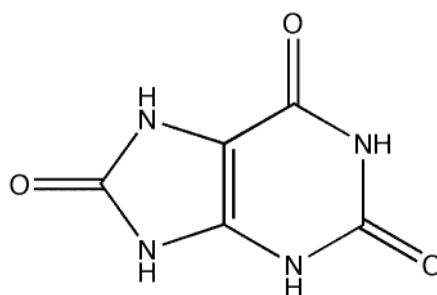


Figure 1.8. Structure of the sex pheromone in *P. dumerilii*, uric acid (Source: Guidechem).

3. L – Ovothiol A

The egg release pheromone, identified as L – Ovothiol A (Figure 1.9) can be isolated from the coelomic fluid of sexually mature male *P. dumerilii* (Rohl et al., 1999). When released into the water column, L-Ovothiol A initiates egg release in swarming females. Interestingly, L - Ovothiol A is stored in male worms as a disulphide, that is not bioactive. To utilize the compound as a sex pheromone it is reduced and released as the free thiol (Röhl et al., 1999), this process uses 2 GSH (glutathione, a linear tripeptide) per molecule (pers. comm. Dr Jörg Hardege).

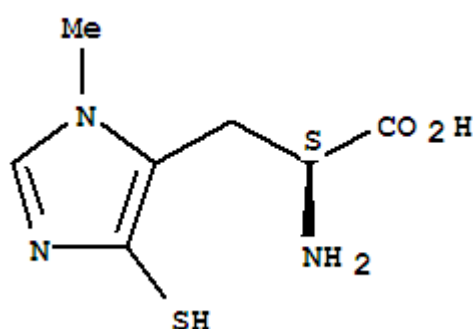


Figure 1.9. Structure of the sex pheromone in *P. dumerilii*, L-Ovothiol A (Source: Guidechem).

1.12.2. *Alitta succinea*

1. Cysteine-glutathione disulphide (CSSG)

Cysteine-glutathione disulphide (‘nereithione’) (CSSG) (Figure 1.10) is a tetra- peptide pheromone released by female *A. succinea* whilst swimming. When released in low concentrations (10^{-9} M), CSSG induces males to significantly increase swimming activity and speed thereby facilitating access to slower swimming females. At high concentrations (in excess of 10^{-6} M) CSSG induces males to release gametes (Ram et al., 1999).

Glutathione (GSH) present within the body fluid and the amino acid cysteine are used to synthesise CSSG on demand. Such production occurs only at the heteronereid stage of the life cycle (Hardege et al., 2004).

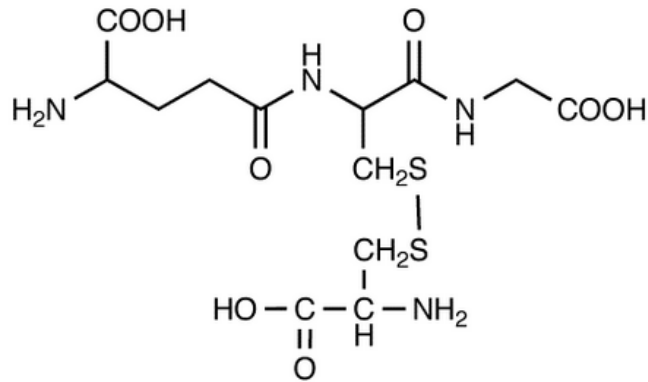


Figure 1.10. Structure of the sex pheromone in *A. succinea*, CSSG (Source: Guidechem).

1.13. Aims and objectives

In a natural environment, animals are exposed to a large number of conflicting chemical cues. Upon detection of such chemicals decisions have to be made that subsequently determine behaviour. If detection of such chemicals is no longer possible due to physical and/or chemical changes to the ecosystem, i.e. chemical nature of the water column, it stands to reason that vital behaviours may be significantly altered. The following investigation aims to assess the effects that ocean acidification (represented by a decrease in ocean pH from 8.2 to 7.8) will have upon the reproductive and feeding activities of the marine polychaetes *P. dumerilii* and *A. succinea* (effects upon reproduction only).

As such this study aims to answer the overall question:

- Does a decrease in pH (as a result of ocean acidification) alter the essential life processes of nereidid polychaetes *Platynereis dumerilii* and *Alitta succinea*?

Objectives

To identify potential differences in the following, when cultured in two different pH conditions (8.2 and 7.8):

Survival and development of juveniles

Gamete production

Male recognition of the female pheromone uric acid/CSSG

Fertilisation (gamete quality)

Larval success

Feeding

Predator avoidance and subsequent feeding

Consumption of food

Chapter 2:

Can nereidid polychaetes survive and reproduce in reduced pH seawater conditions?

2.1. Abstract

For semelparous organisms, a once in a lifetime reproductive event is reliant upon crucial timing. Nereidid polychaetes, like many marine invertebrates, use environmental and endocrine cues to ensure coordinated maturation and precise determination of the time and location of mass spawning events. Chemical signalling via sex pheromones then coordinates their reproductive behaviours that induce the ‘nuptial dance’ reproductive behaviour and the subsequent release of gametes. Pheromones in nereidids include diverse molecules such as volatile lipophilic 5-methyl-3-heptanone, uric acid and small glutathione derived peptides such as cysteine-glutathione disulphide (CSSG).

In recent years, concern has arisen over changing ocean carbonate chemistry as a result of oceanic uptake of anthropogenic carbon dioxide (CO₂). Whilst research to date has focused largely on the impacts upon calcifying organisms, our knowledge of how pH will affect chemoreception and the physiology and fitness of marine organisms is limited. This study evaluates how successful development to maturity and the ability to successfully utilize sex pheromones of nereidid polychaetes, *Platynereis dumerilii* and *Alitta succinea*, are potentially affected. When exposed to pH levels forecasted to occur at ca 2100 (pH 7.8) worms show reduced survival in addition to their ability to reach the sexually mature heteronereis stage. Where maturation occurs, females produce fewer eggs and males show a dramatically reduced response to natural as well as synthetic peptide sex pheromones as exhibited by a significant reduction in swimming speed. Fertilisation and larval success are significantly reduced. In the oceanic environment where chemoreception is widespread, such results indicate disruption to the sensory ability of marine invertebrates may potentially impact upon essential life processes.

2.2. Introduction

2.2.1. Biomarkers to assess fitness

Biomarkers can be defined as detectable biochemical, cellular, physiological or behavioural variations that can be measured in tissue or body fluid samples or at the whole organism level (Depledge et al., 1995). These variations can be used to provide evidence of exposure to and/or the effects of pollutants, being either analytical or behavioural in nature (Depledge et al., 1995). Analytical markers assess the molecular content of cells and tissues whilst behavioural markers use changes in a typical behaviour to be an indicator of exposure to a stressful environmental factor (Depledge et al., 1995).

Assessment of such parameters in key species is often used as an indicator of ecosystem health in marine habitats (Durou et al., 2007). This can be especially useful in areas where exposure to industrial and agricultural effluent is high (Durou et al., 2007).

In addition to their use in the detection of (chemical) pollutant effects, biomarkers are useful tools to determine if an organism has been subject to environmental stress by comparing the responses of healthy, unstressed individuals to conspecifics with known stressors (Depledge et al., 1995). With ever increasing concern regarding the effects of ocean acidification upon marine life, such methods are invaluable as discussed in Hardege et al. (2011).

Changes to seawater chemistry, as a result of ocean acidification, may act directly upon the individual itself. A number of processes predicted to be negatively affected include: growth and development, reproductive output and acid/base regulation (Haye et al., 2012).

2.2.2. Biomarkers to investigate the effects of ocean acidification

A large number of studies to date have focused upon the negative impacts of ocean acidification on calcifying organisms owing to significant reductions in calcium carbonate availability (and thereby calcification) (Bryne, 2011). However, it is now

widely believed that ocean acidification will negatively impact a large suite of marine organisms, including marine invertebrates (Hall-Spencer et al., 2008).

Temperature and pH are among the most important environmental factors controlling the distribution, physiological performance, morphology and behaviour of marine invertebrates (Pörtner et al., 2004). Many marine invertebrates, including *Platynereis dumerilii* and *Alitta succinea*, broadcast-spawn their gametes within the water column for external fertilisation (Byrne, 2011). Studies in recent years have therefore begun to focus upon fertilisation and larval development in an acidified environment due to their sensitivity to changing water chemistry (see Byrne, 2011 for review).

The effects of increased acidification on fertilisation and larval development are varied in studies to date, for example, Byrne et al. (2010) report robustness to ocean acidification in a sea urchin species, *Heliocidaris erythrogramma*, whilst Havenhand et al. (2008) report conflicting results for the same species. For marine invertebrates, sensitive life history stages are of increasing concern as their sensitivity to acidification may present a bottleneck for species persistence (Byrne et al., 2010). It is important, therefore, to gain a wide knowledge of the impacts that ocean acidification will have upon the early life history stages of a large range of species and phyla. This study will be the first to investigate the impacts ocean acidification has upon reproductive fitness in a nereidid polychaete.

This study will assess reproductive output/fitness by comparing a number of ‘biomarkers’ from pH stressed individuals to healthy conspecifics. The ability to reach the mature heteronereis stage will be monitored as will the survival of individuals in each pH treatment over the culture period. Where animals successfully reach maturity; egg production, male swimming speed (an essential behaviour in the reproductive ‘nuptial dance’), fertilisation and larval development will be assessed. These will be deemed to be ‘biomarkers’ of reproductive fitness.

2.2.3. Pheromones mediating broadcast spawning

As well as direct effects upon the individual, ocean acidification may affect the availability and structure of many chemical compounds present within the marine system (Feely et al., 2009). Such chemicals include the pheromones mediating

broadcast spawning in many species, for example the nereidid polychaetes *P. dumerilii* and *A. succinea*.

Semelparous nereidid polychaetes die soon after a once in a lifetime reproductive event. Synchronous spawning has therefore proven essential for their survival and prompted the evolution of mechanisms to eliminate potential gamete wastage in the form of asynchronous gamete release (Hardege et al., 2004). In broadcast spawning events, sex pheromones act as a fine timing mechanism that ensures successful fertilisation (Babcock et al., 1992). These sex pheromones are located in the coelomic fluid of gravid specimens (Townsend, 1939). Exposure of ripe heteronereids to sex pheromone containing body fluid induces the release of gametes and reproductive behaviour, i.e. increase male swimming speed ‘the nuptial dance’ (Hardege et al., 2004). Should ocean acidification alter the structure of such chemicals, worms may no longer be able to successfully detect the chemicals needed for successful reproduction to occur.

2.2.4. Nereidid reproductive behaviours

The reproductive behaviour and chemical nature of sex pheromones of *P. dumerilii* and *A. succinea* have been discussed fully in Chapter 1, section 1.10.

2.3. Research rationale

The life cycle of the nereidid polychaetes *P. dumerilii* and *A. succinea* is relatively short and easy to manipulate within the laboratory. Their ease of culture and accessibility, in addition to the wealth of knowledge available regarding their development and chemical cues (in particular *P. dumerilii*), has led to their selection for the current investigation.

Almost all structurally elucidated aquatic chemical cues are potentially pH dependent (Hay et al., 2009). These include those sex pheromones responsible for the contribution of individuals to subsequent generations and ultimate survival of a species. Chemical cues may be affected by pH either directly through structural or conformational changes (i.e. simple hydrogen bond shifting) or indirectly through altered receptor binding properties (i.e. reduced inter-molecular forces binding constants between receptor and signal molecule) (Hardege et al., 2013, under review). Peptides, thiols, nucleosides and organic acids are known aquatic sex pheromones in nereidid polychaetes (Hardege et al., 2004). It stands to reason that if crucial sex pheromones are negatively impacted by low pH that reproduction and in turn survival of a species may be adversely affected.

There has been much focus on the effects of low pH upon freshwater organisms (Heuschele and Candolin, 2007; Leduc et al., 2004a,b) but few publications detail the effects of pH nereidid polychaetes. Environmental changes may affect an animal's physiology or induce metabolic stress resulting in certain energy-demanding behaviours being allocated a lower priority in favour of maintaining homeostasis (Haye et al., 2012). It is therefore anticipated that pH stress may reduce survival, development and reproductive output. This study will attempt to investigate the effects of low pH upon chemically coordinated reproduction and development in polychaetes.

2.4. Aim

To investigate the effects low pH has upon the reproductive behaviours and survival of *P. dumerilii* and *A. succinea*.

2.4.1. Hypotheses

Hypothesis 1: Survival will be lower in *P. dumerilii* subject to pH stress.

Hypothesis 2: *P. dumerilii* will be less likely to reach maturity when subject to pH stress.

Hypothesis 3: Mature female *P. dumerilii* and *A. succinea* will have reduced egg production when subject to pH stress.

Hypothesis 4: Mature *P. dumerilii* and *A. succinea* will fail to fully respond to sexual chemoreception cues and not perform their normal reproductive behaviour when subject to pH stress.

Hypothesis 5: Fertilisation and larval success will be reduced in *A. succinea* subject to pH stress.

2.5. Materials and Methods

2.5.1. Collection details

Specimens used in this project were collected (with the assistance of J.D. Hardege and other members of the Functional Ecology Research Group) from known populations at: Hinkley Point, Bristol and Cardiff Bay, Wales. *P. dumerilii* conceal themselves amongst seaweed such as *Corallina officinalis* and on the underside of rocks found on the rocky shore at Hinkley Point; they are readily exposed during low tide. *A. succinea* inhabit mussel beds found along the dock walls at Cardiff Bay.

Animals were collected during low tide on the rocky shore in Bristol and from dock walls in Cardiff. Worms were removed on location and transported back to the laboratory in 1 cm depth damp coral sand in small containers to prevent desiccation. Boxes were placed in insulated coolers with appropriate quantities of ice to lower the temperature to between 8 and 12 °C.

2.5.2. Culture conditions

Platynereis dumerilii

Animals were cultured in accordance with procedures detailed by Hauenschild & Fischer (1969). Following collection, juvenile worms were transferred to aerated aquaria, each holding in excess of 50 individuals in a temperature controlled room between 18 and 19 °C. Each tank contained seawater (to 3 cm depth) in one of two pH conditions (8.2 and 7.8). Manual maintenance of pH required the addition of either CO₂ gas (to decrease pH) or liquid NaOH (to increase pH) where needed. Salinity was monitored on a regular basis using a refractometer and maintained at 32.

Commercially available fish diet (Tetramarin®) and minced spinach leaves were used as food for juveniles every 2 weeks and 2 – 3 days respectively. Fish food flakes were ground using a pestle and mortar, and placed in a container with a small amount of seawater. The solution was allowed to ‘settle out’ and the excess liquid decanted to remove dyes which may affect the water quality (this procedure was repeated for both fish food flakes and minced spinach leaves). Food was then distributed sparingly using

a pipette. In both instances, food was rinsed prior to feeding to. The water in these aquaria was changed once a week to remove metabolites, debris and unused food particles. Tanks were covered with lids to prevent fluctuation in salinity due to evaporation.

Artificial illumination was used with a fixed daylight of 16 hours. A low-light moonlight lamp illuminated the animal stock for four consecutive nights in synchrony to the natural lunar phase at 28 day intervals; important for the sexual maturation of juveniles.

Animals were counted Monday to Friday throughout the culture period. Aquaria were checked daily for heteronereids; easily distinguishable by their colouring. Sexually mature individuals were transferred to small crystallising dishes with seawater pasteurised by heating to 80 °C for 30 minutes.

Where successful fertilisations occurred, as determined by observation with a light microscope, eggs were distributed into 2 – 3 crystallising dishes to prevent crowding and oxygen depletion. Mated females were discarded, whilst males were used for additional matings (where possible).

On the seventh day following fertilisation, larvae were fed a few drops of a *Tetraselmis marina* culture. Larval condition was checked daily with the use of a light microscope to establish the need for more *T. marina* (via observation of the larval gut).

After twelve weeks, young worms were carefully transferred to small aquaria. These cultures were left un-aerated for 2 – 3 days to allow the young worms to successfully settle. Henceforth individuals were maintained as detailed above for juveniles. The first water change took place after six weeks. Manual maintenance of pH required the addition of either CO₂ gas (to decrease pH) or liquid NaOH (to increase pH) where needed.

Alitta succinea

Worms were cultured in an adjacent laboratory in a similar manner; 18 (instead of 32) seawater was used, tank dimensions were larger and the only source of food was

Tetramarin ®. Following fertilisation and development to larvae, it proved difficult to sustain larval development (due to problems with larval feeding) and therefore establish F1 and subsequent generations (pers. comm. Dr Jörg Hardege). Larvae were discarded following applicable experiments.

2.5.3. Survival and development

Animals collected from the field were counted Monday to Friday throughout the culture period, the number of individuals reaching the mature heteronereis stage was also ascertained. The number of surviving F1 generation individuals was noted where possible (i.e. upon the production of tubes), every two weeks following fertilisation to obtain sufficient survival data. Three crystallising dishes (maximum) were assessed for each successful fertilisation.

2.5.4. Measures of reproductive fitness

Egg production

The number of eggs released into 40 ml of seawater by mature female *P. dumerilii* (n = 22) and *A. succinea* (n = 30) was used to calculate the number of eggs per ml for each test female. A representative 2 ml sample was assessed and the resulting number divided by two.

Fertilisation and larval success

Female-male *A. succinea* pairs, from the same and opposing culture conditions (i.e. a female and male both cultured in pH 8.2 or 7.8; a female cultured in pH 8.2 and a male cultured in pH 7.8, or vice versa), were assessed for both fertilisation and larval success. Each pair combination (4 in total) was replicated 5 times with different individuals thereby producing mean values for fertilisation and larval success.

Sterile 24 well plates (see Figure 2.1) were prepared prior to the reproductive event. Each row contained one of two seawater pH treatments: 8.2 and 7.8. Each well contained 1 ml pasteurised 18 salinity seawater. This setup allowed for four replicates of each pH treatment per plate.

Males were stimulated to release gametes in 40 ml seawater using low dose (10^{-6} M) CSSG. The sperm was removed using a 0.22 μm filter to leave behind only the egg releasing pheromone and seawater. This 'male water' was then used to stimulate females to spawn in 40 ml 18 pasteurised seawater. 100 μl of eggs were pipetted into each well of the 24 well plate. The test male was again induced to release gametes using high dose CSSG (10^{-4} M) and this time 50 μl of sperm were added to each well plate (See Figure 2.1). Gametes were viable for approximately 20 minutes following release and so this work was carried out carefully and efficiently.

Approximately 2-3 hours following fertilisation, the number of fertilised and unfertilised eggs in each well was counted using a light microscope. Fertilisation success (%) was defined as the percentage of eggs released by a female to be successfully fertilised. Larval success was calculated in much the same way some 48 hours following fertilisation. Larval success (%) was defined as the percentage of fertilised eggs to successfully develop to larvae. Unfertilised eggs (yellow in colour) were easily identifiable by position and a regular circular pattern of lipid droplets around the edge of the eggs. Fertilised eggs (blue or clear in colour) gained a protective surrounding material (jelly matrix) which prevented direct contact with adjacent eggs; instead they formed regular rows equally spaced from one another with lipid droplets in a non-uniform pattern. Successful larval development was characterised by spinning eggs.

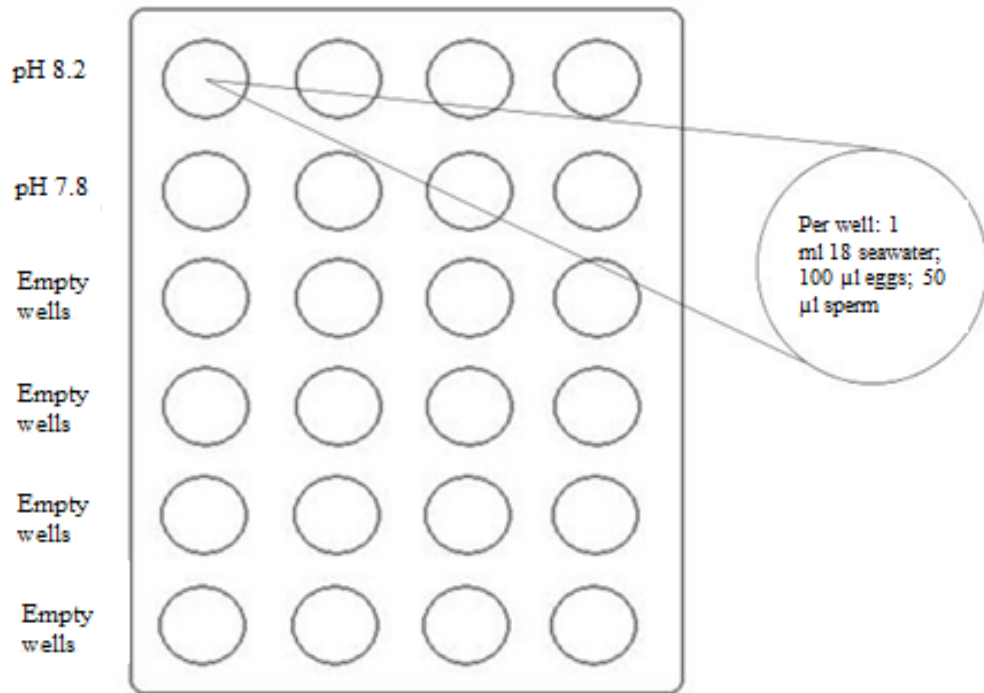


Figure 2.1. Well plate setup for fertilisation and larval success experiments, pH values on the left hand side indicate sea water treatment to be used in the adjacent row.

2.5.5. Behavioural assays

Male ability to detect female pheromone

Uric acid and CSSG (the female produced sex pheromones) induce increased swimming activity in male *P. dumerilii* and *A. succinea*, respectively (Hardege et al., 2004). Behavioural bioassays were carried out to assess the ability of male individuals to detect and respond to uric acid and CSSG when cultured and tested in pH 8.2 and 7.8 seawater. Pheromone detection and response was measured using male swimming speed. Males were exposed to uric acid and CSSG prior to investigation and therefore induced to release gametes. This act ensured each male was fully capable of responding to the female pheromone whilst preventing an increase in response to subsequent exposure by means of pre-adaptation (Wyatt, 2010).

Males were then transferred to a small crystallising dish (approximately 67 mm diameter) containing 40 ml pasteurised 32 (*P. dumerilii*) and 18 (*A. succinea*) salinity seawater. 3 µl of low dose (10^{-6} M) uric acid and CSSG was added and the number of

complete turns around the dish noted over a 30 second time period. This process was repeated for two different seawater pH treatments: 8.2 and 7.8. Males were placed in clean, pH culture, seawater for a 10 minute period between each trial. Swim speeds were calculated as a measure of mm/min utilising the known diameter of the crystallizing dishes used (67 mm) to work out the circumference and therefore the distance travelled per turn. Each male (32 *P. dumerilii*; 40 *A. succinea*) was tested with 10 replicates for each pH treatment. The mean swim speed for each individual was determined at each of the experimental pH treatments.

2.5.6. Data analysis

Data collected was analysed using SigmaPlot 12.0 and R. Tests for normality and equality were used to justify the use of parametric or non-parametric deductive tests. The following statistical analyses were carried out on the respective data sets:

Survival: Gehan-Breslow test.

Development: Chi-Square test.

Egg production: Mann-Whitney test.

Male ability to detect female pheromone: Two-way ANOVA.

Fertilisation and larval success: Two-way ANOVA.

2.6. Results

2.6.1. Survival

Following collection of *P. dumerilii*, the number of individuals was monitored and noted Monday to Friday. The Gehan-Breslow survival test was used to compare multiple survival curves, i.e. individuals cultured at pH 8.2 and 7.8 (Figure 2.2).

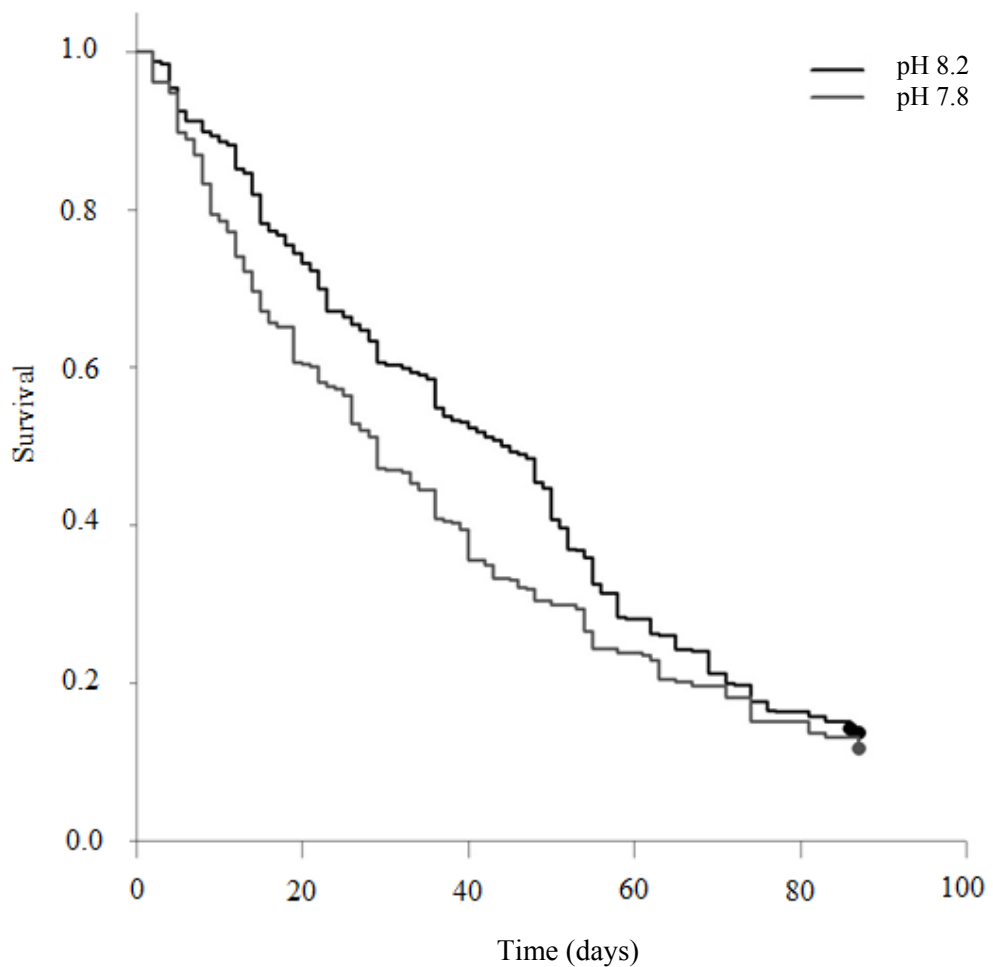


Figure 2.2. Curves to show the survival of *P. dumerilii* cultured in pH 8.2 and 7.8, over a 90 day culture period. $P < 0.001$.

The Gehan-Breslow statistic for the survival curves is greater than would be expected by chance; there is a statistically significant difference between survival curves ($P < 0.001$). As can be seen from Figure 2.2, individuals cultured at pH 7.8 have a lower

survival rate over time. These results suggest that low pH negatively impacts survival of the polychaete *P. dumerilii* in a lifetime.

2.6.2. Development

In addition to monitoring the number of surviving individuals throughout the 90 day culture period, the number of animals to reach maturity in each culture was also noted. There was a statistically significant association between pH culture and the number of animals to reach maturity (Chi-square test, $\chi^2 = 38.50$, $df = 1$, $P < 0.001$) (Figure 2.3). Mature individuals were more significantly associated with pH 8.2 than 7.8.

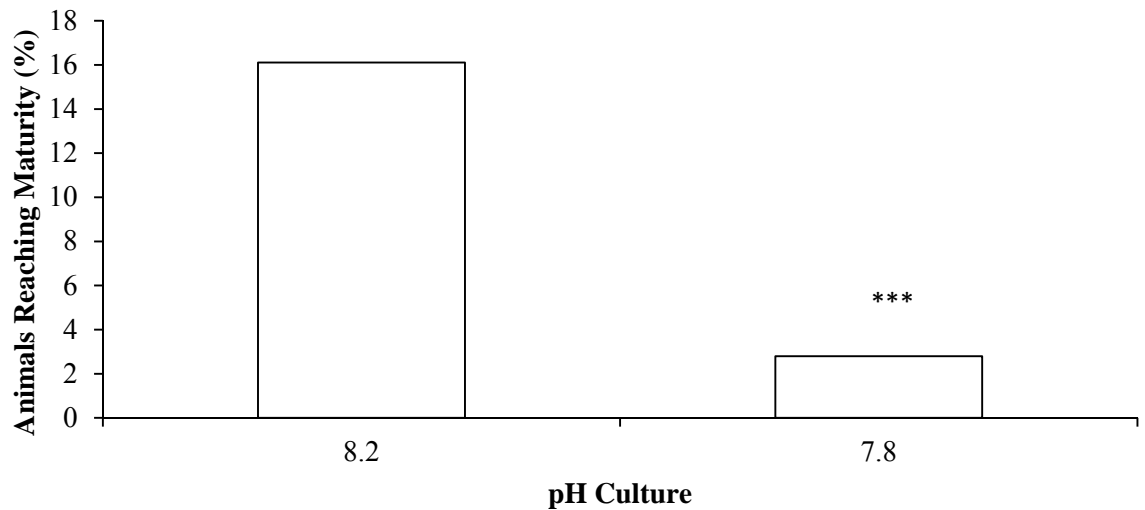


Figure 2.3. Percentage of *P. dumerilii* to reach maturity during the 90 day culture period maintained in one of two pH cultures, 8.2 and 7.8, $n = 565$ individuals per culture. *** = $P < 0.001$.

2.6.3. Egg production

Where mature *P. dumerilii* and *A. succinea* females occurred, they were isolated from their respective pH culture (8.2 and 7.8) and placed in small crystallising dishes. Spawning was allowed to occur and egg production per ml of seawater determined.

Platynereis dumerilii

The number of eggs produced by female *P. dumerilii* for both pH treatments 8.2 and 7.8 conformed to normal distribution (Shapiro-Wilk, $P > 0.15$).

The variances of the two samples could not be considered equal ($P < 0.05$) therefore a Mann-Whitney test was used to test the null hypothesis that there was no significant difference in median egg production between pH treatments 8.2 and 7.8.

There was a statistically significant difference in median number of eggs (Figure 2.4) produced between pH treatments 8.2 and 7.8 (Mann-Whitney, $U = 28.0$, $n_{1,2} = 15,7$, $P < 0.001$) (Figure 2.4).

Alitta succinea

Data failed a Shapiro-Wilk test for normality ($P < 0.05$). The variances of the two samples could not be considered equal ($P < 0.05$) therefore a Mann-Whitney test was used to test the null hypothesis that there was no significant difference in mean egg production between pH treatments 8.2 and 7.8.

There was a statistically significant difference in median number of eggs (Figure 2.5) produced between pH treatments 8.2 and 7.8 (Mann-Whitney, $U = 343.0$, $n_{1,2} = 15$, $P < 0.001$) (Figure 2.5).

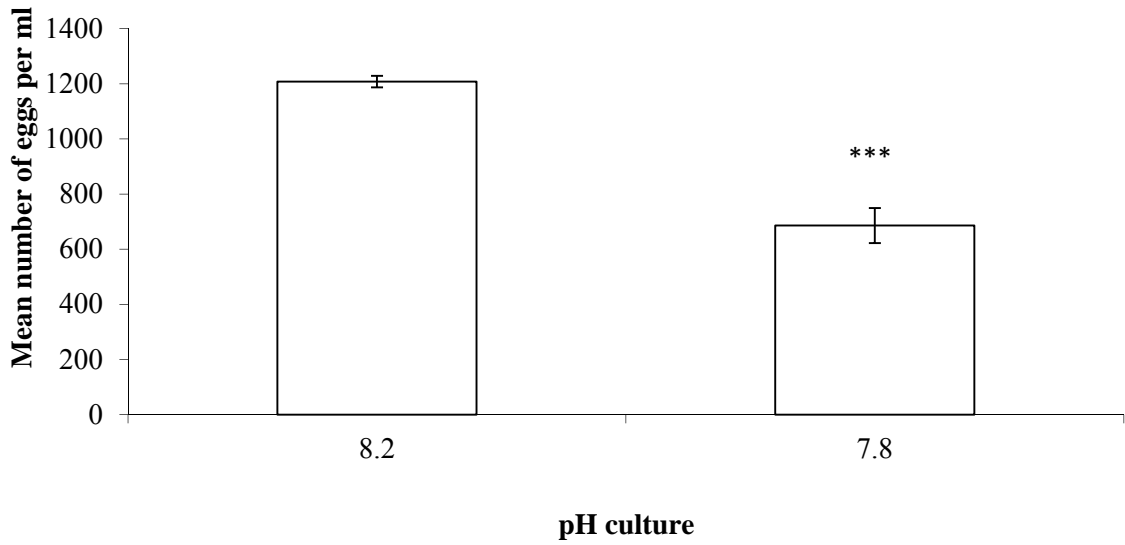


Figure 2.4. Mean number of eggs per ml (\pm SE) released by female *P. dumerilii* maintained in two pH cultures, 8.2 and 7.8, n = 15,7. *** = $P < 0.001$.

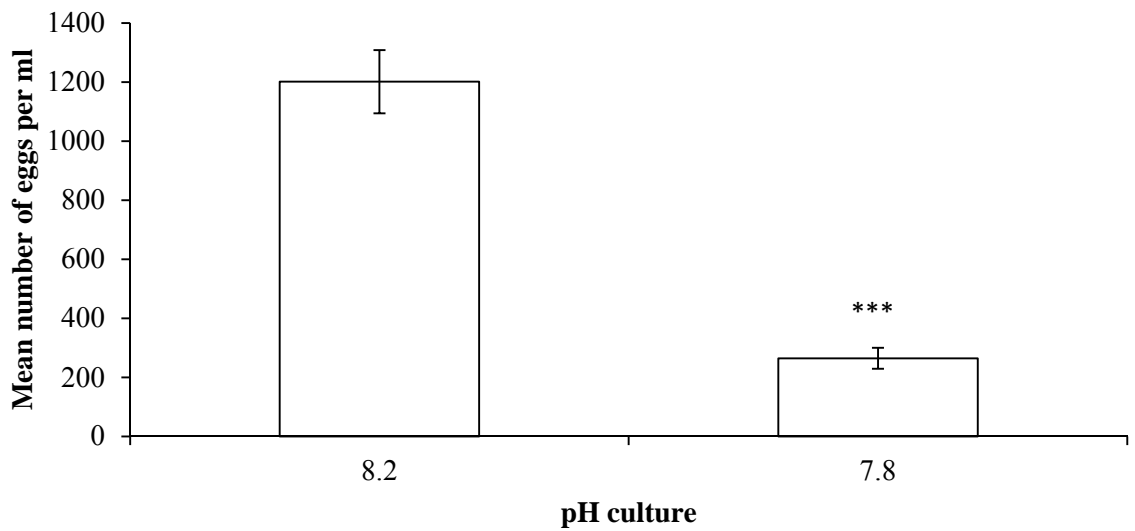


Figure 2.5. Mean number of eggs per ml (\pm SE) released by female *A. succinea* maintained in two pH cultures, 8.2 and 7.8, n = 15 individuals per culture. *** = $P < 0.001$.

2.6.4. Male ability to detect female pheromone

Mature *P. dumerilii* and *A. succinea* males were isolated from their respective cultures and placed in small crystallising dishes. Individuals were induced to swim using 3 μ l of low dose (10^{-6} M) uric acid and CSSG in two seawater pH treatments, 8.2 and 7.8. The number of complete turns around the dish was noted over a 30 second time period. Mean swim speeds were calculated as a measure of mm using the known circumference of the crystallising dish.

Platynereis dumerilii

The two-way ANOVA with interaction for *P. dumerilii* is shown in Table 2.1.

Table 2.1. Two-way ANOVA for the median swim speed of male *P. dumerilii* from pH cultures 8.2 and 7.8, in response to 3 μ l of the female pheromone uric acid (10^{-6} M) in pH 8.2 and 7.8 seawater.

Source	df	SS	MS	F value	Sig.
pH treatment	1	0.9607	0.9607	28.4747	<0.001
pH culture	1	5.3832	5.3832	159.5532	<0.001
pH treatment*pH culture	1	0.2531	0.2531	7.5029	<0.05
Error	28	0.9447	0.0337		

There was a statistically significant interaction between pH treatment and pH culture (ANOVA, $df = 1$, $F = 7.50$, $P < 0.05$) (Table 2.1) for the median swim speed of male *P. dumerilii* (Figure 2.6).

When males are cultured at pH 7.8, mean swim speed is significantly reduced in both pH treatments (Figure 2.6). Mean swim speed is significantly reduced at pH treatment 7.8 regardless of the pH culture that the males originate from (Figure 2.6).

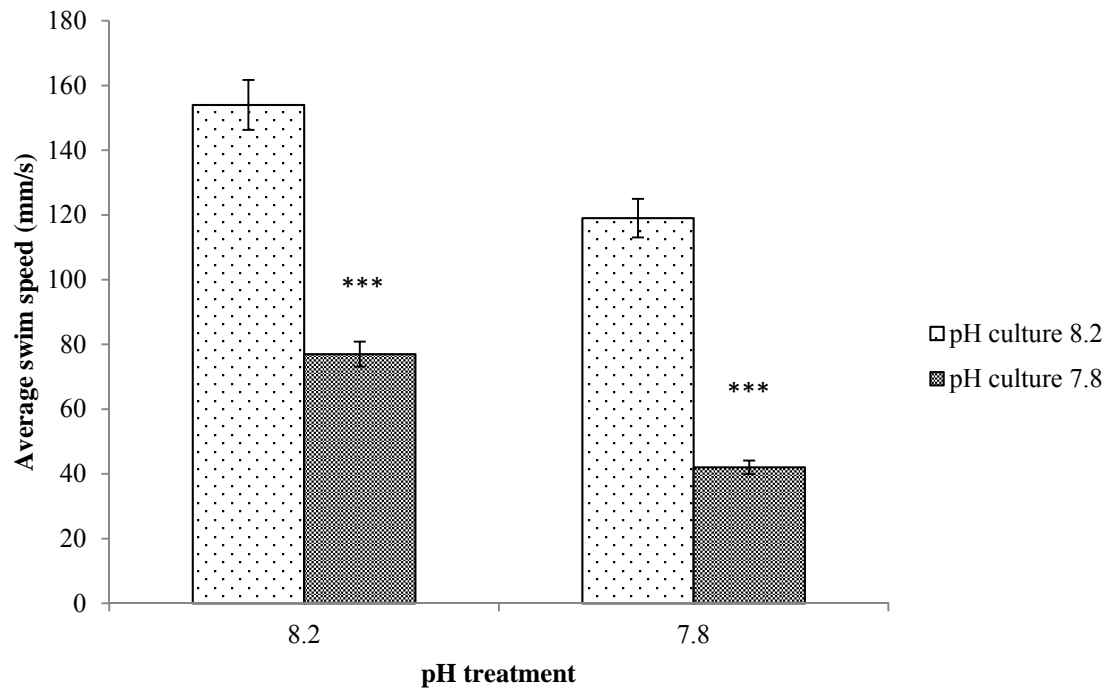


Figure 2.6. Mean swim speed (mm/s) of male *P. dumerilii* (\pm SE) at pH 8.2 and 7.8 n = 15,7. *** = $P < 0.001$.

Alitta succinea

The two-way ANOVA with interaction for *A. succinea* is shown in Table 2.2.

Table 2.2. Two-way ANOVA for the median swim speed of male *A. succinea* from pH cultures 8.2 and 7.8, in response to 3 μ l of the female pheromone CSSG (10^{-6} M) in pH 8.2 and 7.8 seawater.

Source	df	SS	MS	F value	Sig.
pH treatment	1	0.1455	0.1455	0.7661	>0.05
pH culture	1	11.9002	11.9002	62..6585	<0.001
pH treatment*pH culture	1	1.2653	1.2653	6.6623	<0.05
Error	37	6.8371	0.1899		

There was a statistically significant interaction between pH treatment and pH culture (ANOVA, $df = 1$, $F = 6.66$, $P < 0.05$) (Table 2.2) for the median swim speed of male *P. dumerilii* (Figure 2.7).

When males are cultured at pH 7.8, mean swim speed is significantly reduced in both pH treatments (Figure 2.7). Interestingly, mean swim speed is significantly reduced at pH treatment 7.8 when males are cultured at pH 8.2 but not pH 7.8 (Table 2.2, Figure 2.7).

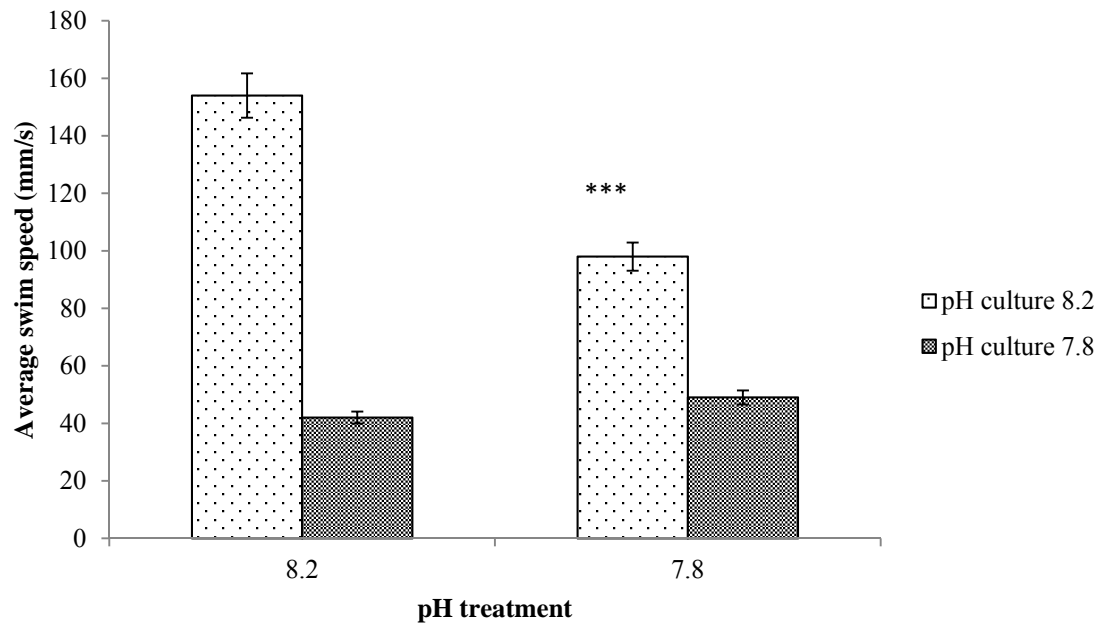


Figure 2.7. Mean swim speed (mm/s) of male *A. succinea* (\pm SE) at pH 8.2 and 7.8 $n = 15,7$. *** = $P < 0.001$.

2.6.5. Fertilisation success

Female and male *A. succinea* were cultured in one of two pH cultures, 8.2 and 7.8. Mature individuals were paired together from the same and opposing cultures to form 4 female-male pair combinations, for example, female 8.2 – male 8.2, female 8.2 – male 7.8 and so on. Individuals were allowed to spawn and eggs and sperm then combined in one of two pH treatments, 8.2 and 7.8. Fertilisation success was determined as the mean number of eggs to be fertilised. The two-way ANOVA with interaction is shown in Table 2.3.

Table 2.3. Two-way ANOVA for the median fertilisation success of *A. succinea* from pH cultures 8.2 and 7.8, in seawater pH 8.2 and 7.8.

Source	df	SS	MS	F value	Sig.
pH treatment	1	2.2756	2.27565	35.9682	<0.001
pH culture	3	9.1519	3.05062	48.2172	<0.001
pH treatment*pH culture	3	1.0300	0.34333	5.4266	<0.01
Error	152	9.6168	0.06327		

There was a statistically significant interaction between pH treatment and pH culture (ANOVA, $df = 3$, $F = 5.43$, $P < 0.01$) (Table 2.3) for the median fertilisation success of *A. succinea*. An interaction plot allows for further investigation (Figure 2.8).

As can be seen from the Figure 2.8, fertilisation success is significantly reduced at pH treatment 7.8 regardless of the pH culture that the mating pairs originate from. When males are cultured at pH 7.8 however, fertilisation success is significantly reduced in both pH treatments. These results suggest low pH has a negative impact upon fertilisation; males appear to be the limiting factor in mean fertilisation success of *A. succinea*.

Tukey's HSD post-hoc multiple comparison test showed that when males originate from the same pH culture, there is no significant difference in fertilisation success at pH treatment 8.2 ($P < 0.001$) (Figure 2.8). Fertilisation success was significantly reduced in males cultured at pH 7.8 versus 8.2. Similarly, at pH treatment 7.8 ($P < 0.05$) (Figure

2.8), fertilisation success was significantly reduced when males were cultured at low pH or females were cultured at high pH. The optimal fertilisation occurred when females were cultured at pH 7.8 and males at pH 8.2. These findings further reinforce the theory that male *A. succinea* dictate and affect fertilisation success.

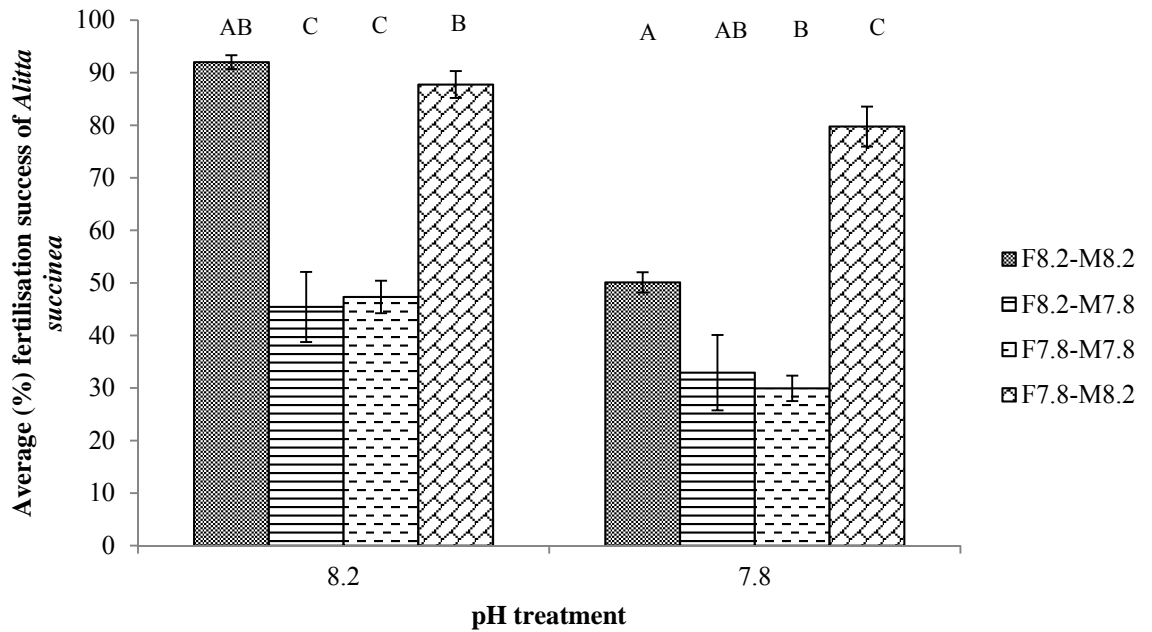


Figure 2.8. Average (%) fertilisation success of *A. succinea* at pH treatment 8.2 and 7.8 when females (F) and males (M) were cultured in the same and opposing pH conditions. Letters represent significance based on separate Tukeys HSD post-hoc analysis tests for each pH treatment.

2.6.6. Larval success

Following fertilisation, fertilised eggs were allowed to develop to larvae. Larval success was determined as the mean number of fertilised eggs to successfully develop to larvae. The two-way ANOVA with interaction is shown in Table 2.4.

Table 2.4. Two-way ANOVA for the median larval success of *A. succinea* from pH cultures 8.2 and 7.8, in seawater pH 8.2 and 7.8.

Source	df	SS	MS	F value	Sig.
pH treatment	1	1.7601	1.76014	62.9136	<0.001
pH culture	3	5.0632	1.68774	60.3255	<0.001
pH treatment*pH culture	3	0.3168	0.10561	3.7747	<0.05
Error	152	4.2525	0.02798		

There was a statistically significant interaction between pH treatment and pH culture (ANOVA, $df = 3$, $F = 3.77$, $P < 0.05$) (Table 2.4) for the median larval success of *A. succinea*. An interaction plot allows for further investigation (Figure 2.10).

As with fertilisation success, larval success is significantly reduced at pH treatment 7.8 regardless of the pH culture that the mating pairs originate from (Figure 2.9). However, in contrast to fertilisation success where it seems males are the limiting factor, results here suggest that females are the limiting factor in successful breeding. When females are cultured at pH 7.8 larval success is significantly reduced in both pH treatments (Figure 2.9).

Tukey's HSD post-hoc multiple comparison test showed that there was a significant difference in larval success when females originated from different pH cultures at pH treatment 8.2 ($P < 0.001$) (Figure 2.9) and 7.8 ($P < 0.001$) (Figure 2.9). Larval success was significantly reduced when females were cultured at pH 7.8 versus 8.2.

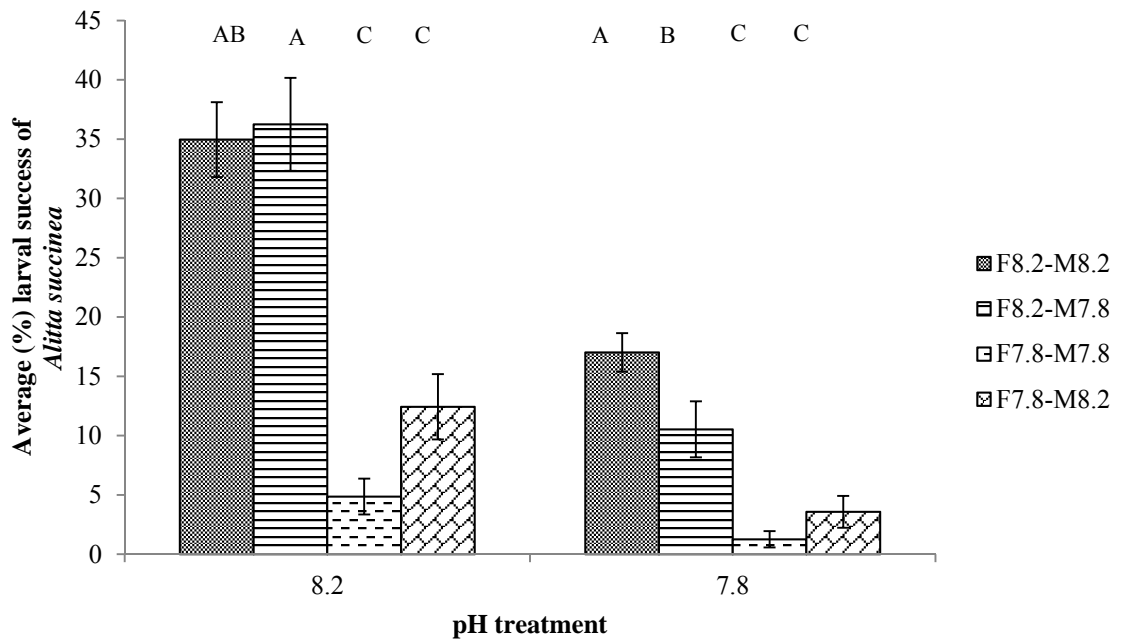


Figure 2.9. Average (%) larval success of *A. succinea* at pH treatment 8.2 and 7.8 when females (F) and males (M) were cultured in the same and opposing pH conditions. Letters represent significance based on separate Tukeys HSD post-hoc analysis tests for each pH treatment.

2.7. Discussion

2.7.1. Survival and development

Survival was markedly decreased at pH 7.8 (dropping to less than 5 % after 8 weeks of culture and 0 % some 4 weeks later) when compared with the control group, pH 8.2 (survival decreased to 20 % and 6 % respectively) (see Figure 2.2). These results coincide with survival data collected (within the research group) in similar investigations with a closely related species, *A. succinea* (unpublished data, 2008; 2009, 2010). Whilst lower at pH 7.8, survival was also low at pH 8.

Metamorphosis to the swimming heteronereid stage is critical in order for reproduction to occur. It is only at this stage that organisms are able to produce gametes to store in the body cavity, swim to the water surface and engage in the nuptial dance required for reproduction (Hardege et al., 2004). A significant number of individuals within a population need to reach this stage in order to contribute to the next generation and maintain a healthy population. If organisms fail to reach maturity in the marine environment populations may cease to exist. The percentage of animals reaching the heteronereid stage was significantly reduced following prolonged exposure to pH 7.8 (see Figure 2.3). Fewer heteronereids means fewer opportunities for reproduction.

Whilst body size (length in mm) of juvenile and adult *P. dumerilii* collected from the field was not obtained, general observations during culture noted an apparent reduction in growth between pH cultures with smaller individuals at pH 7.8. Growth data collected (within the research group) on *A. succinea* confirms these observations (unpublished data, 2008, 2009, 2010). These findings coincide with the low number of organisms reaching maturity and fertilisations observed within this investigation. It was the latter that led to the need for fertilisations to be carried out using the closely related species, *A. succinea* that exhibits an almost identical reproductive behaviour (Hardege, 2004).

2.7.2. Measures of reproductive fitness

Egg production

Female *P. dumerilii* and *A. succinea* cultured in pH 7.8 released significantly fewer eggs than the control pH 8.2 (see Figure 2.4, 2.5). As a semelparous broadcast spawner, both males and females need to produce a large number of gametes in order to increase the probability of contact and subsequent fertilisation (Hardege, 1999).

Stressor induced fecundity reduction as seen in a number of studies (Tominaga et al., 2004; Hansen et al., 2003; Saha et al., 1999), including this investigation, will reduce the number of offspring able to reproduce, contribute to the next generation and ultimately survival of the species. The toxic haptophyte *Prymnesium parvum* has been shown to reduce fecundity in the calanoid copepods *Eurytemora affinis* and *Acartia bifilosa* (Sopanen et al., 2006). Similarly, copepod fecundity has also been shown to be negatively impacted as a result of ocean acidification (Fitzer et al., 2012). Whilst the cause of this reduction has not yet been proven it is suggested that ocean acidification suppresses metabolic activity through reduced protein synthesis which in turn leads to a depressed reproductive capability (Fitzer et al., 2012).

A number of studies have hypothesised that body size may directly influence fecundity (Chatzinikolaou and Richardson, 2010). As discussed above, worms cultured at pH 7.8 appeared to exhibit reduced growth. Whilst these preliminary observations were not translated into data, egg production was significantly reduced in pH stress conditions. This may suggest a direct correlation between body size and fecundity. However, Rettob (2012) found that only 26% of *Perenereis cultrifera* appeared to be influenced by body length. A (presumed) reduction in somatic growth as a result of pH stress may therefore not be responsible for a reduction in egg production as seen here. Conversely, Olive et al (1997) found the gonad index (defined as the ratio of gonad biomass to total biomass) of *Nephtys hombergi* to be age/size dependent.

Further study by Schreck et al (2001) has shown other environmental stressors, in particular nutrition, were found to affect fecundity and gamete quality of rainbow trout, *Oncorhynchus mykiss*. Red abalone gamete production (both sperm and egg) has since been shown to be negatively impacted by warm water and starvation stressors (Rogers-

Bennett et al., 2010). Females in ‘ideal’ conditions produced between 3 and 21 million mature oocytes whilst those exposed to warm waters produced only 400,000; those subject to starvation produced none. Should ocean acidification negatively impact prey availability, said stressor will combine with that of the acidified conditions to further exacerbate a reduction in egg production.

Some marine organisms may be more resilient to acidification stressors; CO₂-induced seawater acidification (pH 7.6) appeared to have no significant effect upon *Calanus glacialis*, an Arctic shelf-water copepod, in a study by Weydmann et al., 2012. Reduction in pH seawater to 6.9 did, however, significantly delay hatching and may therefore have impacted upon overall hatching success (Weydmann et al., 2012).

Sex pheromones

The detection of chemicals in the marine environment is crucial for the functioning of the reproductive event. As broadcast spawning species which release gametes into the water column, *P. dumerilii* and *A. succinea* rely heavily upon chemical communication (Hardege, 1999). Disruption to the detection of sex pheromones will have a significant impact upon their ability to successfully reproduce and in turn maintain a healthy population for survival.

A significant decrease in *P. dumerilii* and *A. succinea* swim speed was noted when males were cultured at pH 7.8 (see Figure 2.6, 2.7). Such responses may indicate an impaired ability to detect and adequately respond to female pheromones or result from poor swimming ability as low pH impacts upon the fitness of the worm itself.

It has been hypothesised that pheromone detection is potentially pH dependent, affected directly through structural or conformational changes to the peptide pheromone molecule, i.e. simple hydrogen bonding shifting, or indirectly through alteration to the receptor (Hardege et al., 2011). Chemoreceptors, required for the detection of chemical stimuli including pheromones, are membrane bound proteins. The chemoreceptors of male *A. succinea* (also used in the detection of food) are located on the cirri of parapodia to the front and rear of the organism (Hardege, 1999). Little is known, however, with regards to the receptor structure and method of signal transduction.

When signal molecules bind with the receptor a series of internal events are initiated leading to a specific developmental or behavioural response (Wyatt, 2003). Signal molecules have specific 3D structures recognisable by the chemoreceptor. Changes in pH alter the acid dissociation (pKa), charge, shape and structure of molecules (Hardege et al., 2013, under review). Sufficient changes in the receptor and/or substrate may prevent the successful binding of the two thereby preventing the developmental or behavioural response. This was shown to be the case in a study carried out by Xu et al. (2010). At a reduced pH of 4.5 the pheromone binding protein (AtraPBP1) of the naval orange worm moth, *Amyebis transitella*, was no longer able to successfully bind with the receptor due to the formation of a C-terminal helix within the receptor itself.

When male *A. succinea* were cultured at pH 7.8, swimming speed was slightly higher at pH treatment 7.8 than 8.2 (the opposite was true when males were cultured at pH 8.2) (see Figure 2.7). This may be indicative of some means of acclimatisation to lower pH. However, when compared to the control culture, pH 8.2, swimming speed was significantly lower in all instances (see Figure 2.7). Similarly, when male *P. dumerilii* were cultured at pH 7.8, swimming speed was significantly lower than those worms cultured at pH 8.2. Swimming speed was significantly lower at pH treatment 7.8 than 8.2 (see Figure 2.6) when worms were cultured at pH 8.2 and 7.8.

It seems more likely therefore that lowered swim speed is a result of poor swimming performance, indicative of reduced fitness, i.e. a reduction in swimming ability due to impaired muscle function (in individuals cultured at low pH) as opposed to disruption of chemical reception. If the sense of smell were affected as a result of lowered pH the effect upon swim speed would be all-or-nothing, i.e. males would simply not alter their swim speed.

Worm size is directly linked to swimming ability (Ram & Hardege, 2005). As individuals cultured at low pH appeared to be smaller, it is possible that whilst able to detect the chemical signal they were in too poor a condition to adequately respond with an increase in swimming speed. It should be noted, all males were shown to be capable of responding to the female pheromones, uric acid and CSSG, prior to experimentation through treatment with synthetic uric acid and CSSG. As such all individuals tested were capable of performing the desired behavioural response and the related higher

swim speeds. Their subsequent inability to respond in trials suggests a reduction in fitness.

Due to the ubiquity of chemical communication in the marine environment and its importance in many processes needed for survival, a reduction in the ability to detect chemical signals could have devastating impacts upon the marine ecosystem. The effects upon such processes have been studied in freshwater ecosystems (Leduc et al., 2004a) and are now beginning to emerge in the marine counterpart (Munday et al., 2009a; Dixon et al., 2010).

Fertilisation and larval success

Fertilisation is essential for the development and survival of any species. Many marine invertebrates, including *Platynereis dumerilii* and *Alitta succinea* rely upon fertilisation in the water column via free spawning gametes (Hardege et al., 1999). These gametes are particularly sensitive to changes in the environment and as such have been used in the assessment of toxicants for many years (Dinnel et al. 1987; Ringwood 1992; Bay et al 1993; Carr et al 2006). As the ocean continues to increase in both temperature and PCO₂ (Caldeira and Wickett 2003; IPCC 2007; Fabry et al. 2009; Pörtner 2008), gametes will undoubtedly be subject to further stressors. It stands to reason, therefore, that if fertilisation is negatively impacted upon, the survival of many species will be at risk.

Recent fertilisation studies (Reviews: Kurihara 2008; Bryne 2010) in which six echinoderm and mollusc species were maintained at pH 7.6, indicated some degree of robustness to climate change stressors. Negative effects were experienced only in more extreme conditions (e.g. 2300 and beyond, pH 7.4, PCO₂ 2000+ ppm). Of the six species only *Heliocidaris tuberculata* showed slight sensitivity to acidification. Similar results were obtained by Ericson et al. (2010) whilst, conversely, data obtained by Havenhand et al (2008) showed statistically significant reductions in sperm swimming speed and percent sperm motility of the sea urchin *Heliocidaris erthrogramma* at pH 7.7. Incorporating these results into a fertilisation kinetics model (Styan 1998) predicted an acidification-induced reduction in fertilisation success of 24.9 %.

Fertilisation in *A. succinea* appears to be somewhat more sensitive to pH stress than the species detailed above. Fertilisation was significantly reduced at pH treatment 7.8 regardless of the pH culture the mating pairs originated from (see Figure 2.8). Moreover, fertilisation success was significantly lower when males were cultured at pH 7.8 (see Figure 2.8). These results suggest males become a limiting factor impacting fertilisation success when cultured and tested at low pH (7.8). *A. succinea* live in self-built sediment cores, U-tubes, and as such create their own microclimate. The pH of sediment is potentially much more stable than that of the surrounding water column. Organisms that live within it may be more sensitive to pH fluctuations, and therefore more at risk to levels forecasted to occur by 2100. *A. succinea* are, however, a brackish water species and as such may be capable of tolerating fluctuating salinity. As can clearly be seen, it is difficult to predict resilience to pH change when incorporating a number of factors.

Interestingly, when females were cultured at pH 7.8 fertilisation success was higher at pH treatment 7.8 than those females cultured at pH 8.2 (see Figure 2.8). This may suggest a degree of tolerance or adaptation to pH stress following prolonged exposure to low pH as has been shown in previous investigations, for example, Byrne et al. (2010). Conversely, these results may be explained by a simple egg to sperm ratio. While numbers of eggs produced was reduced at pH 7.8 the same measure of sperm (50 μ l) was added each time thereby increasing the probability of contact and fertilisation regardless of pH treatment.

These findings have important implications for the detection of chemical cues in the marine environment; the behavioural and developmental processes which they govern and most importantly the survival of a number of species. Further research into how chemical signals may be affected is urgently required, a view further emphasised by the variability in results between related investigations on ocean acidification effects upon reproduction.

Larvae are subject to many stressors, including pH, in the marine environment and it stands to reason therefore that not all fertilised eggs will successfully develop into juvenile individuals. High fertilisation success is therefore not indicative of a high F1 generation. It is important to assess larval success in conjunction with fertilisation

success as neither alone can be used as a cleared indicator of the effects of reduced pH upon reproductive output.

Like fertilisation success, larval success was significantly reduced at pH treatment 7.8 regardless of the pH culture the mating pairs originated from (see Figure 2.10). When females were cultured at pH 7.8, larval success was significantly lower in all instances (Figure 2.10). These results suggest that this time it is the female that becomes the limiting factor impacting larval success when maintained and tested at low pH. Previous data sets (within this investigation) have shown that egg production is significantly reduced at low pH. It seems likely that those eggs produced are of a lower quality and therefore negatively impact upon successful larval development. Sea urchins and their larval stages are regarded as one of the more sensitive taxa due to their carbonate skeletons (Stumpp et al., 2011). *Strongylocentrotus purpuratus* embryos were raised in control (pH 8.1) and CO₂ acidified seawater (pH 7.7) growth and development were assessed through the measurement of a number of parameters. Following one week of development, comparison of these parameters between treatments suggests larvae suffer a developmental delay (Stumpp et al., 2011)

As with fertilisation, Havenhand et al. (2008) found larval development of the sea urchin *H. erythrogramma* to be significantly reduced at pH 7.7 when compared with those maintained at pH 8.2. Once again, these results contradict those of Byrne et al (2010) which indicated robustness to climate change stressors. Negative effects were observed only in much more extreme conditions predicted for the year 2300 and beyond, a pH of 7.4. More recently Martin et al. (2011) found the sea urchin *Paracentrotus lividus* appears to be extremely resistant to low pH with no significant effect upon fertilisation or larval success. It was noted, however, that larval development whilst normal was slowed at low pH.

The above studies focus upon organisms from a marine environment, with few studies investigating the more variable coastal ecosystems. In a study by Pansch et al. (2012), barnacles *Amphibalanus improvisus* from the variable Kiel Fjord were subject to simulated warming and ocean acidification during early development. None of the CO₂ induced acidification treatments significantly impacted larval development suggesting tolerance, perhaps in part due to the variable environment in which they naturally reside (Pansch et al., 2012). These results reinforce the previous hypothesis that species such

as *A. succinea* (which also reside in brackish water environments) may be more resilient to future fluctuations in pH than a fully marine species such as *P. dumerilii*.

2.7.3. Evolutionary context

It is unclear whether marine communication systems have the plasticity to adapt to pH changes predicted for the coming century. Rapid evolution of olfactory systems is potentially viable in species where structural differences can result from biochemical modifications, or where signalling bouquets are involved. Cuticular hydrocarbon profiles (CHC's) in fruit flies, *Drosophila melanogaster*, are to date, the only experimental proof for co-evolutionary changes of lock-key pheromone systems (Shirangi et al., 2009).

Pheromone evolution can occur via alteration to the ratio of compounds comprising the pheromonal bouquet (Lofstedt, 1993). This evolutionary mechanism does not alter the structure of the pheromone or receptor, merely the amount at which the pheromone molecules occur. As the female pheromones of *P. dumerilii* and *A. succinea* comprise only one pheromone molecule this method of evolution is not possible.

External and internal pressures favour structurally different enantiomers or isomers in pheromonal systems. The bark beetle, *Ips pini*, emit an aggregation pheromone ipsdienol. Predators are able to detect a specific chiral enantiomer of this molecule to locate and prey upon them (Raffa and Klepzig, 1989). In response to this selection pressure *I. pini* emit the opposite chiral enantiomer allowing them to remain in the environment undetected. The molecule itself remains largely unchanged whilst in a different orientation. If a similar change were to occur to the sex pheromone of *P. dumerilii* and *A. succinea*, it would be of little benefit as the molecule itself would still be susceptible to more damaging structural changes potentially preventing binding with the receptor molecule.

Chapter 3:

Are essential behavioural responses affected by short term and long term exposure to low pH?

3.1. Abstract

As with many marine organisms, *Platynereis dumerilii* adapt their behaviour in accordance with chemical cues encountered. Owing to the complex chemical background noise associated with an aquatic environment, animals need to distinguish between specific cues within a number of odours. Amongst the essential behaviours controlled by chemoreception are feeding and the detection of predators. Abiotic disruptions to such life processes could have serious impacts upon the survival and fitness of aquatic individuals.

In recent years, concern has arisen over changing ocean carbonate chemistry as a result of oceanic uptake of anthropogenic carbon dioxide (CO₂). Our knowledge of how pH will affect chemoreception is limited. This study shows that upon short term and long term exposure to low pH, *P. dumerilii* fails to respond effectively to food and chemical stimulants glycine and taurine. The effects of this are potentially severe, increased foraging times needed to compensate for failed attempts to locate food will result in higher predation levels as individuals spend longer exposed and take higher risks. When presented with predator odour *P. dumerilii* fails to respond and exhibit predator avoidance behaviour. Consumption of spinach at 24 and 48 hours post feeding is significantly reduced when individuals are cultured at pH 7.8. Coupled with the need for increased foraging times such effects upon chemo-responsiveness could have major impacts to the individual, population and survival of the species.

3.2. Introduction

3.2.1. Chemoreception

Chemical senses are utilised by organisms throughout the animal kingdom; chemical signals bind to receptor sites which then transfer appropriate information to the brain (Wyatt, 2010). Chemoreception can be categorised as gustatory (taste) or olfactory (smell) (Drickamer et al., 1996). Chemoreceptors responsible for chemoreception allow not only distinction of kin and gender in conspecifics; but are highly sensitised allowing animals to detect species-specific chemicals when concentration may be as low as several molecules per million (Bronmark and Hansson, 2012).

In a complex aquatic environment, chemoreceptors allow organisms to perceive and process information where visual or physical cues are not always available (Bronmark and Hansson, 2012). Chemical signals are crucial in eliciting essential behaviours such as reproduction (as discussed in Chapter 2), feeding and predator avoidance in many aquatic organisms (Dunham, 1978). Chemical cues and signals must be produced, released, received and processed by the receiver to be a successful means of communication (Wyatt, 2003). The term ‘signal disruption’ denotes interference with any of these steps; such disruption may lead to reduced efficiency of information transfer (Wyatt, 2003).

The majority of chemical signals act at very low concentrations and degrade rapidly within the aquatic environment (Bronmark and Hansson, 2012). Furthermore, the aquatic environment comprises a cocktail of ambient chemicals and organisms must therefore be able to distinguish between odours (Derby and Sorenson, 2008). Chemical signals often occur in complex blends, instead of pure compounds (Wyatt, 2010). Over evolutionary time, organisms have therefore developed a very finely tuned chemosensory system to detect and react to these compounds (Bronmark and Hansson, 2012). If the ability to detect and successfully identify odours is impaired via ‘signal disruption’, this may have serious consequences for the survival and fitness of animals that rely heavily upon chemoreception (Wisenden, 2000).

3.2.2. Feeding stimulants and behaviour

The behavioural mechanisms by which marine animals find food vary widely. Some organisms use visual receptors to detect the colour, size and shape of food items whilst others locate food by mechanoreceptors. Mechanoreceptors sense hydrodynamic disturbances within the water column caused by moving prey. Sea anemones, such as *Haliplanella luciae*, possess thousands of hair bundle mechanoreceptors upon their tentacles which respond when adequately stimulated, i.e. water vibrations caused by small swimming animals (Watson and Hessinger, 1989). However, in a complex aquatic environment where visual location of food is not always possible, a large number of organisms rely upon chemoreception, using a complex mixture of chemical cues to stimulate appropriate sensory organs and locate food (Bronmark and Hansson, 2000). Chemoreception is also important for discriminating between food choices, for example, between distasteful or harmful substances and nutritious ones (Wyatt, 2003).

Chemicals affecting feeding behaviour are classified into those which exert their effect at a distance (attractants, arrestants and repellents) and those which require contact (incitants and suppressants, stimulants and deterrents) (Bronmark and Hansson, 2012). Fish are able to detect food at a distance either visually or chemically; in all instances it is gustation that determines the final decision to ingest food or not (Bronmark and Hansson, 2012). Smaller organisms, such as nereidid polychaetes, are able to detect food visually at much shorter distances and therefore rely heavily upon chemical detection within the water column.

Copeland and Wieman (1924) were one of the first to study and detail the feeding behaviour of a polychaete, *Nereis virens*. When exposed to crushed periwinkles and other animal food *N. virens* would emerge from its tube. Exploiting this feeding behaviour, Watson et al. (2005) exposed *N. virens* to whole-body extracts of conspecifics, polychaete prey species and flatfish muscle (predator). Feeding and foraging activity were significantly reduced when *N. virens* were exposed to whole-body extracts of conspecifics; these extracts were deemed to act as an alarm signal. Extracts of flatfish muscle reduced the number of worms feeding but did not impact upon activity outside the burrow (Watson et al., 2005).

Similar experiments were carried out by Magnum and Cox (1971); extracts of 32 marine organisms and chemical substances (including amino acids found in extracts of bivalve flesh) were used to describe and monitor feeding behaviour of the onuphid polychaete, *Diopatra cuprea* (Lindsay, 2009). Detection of such stimulants may be controlled by the nuchal organs, generally considered the primary chemoreceptive organ in polychaetes (Lindsay, 2009).

Whilst the feeding behaviour and ecology of polychaetes has been studied for many years, there is a wealth of data in comparison for both fish and crustaceans (Derby et al., 1996; Ache, 1982; Hara, 1975; Carr, 1982; Mackie and Grant, 1974).

Fishes

The successful acquisition of food by fishes begins with foraging followed by detection, capture and finally digestion (Hara, 2006). A number of sensory systems contribute to the feeding process with the chemosensory system playing a dominant role in many fish species (Hara, 2006). Naturally occurring amino acids are effective olfactory stimuli (Yamashita et al., 2006). Cell organization of the sensory epithelia and characteristic response to these chemicals is fairly uniform throughout fish species (Hara, 2006). In contrast, the specialised end organ of the gustatory system, i.e. taste buds, differs from species to species. Fishes are loosely classified into those that are broadly tuned, responding to most naturally occurring amino acids and those that are narrowly tuned, responding to a few amino acids only (Hara, 1994).

Crustaceans

In marine crustaceans, feeding cues generally consist of simple organic molecules that can be easily replaced with comparable synthetic mixtures based on naturally-occurring ones (Bronmark and Hansson, 2012). In response to chemical stimulus, antennular flicking will occur in many species, for example, *Carcinus maenas*. This is a sharp downward movement of the filament to allow increased exposure to the surrounding chemical environment (Schmitt and Ache, 1979). Whilst effective, synthetic chemicals have not proven to be as great a stimulant as natural foods (Shelton and Mackie, 1971).

Amino acids and amines are highly excitatory to crustacean receptor cells (Derby and Ache, 1984). Furthermore they have been shown to be effective chemical feeding stimuli in a number of species (Tierney and Atema, 1987; Hara, 2006). Glycine commonly occurs in the tissues and excretory products of many invertebrates, a key component of many crustaceans diets (Hayden et al., 2007). It has been found to elicit feeding movements and significantly increase feeding behaviours in the crayfish, *Orconectes virilis* and *Orconectes rusticus* (Tierney and Atema, 1998). Chemosensitivity to specific compounds correlates directly to the diet of the species. Glycine and glutamate are two of the most abundant amino acids in the cuticle of crustaceans, and sensitivity to these compounds can change dependent upon location and specific diet preferences (Tierney and Atema, 1998).

Just as chemoreception allows aquatic animals to discriminate between food and non-food sources, it is also this sensory system that allows individuals to detect predator and prey within the water column. Studies previously discussed here (Munday et al., 2009a) have shown ocean acidification induced changes to seawater chemistry to negatively impact the ability of fish to detect predators.

3.2.3. Potential for acclimatisation and adaptation in a changing ocean

Marine invertebrate studies predict species will (1) tolerate change due to their existing phenotypic plasticity; (2) adapt genetically; (3) migrate or (4) undergo extinction or extirpation (Peck, 2005; Sultan, 2007; Przeslawski et al., 2008; Visser, 2008; Wetthey & Woodin, 2008). These responses will influence the outcome for species populations (Byrne, 2011).

The oceanic environment has been changing gradually for decades, with some regions changing more than others (IPCC, 2007). It seems likely therefore that some species and populations may have experienced some degree of phenotypic and genetic change already (Byrne, 2011). It is worth noting that the ocean is changing at a much faster pace than in the geological past; it is not known if adaptive genetic change can occur at a rate that will avoid extirpation and species extinctions.

There are significant differences between species and life history stages in tolerance to ocean change stressors such as ocean acidification (Byrne, 2011). Short-lived species

with fast generation times are more likely to be capable of evolutionary adaptation to climate change stressors than slow-developing species (Fabry et al., 2009). It is possible therefore that nereidids such as *Platynereis dumerilii* and *Alitta succinea* may have a greater capacity for adaptation than larger individuals owing to their short life cycle and generation time.

3.3. Research rationale

Whilst there have been numerous investigations regarding chemoreception, feeding behaviour and predator avoidance in crustaceans and fishes there have been few studies carried out with nereidid polychaetes, in particular *Platynereis dumerilii*. The potential negative impact(s) ocean acidification may have upon chemoreception is also yet to be explored.

P. dumerilii are maintained in the laboratory on a diet of spinach and commercial fish food. Preliminary tests using the amino acids glycine and taurine induced sufficient feeding behaviour indicating potential for subsequent behavioural tests utilising these amino acids. Sensitive chemoreceptors allow for the detection of such molecules. A reduction in oceanic pH could negatively affect the functioning of chemoreceptors and thereby detection of essential food molecules and predators to be avoided.

Ocean acidification studies to date have focused exposure to low pH over relatively short periods (Dupont et al., 2010). These studies have come under criticism in recent years; acute exposure to reduced pH is not realistic of the challenges organisms will face in the coming century and ultimately overlook the potential for acclimatisation and adaptation entirely (Fabricus et al., 2011). The following data set(s) will aim to show the potential effects short term and long term exposure to low pH has upon the detection of individual chemicals, a complex food ‘cocktail’, i.e. spinach and predators.

For the purposes of this investigation short term exposure to low pH is defined as those animals exposed to low pH for a short period of time following culture in ‘normal’ pH, i.e. 8.2. Long term exposure to low pH is defined as those animals culture in low pH, i.e. 7.8.

3.4. Aim

To investigate the effects short term and long term exposure to low pH has upon the detection of feeding stimuli and predators in *P. dumerilii*.

3.4.1. Hypotheses

Hypothesis 1: *P. dumerilii* will fail to fully respond to chemoreception cues and not perform the following normal behaviours when subject to acute pH stress:

- a) feeding response
- b) predator avoidance
- c) feeding consumption

Hypothesis 2: *P. dumerilii* will fail to fully respond to olfactory cues and not perform the following normal behaviours when subject to long term pH stress:

- a) feeding response
- b) predator avoidance
- c) feeding consumption

3.5. Materials and Methods

3.5.1. Collection details

Animals used in this project were collected (with the assistance of J.D. Hardege and other members of the Functional Ecology Research Group) during low tide on the rocky shore in Bristol, UK. Specimens were transported back to the laboratory in 1 cm depth damp coral sand in small containers to prevent desiccation. Boxes were placed in insulated coolers with appropriate quantities of ice to lower the temperature to between 8 and 12 °C.

3.5.2. Culture conditions

Animals were cultured in accordance with procedures detailed by Hauenschild & Fischer (1969). Following collection, juvenile worms were transferred to aerated aquaria, each holding in excess of 50 individuals in a temperature controlled room between 18 and 19 °C. Each tank contained seawater (to 3 cm depth) at pH 8.2. Manual maintenance of pH required the addition of either CO₂ gas (to decrease pH) or liquid NaOH (to increase pH) where needed. Salinity was monitored on a regular basis using a refractometer and maintained at 32‰.

Commercially available fish diet (Tetramarin®) and minced spinach leaves were used as food for juveniles every 2 weeks and 2 – 3 days respectively. Fish food flakes were ground using a pestle and mortar, made into liquid form using distilled water and distributed sparingly with a pipette. In both instances, food was rinsed prior to feeding to remove dyes which would otherwise affect the water quality. The water in these aquaria was changed once a week to remove metabolites, debris and unused food particles. Tanks were covered with lids to prevent fluctuation in salinity due to evaporation.

Artificial illumination was used with a fixed daylight of 16 hours. A low-light moonlight lamp illuminated the animal stock for four consecutive nights in synchrony to the natural lunar phase at 28 day intervals; important for the sexual maturation of juveniles.

3.5.3. Behavioural assays

Feeding response

Twenty five *P. dumerilii* were transferred from each pH culture (8.2 and 7.8) to crystallising dishes (approximately 67 mm diameter) containing pH 8.2 and 7.8 seawater (relevant to the pH culture they originated from) (to 1 cm depth). Individuals were starved for 48 hours prior to investigations. They were then presented with a food source (in this case minced spinach leaves). Following a 10 second observation period, the presence/absence of feeding was noted. An animal was said to exhibit a feeding response when it actively moved toward the food source and visible feeding behaviour, i.e. extension of the jaw, took place. Following a further 48 hours starvation the above procedure was repeated in the opposing pH seawater, i.e. if an animal was first tested in pH 8.2 seawater, the above was repeated using pH 7.8 seawater and vice versa. Each animal was tested in pH 8.2 and 7.8.

Escape and feeding response in ‘presence’ of predator (odour) (*Rhithropanopeus harrisi*)

Two small *Rhithropanopeus harrisi*, an estuarine mud crab, were placed in a glass beaker containing 30 ml seawater one hour prior to investigation to generate a sample of crab conditioned water.

As above, 25 *P. dumerilii* were transferred individually from each pH culture (8.2 and 7.8) to crystallising dishes (approximately 67 mm diameter) containing pH 8.2 and 7.8 seawater (relevant to the pH culture they originated from) (to 1 cm depth). Individuals were exposed to 0.25 ml of crab conditioned water (injected into the water column using a syringe). Following a 10 second observation period, the presence/absence of an escape response was noted. An animal was said to exhibit an escape response when it actively swam away from the site where crab conditioned water was injected. A further 5 minutes later, animals were presented with a food source (minced spinach leaves), as in the previous investigation, to test feeding response in a simulated predator presence. The presence/absence of feeding was noted. Following a further 48 hours starvation the above procedure was repeated in the opposing pH seawater, i.e. if an animal was first

tested in pH 8.2 seawater, the above was repeated using pH 7.8 seawater and vice versa. Each animal was tested in pH 8.2 and 7.8.

Amino acid stimulants

Preliminary tests were carried out to establish the concentrations at which *P. dumerilii* exhibit excitatory behaviour in response to the addition of the amino acids glycine and taurine. For worms cultured at pH culture 8.2 and 7.8, concentration thresholds of 10^{-3} M and 10^{-4} M were established for glycine and taurine respectively.

Ten *P. dumerilii* were transferred from each pH culture (8.2 and 7.8) to small crystallising dishes containing pH 8.2 and 7.8 seawater (relevant to the pH culture they originated from). Using a pipette, 30 μ l of the amino acid glycine was added to the water column to stimulate individuals. Following a 10 second observation period, the presence/absence of excitatory behaviour was noted and deemed to indicate a stimulation of feeding behaviour. Excitatory behaviour included a marked increase in swimming activity, movement toward the injection site and visible extension of the jaw. Each animal was tested in pH 8.2 and then 7.8.

The above experiment was repeated with a further 10 *P. dumerilii* (from each pH culture) using the amino acid taurine. Again, each animal was tested in pH 8.2 and then 7.8.

During all feeding trials, worms were transferred to clean crystallising dishes containing fresh seawater and allowed to acclimate for 10 minutes between replicates; this was deemed adequate time for desensitization to the previous cue (pers. comm. Dr. Jörg Hardege). Visual stimulation was kept to a minimum using cardboard sheeting to obstruct the experimenter from view. The influence of visual stimulation was controlled for by repeating the experiments detailed above using 30 μ l seawater (only) as a negative control.

Consumption of food

Ten *P. dumerilii* were transferred from each pH culture (8.2 and 7.8) to small crystallising dishes. Minced spinach leaves were soaked in water for a 5 minute period; moist flakes were transferred to paper towelling to remove excess water and 0.02 g weighed. 0.02 g spinach leaves were added to each crystallising dish. After 24 hours the remaining spinach leaves were removed, transferred to paper towelling to remove excess water and again weighed. The remaining spinach leaves were returned to each dish. Following a further 24 hours the spinach leaves were reweighed.

3.5.4. Data analysis

Data collected was analysed using SigmaPlot 12.0. Tests for normality and equality were used to justify the use of parametric and non-parametric deductive tests. The following statistical analyses were carried out on the respective data sets:

Feeding response: Wilcoxon Signed-rank test.

Escape response: Wilcoxon Signed-rank test and paired t-test.

Feeding response in 'presence' of predator (odour): Paired t-test and Wilcoxon Signed-rank test.

Response to glycine: Paired t-test.

Response to taurine: Paired t-test.

Consumption of food: Onw-way ANOVA.

3.6. Results

3.6.1. Feeding response

Animals were isolated and starved for 48 hours prior to experimentation; individuals were then presented with a food source (spinach) in pH treatment 8.2 and 7.8. The presence/absence of feeding was noted. Figure 3.1 below shows the mean (%) feeding response to spinach in pH treatment 8.2 and 7.8.

When worms were cultured at pH 8.2 data failed a Shapiro-Wilk test for normality ($P < 0.05$). The medians of pH treatment 8.2 and 7.8 were 4.0 and 2.0, respectively. A Wilcoxon Signed-rank test indicated a significant reduction (Figure 3.1) in feeding response at pH treatment 7.8 ($Z = -3.720, P < 0.001$).

When worms were cultured at pH 7.8 data failed a Shapiro-Wilk test for normality ($P < 0.05$). The medians of pH treatment 8.2 and 7.8 were 2.0 and 1.0, respectively. A Wilcoxon Signed-rank test indicated a significant reduction (Figure 3.1) in feeding response at pH treatment 7.8 ($Z = -3.186, P < 0.001$).

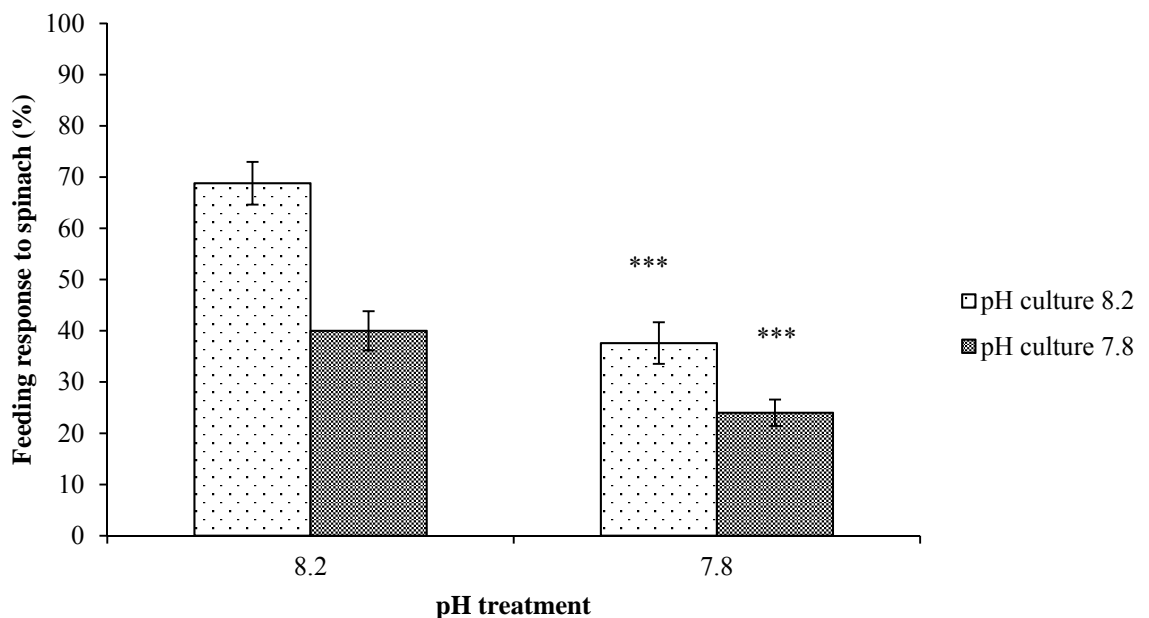


Figure 3.1. Mean feeding response (\pm SE) of *P. dumerilii* to spinach at pH 8.2 and 7.8, $n = 25$. *** = $P < 0.001$.

3.6.2. Escape response

Animals were isolated prior to experimentation; crab conditioned water (odour) was injected into the water column in pH treatment 8.2 and 7.8. The presence/absence of an escape response was noted. Figure 3.2 below shows the mean (%) escape response to crab conditioned water in pH treatment 8.2 and 7.8.

When worms were cultured at pH 8.2 data failed a Shapiro-Wilk test for normality ($P < 0.05$). The medians of pH treatment 8.2 and 7.8 were 3.0 and 1.0, respectively. A Wilcoxon Signed-rank test indicated a significant reduction (Figure 3.2) in escape response at pH treatment 7.8 ($Z = -4.352$, $P < 0.001$).

When worms were cultured at pH 7.8 Data passed a Shapiro-Wilk test for normality ($P > 0.05$). There was a statistically significant reduction (Figure 3.2) in mean escape response at pH treatment 7.8 (mean = 0.640 ± 0.757 S.D.) (t-test, $t = 4.769$, $df = 24$, $P < 0.001$).

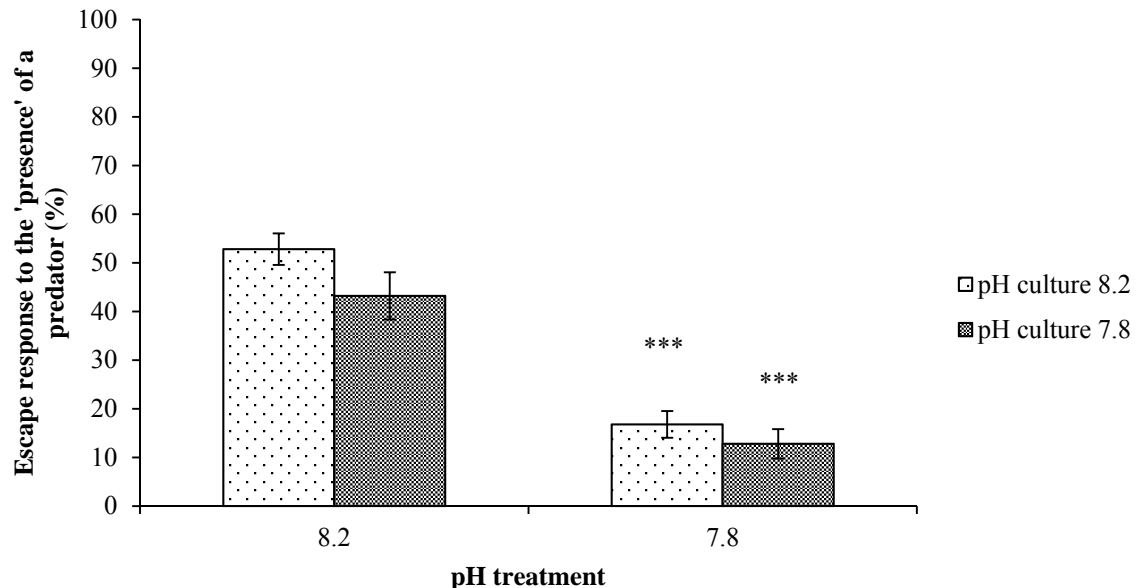


Figure 3.2. Mean escape response (\pm SE) of *P. dumerilii* to the 'presence' of a predator (*R. harrisii* odour) at pH 8.2 and 7.8, $n = 25$. *** = $P < 0.001$.

3.6.3. Feeding response in ‘presence’ of predator (odour)

Animals were isolated and starved for 48 hours prior to experimentation; crab conditioned water (odour) was injected into the water column. Following a 5 minute period, individuals were presented with a food source (spinach) and the presence/absence of feeding was noted. Tests were carried out in pH treatment 8.2 and 7.8. Figure 3.3 below shows the mean (%) feeding response in the presence of a predator (crab conditioned water, i.e. odour) in pH treatment 8.2 and 7.8.

When worms were cultured at pH 8.2 data passed a Shapiro-Wilk test for normality ($P > 0.05$). There was a statistically significant reduction (Figure 3.3) in mean feeding response in the ‘presence’ of a predator at pH treatment 7.8 (mean = 1.600 ± 0.707 S.D.) (t-test, $t = 2.568$, $df = 24$, $P < 0.05$).

When worms were cultured at pH 7.8 data failed a Shapiro-Wilk test for normality ($P < 0.05$). The medians of pH treatment 8.2 and 7.8 were 1.0 and 0.0, respectively. A Wilcoxon Signed-rank test indicated a significant reduction (Figure 3.3) in escape response at pH treatment 7.8 ($Z = -3.042$, $P < 0.01$).

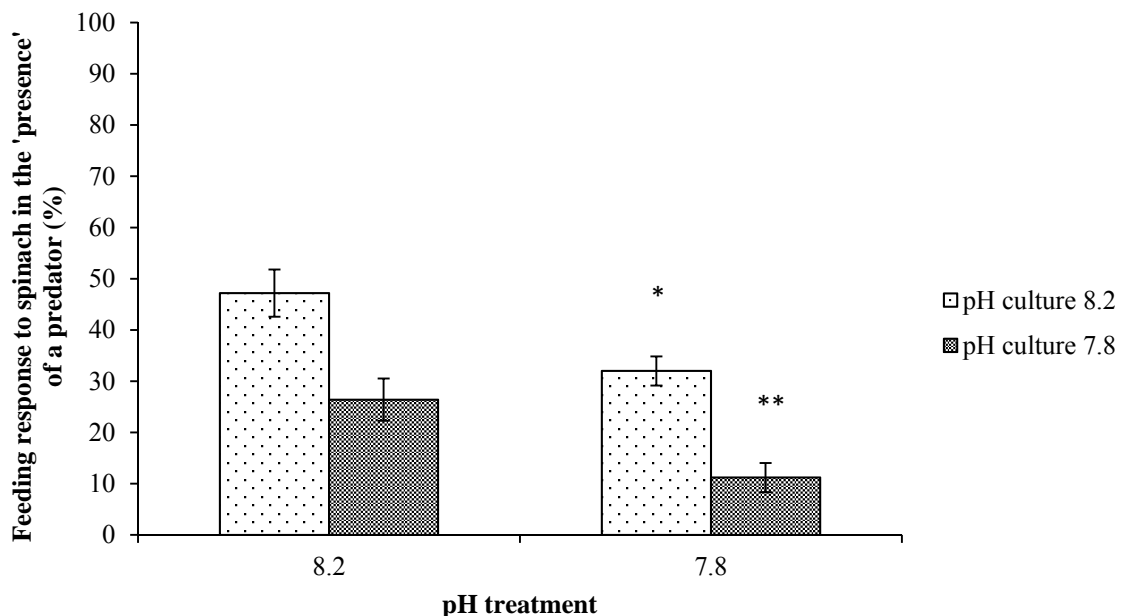


Figure 3.3. Mean feeding response (\pm SE) of *P. dumerilii* to spinach in the ‘presence’ of a predator (*R. harrisii* odour) at pH 8.2 and 7.8, $n = 25$. ** = $P < 0.05$.

3.6.4. Response to glycine

Animals were isolated prior to experimentation. Glycine solution was then injected into the water column at pH treatment 8.2 and 7.8. The presence/absence of a feeding response was noted. Figure 3.4 below shows the mean (%) feeding response to glycine in pH treatment 8.2 and 7.8 for those organisms maintained at pH culture 8.2 and 8.2 F1 (generation) and pH culture 7.8 and 7.8 F1 (generation), respectively.

When worms were cultured at pH 8.2 data from field and laboratory-bred (i.e. F1 generation) cultures passed a Shapiro-Wilk test for normality ($P > 0.05$). There was a statistically significant reduction (Figure 3.4) in feeding response to the stimulant glycine at pH treatment 7.8 (mean = 2.920 ± 0.954 S.D.) (t-test, $t = 16.366$, $df = 24$, $P < 0.001$) when cultures were collected from the field (pH culture 8.2).

Likewise, there was a statistically significant reduction (Figure 3.4) in mean feeding response to the stimulant glycine at pH treatment 7.8 (mean = 3.400 ± 1.174 S.D.) (t-test, $t = 3.628$, $df = 9$, $P < 0.01$) when cultures were laboratory bred (pH culture 8.2 F1).

When worms were cultured at pH 7.8 data from field and laboratory bred cultures passed a Shapiro-Wilk test for normality ($P > 0.05$). There was a statistically significant reduction (Figure 3.4) in mean feeding response to the stimulant glycine at pH treatment 7.8 (mean = 3.840 ± 3.051 S.D.) (t-test, $t = 3.597$, $df = 24$, $P < 0.001$) when cultures were collected from the field (pH culture 7.8).

Likewise, there was a statistically significant reduction (Figure 3.4) in mean feeding response to the stimulant glycine at pH treatment 7.8 (mean = 2.100 ± 1.370 S.D.) (t-test, $t = 2.293$, $df = 9$, $P < 0.05$) when cultures were laboratory bred (pH culture 7.8 F1).

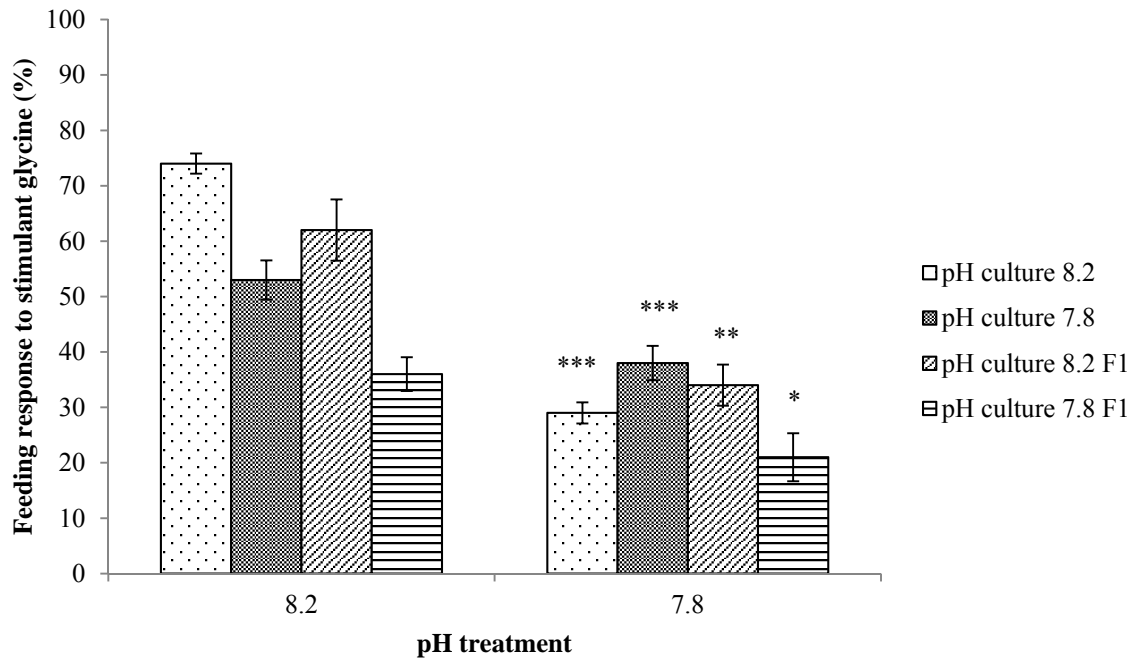


Figure 3.4. Mean feeding response (\pm SE) of *P. dumerilii* to the stimulant glycine at pH 8.2 and 7.8, $n = 25$. *** = $P < 0.001$.

3.6.5. Response to taurine

Animals were isolated prior to experimentation; taurine solution was injected into the water column in pH treatment 8.2 and 7.8. The presence/absence of a feeding response was noted. Figure 3.5 below shows the mean (%) feeding response to taurine in pH treatment 8.2 and 7.8 for those organisms maintained at pH culture 8.2 and 8.2 F1 (generation) and pH culture 7.8 and 7.8 F1 (generation) respectively.

When worms were cultured at pH 8.2 data from field and laboratory bred (i.e. F1 generation) cultures passed a Shapiro-Wilk test for normality ($P > 0.05$). There was a statistically significant reduction (Figure 3.5) in mean feeding response to the stimulant taurine at pH treatment 7.8 (mean = 2.480 ± 1.636 S.D.) (t-test, $t = 15.074$, $df = 24$, $P < 0.001$) when cultures were collected from the field (pH culture 8.2).

Conversely, there was no statistically significant difference (Figure 3.5) in mean feeding response to the stimulant taurine between pH treatment 8.2 (mean = 7.000 ± 0.816 S.D.) and 7.8 (mean = 6.100 ± 1.524 S.D.) (t-test, $t = 1.588$, $df = 9$, $P > 0.05$) when cultures were laboratory bred (pH culture 8.2 F1).

When worms were cultured at pH 7.8 data from field and laboratory bred cultures passed a Shapiro-Wilk test for normality ($P > 0.05$). There was a statistically significant reduction (Figure 3.5) in mean feeding response to the stimulant taurine at pH treatment 7.8 (mean = 3.600 ± 2.944 S.D.) (t-test, $t = 4.843$, $df = 24$, $P < 0.001$) when cultures were collected from the field (pH culture 7.8).

Likewise, there was a statistically significant reduction (Figure 3.5) in mean feeding response to the stimulant taurine at pH treatment 7.8 (mean = 2.000 ± 0.816 S.D.) (t-test, $t = 4.311$, $df = 9$, $P < 0.01$) when cultures were laboratory bred (pH culture 7.8 F1).

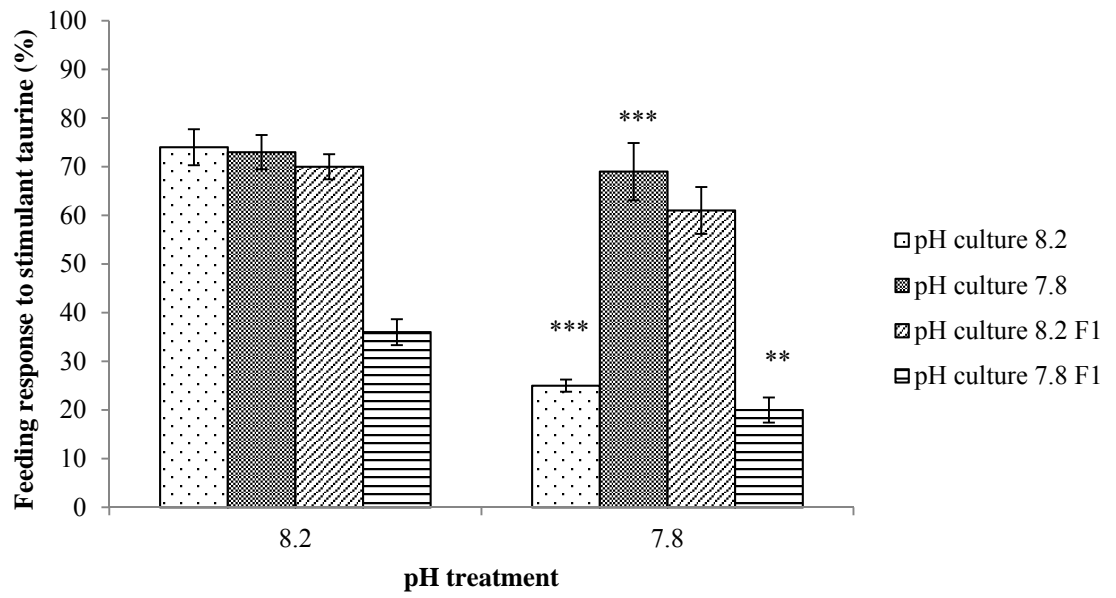


Figure 3.5. Mean feeding response (\pm SE) of *P. dumerilii* to the stimulant taurine at pH 8.2 and 7.8, $n = 25$. *** = $P < 0.001$.

3.6.6. Consumption of food

P. dumerilii from pH culture 8.2 and 7.8 were fed a defined amount of minced spinach leaves (0.02 g). Worms were allowed to feed. After 24 hours the remaining spinach leaves were removed, transferred to paper towelling to remove excess water and weighed. This procedure was repeated and spinach leaves reweighed following a further 24 hours.

Data passed a Shapiro-Wilk test for normality ($P > 0.05$). There was a statistically significant interaction between pH culture and time post feeding (ANOVA, $df = 2$, $F = 40.0$, $P < 0.001$) (Figure 3.6). There was significantly more spinach remaining 24 hours and 48 hours post feeding when animals were cultured at pH 7.8; these results indicate feeding is significantly reduced at low pH (Figure 3.6).

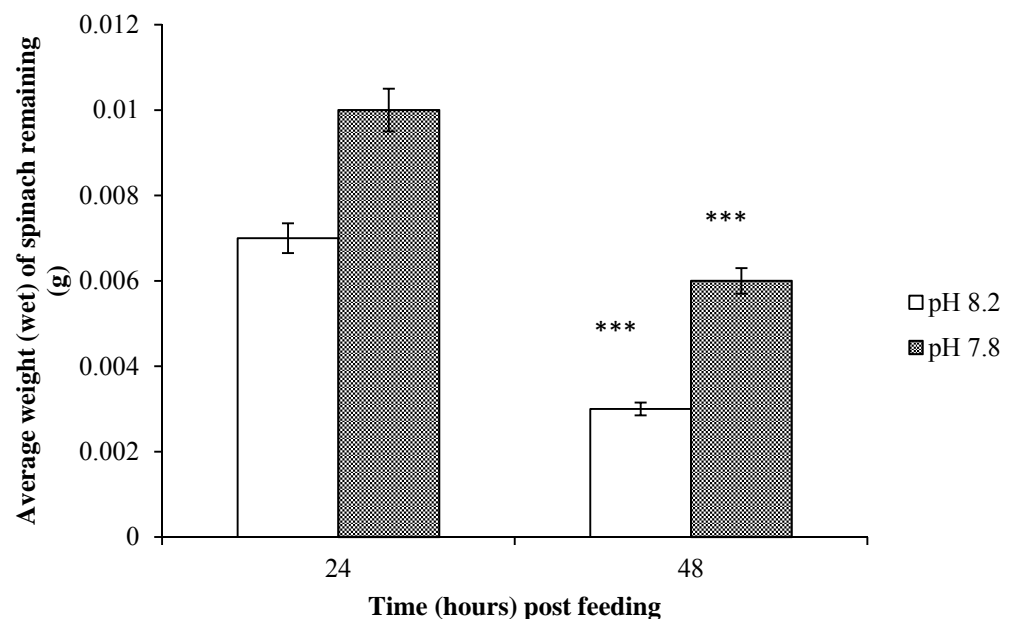


Figure 3.6. Average weight (wet) of spinach remaining 24 and 48 hours post feeding when *P. dumerilii* maintained in pH culture 8.2 and 7.8 were fed 0.02 g spinach, $n=10$. *** = $P < 0.001$.

3.7. Discussion

The successful acquisition of information is essential for the survival and fitness of organisms throughout the animal kingdom. Information gathering can be mediated via visual, tactile, auditory and olfactory senses (Schmitt and Ache, 1979). In the complex aquatic environment, the olfactory sense of an animal is often heightened in order to compensate for a reduction in visibility, for example, to increased turbidity (Schmidt et al., 2010).

Many reports describing the chemical mediation of essential behaviours in the aquatic environment have focused upon feeding (Derby et al., 1996; Ache, 1982; Hara, 1975; Carr, 1982; Mackie and Grant, 1974; Haye et al., 2009). It should be noted, however, that the majority of these studies focus upon feeding in fish and crustaceans. Studies focusing upon the effects of low pH upon feeding are few in number. A study investigating the effect of low pH upon feeding in a polychaete species is absent from the field and the data obtained in this chapter are, therefore novel.

The majority of studies concerning ocean acidification to date have focused upon the performance and survival of individual species in short-term studies (Hall-Spencer et al. 2008). These studies make the assumption that change to an individuals' performance will influence ecosystem function. Controlled experimental studies such as these are limited in space and time; it is likely that important processes (e.g. acclimatisation and adaptation) may not be observed.

Long term studies which utilize long-lived species, e.g. fish, are still problematic. These cultures and experiments are unlikely to allow adequate time for potential adaptation (through multiple generations) to occur (Barry et al., 2010). Short-lived species, such as *P. dumerilii*, are ideal for such purposes due to their short generation time; multiple generations can be cultured within the laboratory allowing for the possibility of potential adaptation in long term studies. Fitzner et al. (2012) show a multi-generational decline in copepod naupliar reproduction exposed to acidified conditions. This study is one such example of utilising a short lived species to investigate the potential for adaptation.

3.7.1. The effects of ocean acidification upon organisms

A number of physiological processes, for example, photosynthesis, calcification and acid-base homeostasis, to name but a few can be influenced by changes to ocean carbonate chemistry (Gattuso et al., 1998; Seibel and Walsh, 2003). As to be expected, there will be ‘winners’ and ‘losers’ in response to ocean acidification. It is important to remember that the effects of ocean acidification will be both direct and indirect. For example, *Acropora* spp. provide habitat for a vast range of organisms in coral reef ecosystems (Bellwood et al., 2004). Negative impacts upon coral may therefore influence biodiversity and ecosystem function due to the loss of critical habitat for various coral-associated taxa.

Taxa, such as cyanobacteria, that are currently exposed to carbon-limited environments may be ‘winners’ in a future high-CO₂ ocean (Barry et al., 2010). A number of experiments upon marine phytoplankton have shown coccolithophores to exhibit reduced rates of calcification (Ridgwell et al., 2009). Similarly, reduced calcification is the most common and consistently observed effect of ocean acidification and has been noted in coral and mollusc species (Michaelidis et al., 2005; Kuffner et al., 2008; Doney et al., 2009). Calcification has been the focus of many ocean acidification studies to date utilising short-term exposure to low pH; such exposure times may be too short to adequately detect acclimatisation. Acclimatisation rates differ between species and this should be taken into account when designing a low pH investigation.

Changes to the ‘cost of living’ are expected to result in reallocation of energy for growth and reproduction. For taxa negatively impacted by ocean acidification, this could result in individuals subject to reduced growth, size, reproductive output and ultimately survival. Physiological stresses such as these (upon individuals) will have consequences for populations and species putting them at risk of reduced abundance, productivity and ultimately extinction. In a study by Melatunan et al. (2013), low pH (as a result of ocean acidification) was shown to disrupt the overall investment of *Littorina littorea* in shell material; essential for protection and ultimately survival.

3.7.2. Effects of short term and long term exposure to low pH

This study is the first to demonstrate that a reduction in sea water pH (both short and long term exposure) has an adverse effect upon essential chemo-sensory behaviours of a polychaete, *P. dumerilii*. Animals exhibited significantly reduced feeding and predator avoidance behaviours when exposed to food (spinach) and predator odour (crab conditioned water) in acidified seawater (pH 7.8). Feeding in the ‘presence’ of a predator was also significantly reduced in acidified seawater, though it should be noted that statistical significance was slight in comparison to the previously mentioned results.

Consumption of food was significantly reduced when animals were cultured at pH 7.8; significantly more spinach (food source) remained 24 and 48 hours post feeding. This experiment was basic in design and results should be interpreted with caution due to the variability in wet weight, there is a potential source of error in measuring food. These results do however provide preliminary findings which could be applied to future investigation.

Short term exposure to low pH resulted in organisms obtained from the field and those bred within the laboratory (i.e. the F1 generation) exhibiting a significantly reduced feeding response when exposed to an organic compound, glycine. Conversely, organisms obtained from the field exhibited a significantly reduced feeding response when exposed to taurine in acidified seawater; those bred within the laboratory did not exhibit significantly different responses between seawater treatments. Similar findings have been noted in *Alitta succinea* investigations within the research group (unpublished data, 2013). Long term exposure to low pH resulted in organisms obtained from the field and bred within the laboratory (i.e. the F1 generation) exhibiting a significantly reduced feeding response when exposed to organic compounds, glycine and taurine. In both instances reduction was less significant in laboratory bred cultures (i.e. the F1 generation); this may suggest some degree of acclimatisation over time. They appear more tolerant to pH stress.

As previously discussed, chemical mediation of behaviours is well studied in fish. In a study by Brown et al. (2002), salmonids failed to respond to a conspecific alarm cue in acidified seawater. This change in behaviour was due to a non-reversible degradation of the cue, a possible result of a covalent change in the alarm pheromone molecule. These

results coincide with those obtained within this study. Similarly, Haye et al. (2012) demonstrated a reduction in feeding when the hermit crab *Pagurus bernhardus* was exposed to low pH seawater treatments.

Freshwater and terrestrial invertebrates have been shown to resist environment stressors (Bridle & Vines, 2006; Derry & Arnott, 2007), providing hope that marine species may be equally capable of adaptive evolution to climate change stressors. Copepods inhabiting lakes acidified to pH 6.0 over a period of 6 to 8 years (due to SO₂) are capable of rapid genetic-based adaptation (Derry & Arnott, 2007). Furthermore, contemporary evolution of stress tolerance can be seen in the genetically based toxicant resistant marine oligochaete (Levinton et al., 2003) and a number of other organisms (Klerks & Weis, 1987; Daborn et al., 2002).

Data collected from freshwater organisms should be interpreted with caution due to the unreliability of pH in freshwater generally. Preliminary unpublished data from within the research group reinforce the findings within this investigation. *Alitta succinea*, a brackish water polychaete exhibited similar reduced feeding responses at low pH. Brackish water organisms are accustomed to fluctuating environmental conditions, including pH, and may be more tolerant to future CO₂ driven ocean acidification than the fully marine species, *P. dumerilii*.

3.7.3. Ocean acidification and ‘signal disruption’

Chemical ‘signal disruption’ can occur at many stages in the signalling process: signal production, transmission, reception, transduction and behavioural or physiological response (Hardege et al., 2013, under review). Four mechanisms by which chemical reception in the marine environment may be disrupted by ocean acidification have been proposed (Haye et al., 2009):

1. Changes to the charge distribution of odour molecules disrupt receptor-ligand interactions.
2. Changes to the charge distribution of the odour receptors disrupt receptor-ligand interactions.
3. Physical damage to the sensory organs.

4. Reduction in motivation associated with increased metabolic load (to maintain acid-base balance in low pH conditions).

Chemical cues bind with structurally specific receptors (Hardege et al., 2011). Chemoreceptors generally consist of membrane bound receptor proteins which, when bound to signal molecules, initiate a series of internal events leading to a developmental or behavioural response (Wyatt, 2003). Changes to the pH of seawater may alter the charge distribution of signal molecules, changing the overall charge and hydrogen content according to the acid dissociation content (pKa) of the molecule affected (Hardege et al., 2011). Changes to the structure of the receptor or signal molecule may therefore prevent successful receptor-ligand binding (Haye et al., 2009). This may result in altered or absent behavioural responses (Hardege et al., 2011).

The extent to which receptor efficiency is impacted will be dependent upon the pKa of ionizable groups around the active site of the receptor protein (Tierney and Atema, 1988). Susceptible groups may repel the cue ligand due to structural changes or changes in charge at the active site (Hardege et al., 2013, under review). Such changes have been shown to occur in the navel orange worm moth, *Amyeibis transitella* (Xu et al., 2010). At low pH (4.5) it was found that a C-Terminal helix formed within the receptor thereby blocking the active site, the appropriate behavioural response was no longer possible (Xu et al., 2010).

Detection of stimulants in *P. dumerilii* may be controlled by the nuchal organs, generally considered the primary chemoreceptive organ in polychaetes (Lindsay, 2009). Other chemosensory structures have also been described. Activity-dependent cell-labeling studies have shown sensory cells in the feeding palps of *Dipolydora quadrilobata* to respond to a chemical known to induce feeding (Riordan and Lindsay, 2002). Whilst the signal transduction pathway has not yet been fully understood, it is believed that G-proteins may be involved (Tsie et al., 2008). Until these processes are better understood it is difficult to predict the effects low pH will have upon chemoreception with respect to feeding.

Amino acids, such as glycine and taurine, may suffer the same fate as receptor-ligand structures, described above, when subject to pH stress. Glycine and taurine possess pKa values, 9.60 and 9.06 respectively, in a region sensitive to pH stress. Hardege et al.,

(2011) have suggested that the majority of information chemicals in the marine environment will be potentially susceptible to pH dependent structural change. These chemicals are responsible not only for feeding but predator detection and avoidance, settlement, mate choice and reproductive behaviours.

3.7.4. Concluding remarks and future work

This study indicates that *P. dumerilii* are unable to acclimatise to low pH following long term exposure to acidified seawater. Worms do, however, appear more resilient to pH stress than those organisms exposed to low pH over a shorter period of time. Organisms fail to successfully respond to chemical cues responsible for many essential life processes, e.g. feeding and predator avoidance, in reduced pH as with short-term experiments in the previous chapter. Should these results translate to the natural environment it can be reasoned that the general behaviour of the species will change. *P. dumerilii* are likely to spend more time foraging for food leaving them at greater risk of predation. This study indicates *P. dumerilii* are less likely to detect predators at low pH and this will therefore have greater impact upon an individual's likelihood to survive.

Chemoresponsiveness is negatively impacted by pH-induced disruption in polychaetes, such implications may be experienced by other taxa. It is likely that effects will differ between species dependent upon their tolerance to pH changes, i.e. brackish water species may be more tolerant than marine as previously mentioned. Chemical cues differ in their structures and the effects of pH upon such structures will differ. Such widespread, variable and unpredictable changes will undoubtedly have the potential to re-order aquatic community dynamics and structure.

Further studies are needed to draw further conclusions and better understand this complex and new topic. Relatively limited measurements of general fitness exist and future studies should aim to link physiological biomarkers for fitness to ecological stress.

Such studies would benefit from the incorporation of animals from naturally occurring CO₂ vents in Ischia, here polychaetes are present and somehow (apparently) adapted to the low pH environment. Interestingly, recent work carried out on *P. dumerilii* obtained from these vent systems by Calosi et al. (2013) found worms were subject to respiratory

distress when transferred from the vent to untreated seawater (pH 8.2). These results were mirrored when organisms from untreated seawater were transferred to vent seawater (pH 7.8).

It is clear therefore that further tests are needed to better understand the effects stress has upon the physiology of these organisms; this may take the form of intracellular glutathione (GSH) analysis. GSH has key functioning in detoxification and maintenance of cell oxidative stress (Kirlin et al., 1999; Pastore et al., 2003). Due to its detoxifying properties, reduced levels of GSH may be an indicator of environmental stress. Furthermore, GSH plays an additional role in the polychaete *A. succinea* acting as a precursor to synthesis of the female sex pheromone cysteine glutathione disulphide (CSSG) (Hardege et al., 2004). Stress upon these organisms may present a potential trade-off between maintaining cell oxidative stress and pheromone production.

Chapter 4:

Are *Platynereis dumerilii* found at a naturally occurring CO₂ vent in Ischia different to other known *P. dumerilii* populations?

4.1. Abstract

Surface ocean pH is expected to decrease by a further 0.3 units by 2100 as a result of anthropogenic CO₂ from the atmosphere (Orr et al., 2005). Short term investigations have focused largely upon calcification rates in planktonic and benthic organisms (Orr et al., 2005). These experiments are not capable of detecting acclimatisation or adaptation (inheritance of desirable traits, i.e. tolerance to pH) and results obtained may be a result of pH shock.

Naturally occurring CO₂ vents have become the focus of a number of studies in recent years (Hall-Spencer et al., 2008; Calosi et al., 2013). These low pH sites, present for thousands of years, provide ideal habitats in which to study the potential effects of future ocean acidification.

This study shows that *Platynereis dumerilii* sampled and sequenced from the CO₂ vent site of Ischia are genetically different from known populations of *P. dumerilii* throughout Europe. Such results could indicate a cryptic species. These individuals are, however, clearly adapted to life at low pH. Individuals appear to show signs of adaptation in behavioural trials with few significant differences between pH treatments 8.2 and 7.8. Future studies are needed to better understand the mechanisms for adaptation to low pH in this and other species.

4.2. Introduction

4.2.1. Ocean acidification

Increasing atmospheric CO₂ concentrations are driving additional CO₂ into the surface waters of the ocean leading to a rise in *p*CO₂ concentrations at the ocean surface (Caldeira and Wickett, 2003). As CO₂ dissolves in the surface waters, it combines with water to form a weak carbonic acid (H₂CO₃), this then readily dissociates to bicarbonate (HCO₃⁻), carbonate ions (CO₃²⁻) and protons (H⁺).

These changes to seawater chemistry result in a decrease in pH and are often referred to as ‘ocean acidification’ (IPCC, 2007). Such changes, already occurring today, are expected to increase in intensity in the future. It is predicted that the pH of surface seawater will drop by a further 0.2-0.4 units by the year 2100 (Orr et al., 2005). Perhaps one of the most obvious impacts of acidified waters will be the affect that it has upon calcifying organisms, making it more difficult for them to successfully build and maintain their carbonate skeletons (Raven et al., 2005). A large number of studies to date have focused upon the effects of ocean acidification upon calcifying organisms (Kleypas et al., 2006; Fabry et al., 2008; Wood et al., 2008; Ries et al., 2009).

Potential effects of ocean acidification

The effects of ocean acidification are likely to be much more widespread than previously anticipated. Changes to seawater chemistry may impact a large number of diverse chemicals, included in these, important chemical signalling compounds (Hay, 2009). Studies on such chemicals are to date few in number. Similarly, the effects of ocean acidification upon larval pelagic stages of invertebrates are still largely unknown (Hall-Spencer et al., 2008).

The behavioural mechanisms by which aquatic animals find food vary widely. In a complex aquatic environment where visual location of food is not always possible, a large number of organisms rely upon chemoreception (Watson et al., 2005). Individuals use a complex mixture of chemical cues to stimulate appropriate sensory organs and locate food (Mackie et al., 1980). Should ocean acidification negatively impact an

organisms ability to detect food within the water column this could have severe consequences for the individual, population and ultimately species.

The majority of studies to date have investigated the potential effects of ocean acidification in short term investigations (Barry et al., 2010). This allows only for the assessment of acclimation and acclimatisation but not adaptation. Virtually nothing is known of the scope for adaptation to the elevated CO₂ conditions predicted to occur in the future; the life cycle of an organism is too long in most model species. There is therefore urgent need for appropriate studies utilising animals with a short life cycle. The polychaete *Platynereis dumerilii* appears to be an ideal species for long term ocean acidification studies owing to their short life cycle and subsequent generation time.

4.2.2. CO₂ vent systems

Hall-Spencer et al. (2008) have shown that naturally occurring CO₂ venting sites can be useful in the assessment of the long-term effects of ocean acidification on benthic biota and sea-floor ecosystems. These ecosystems are able to provide us with a first insight into the effects high-CO₂ may have on both spatial and temporal scales as they are thousands (at least) of years old. It should be noted, that whilst not a precise portrayal of global-scale ocean acidification, they allow us to investigate topics which would otherwise be difficult to address and limited in the laboratory.

The CO₂ vents of Ischia (Naples, Italy) are just one example of a naturally occurring CO₂ venting site. They have been used extensively to investigate the effects of low pH/high *p*CO₂ conditions on marine communities (Hall-Spencer et al., 2008; Cigliano et al., 2010; Kroecker et al., 2012). In 2010, Cigliano et al. found invertebrate species specific patterns of settlement along CO₂ gradients in the aforementioned vent system. Such data may indicate the potential for acclimatisation or adaptation.

Invertebrates at these vent sites include polychaete species such as *Platynereis dumerilii* and *Amphiglena mediterranea*. Some polychaete species maintain or increase their densities along CO₂ gradients toward the vent sites whilst others decrease in density and/or are absent from the high CO₂ vent areas entirely. Indeed, *P. dumerilii* are one such species found both around and within the vent areas. They are one of three species said to ‘dominate’ the most intense venting areas (Cigliano et al., 2010; Kroecker et al.,

2011) and are commonly associated with rocky, shallow, vegetated areas of the Mediterranean (Gambi et al., 1992; Giangrande et al., 2003).

P. dumerilii is a model species for evolution and development (Fischer et al., 2010) and as such a potentially excellent species to study for adaptive ability.

4.2.3 Study site

The CO₂ vents of Ischia (Naples, Italy) are located on the north and south sides of Castello Aragonese d'Ischia (Figure 4.1). These shallow water vents occur at the north-eastern side of Ischia Island and are part of a volcano system present for approximately 132,000 years (Hall-Spencer et al., 2008).

Seawater is acidified by gas bubbling out of the rocky substrate comprising 90.1-95.3 % CO₂, 3.2-6.6 % N₂, 0.6-0.8 % O₂, 0.08-0.1 % Ar and 0.2-0.8 % CH₄ (Hall-Spencer et al., 2008). Mean pH values range from 8.14 to 6.57 along a 300 m gradient running parallel to the rocky shore on both the north and south side of the Castello (Hall-Spencer et al., 2008). The site is microtidal (0.30-0.50 m range) and lacks sulphur (Hall-Spencer et al., 2008). Annual temperature ranges from 13-25 °C whilst the salinity of the water (38 ‰) and total alkalinity (2.5 mequiv. kg⁻¹) remains the same across the sampling stations (Hall-Spencer et al., 2008).

Previous studies carried out by Hall-Spencer et al. (2008) reported a total of 64 megabenthic taxa along the vent area gradient. Reductions in the diversity of adult populations were caused in part by the dissolution of calcified species at low pH (Martin et al., 2008). Hall-Spencer et al. (2008) reported the presence of *P. dumerilii* at the vent sites. *P. dumerilii* samples were obtained from vent sites N3 and S3 for the purposes of this investigation.

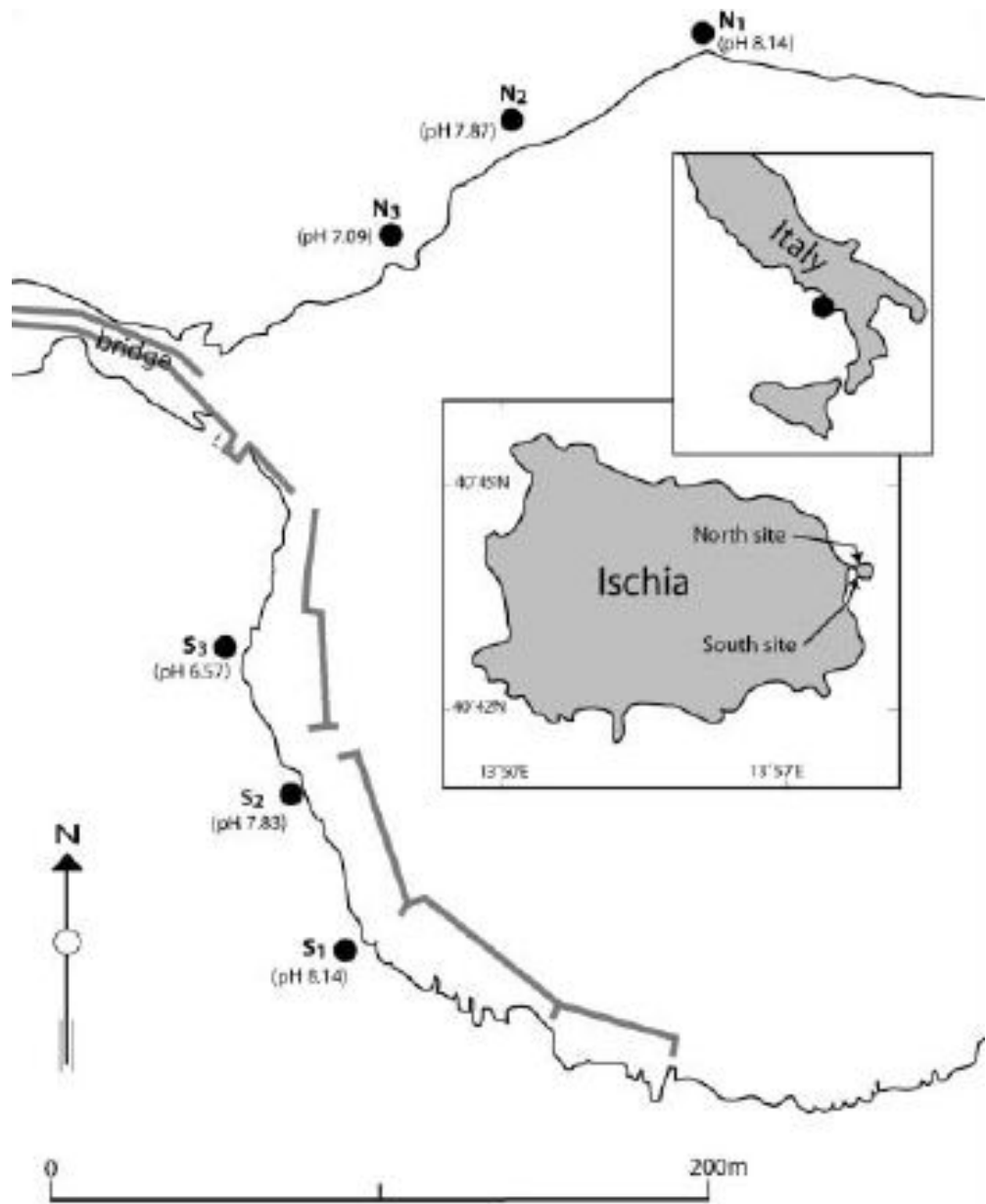


Figure 4.1. Map of the study area at Castello Aragonese, Ischia (Naples, Italy) including location of the sampling stations on the north and south sides along a pH gradient from normal (N1, S1) to acidified (N3, S3) conditions. Molecular analysis was carried out on samples from S3 only.

4.3. Research rationale

Ocean acidification is predicted to pose a great threat to aquatic ecosystems in the coming century; research into this plight has increased within recent decades (IPCC, 2000; Watson et al., 2005; Widdicombe and Spencer, 2008). Calcification studies are large in number (Cohen and Holcomb, 2009) whilst few studies have addressed the effects acidification will have upon larval pelagic life stages and chemical communication (see Byrne, 2011 for review). These processes are essential to the survival of many aquatic organisms (Byrne, 2011). Previous studies within this thesis have attempted to investigate these topics and draw adequate conclusions.

Furthermore, studies to date have investigated acclimation and acclimatisation; little is known of the scope for adaptation to the elevated CO₂ conditions predicted by 2100 (Collins and Bell, 2006). Laboratory experiments are somewhat limited as multiple generations are needed to investigate potential adaptation. Most model species have long life spans making it difficult to breed multiple generations needed. *P. dumerilii* are known to occur around and within the CO₂ vent system in Ischia (Naples, Italy). Their presence within the vent itself suggests potential for adaptation.

Investigations into the mechanisms by which this population are adapted are out of the scope of this investigation. It is important, however, to first determine if they are the same species. Whilst they appear morphologically identical, cryptic species are known to occur within polychaetes. It is also important to consider the planktonic larvae they possess; as an open system with free moving larvae recruitment from outside the vent area is possible. Presence at the vent does not mean that the complete life cycle and reproductive event of this species occurs within the vent.

The following chapter will therefore use molecular analysis to determine the relatedness of seemingly identical populations of *P. dumerilii* collected at increasing distance from the vent systems. Behavioural bioassays will be conducted with organisms collected from a normal pH control site (Forio) and the low pH CO₂ vent of Ischia (Naples, Italy) to investigate potential differences in chemoreception between these populations.

4.4. Aim

To investigate levels of relatedness and phylogeographic patterns in *P. dumerilii* populations collected at increasing distance from naturally occurring CO₂ vents in Ischia (Naples, Italy). To investigate the effects exposure to low pH vent sites has upon the detection of feeding stimuli and predators in *P. dumerilii* and the potential for adaptation.

4.4.1. Hypotheses

Hypothesis 1: If adapted to a low pH environment, *P. dumerilii* obtained from the CO₂ vents of Ischia (Naples, Italy) will potentially be genetically different from *P. dumerilii* obtained from non-vent sites throughout Europe.

Hypothesis 2: *P. dumerilii* obtained from the CO₂ vents of Ischia (Naples, Italy) will respond to chemoreception cues and perform the following normal behaviours when subject to pH stress:

- a) feeding response
- b) predator avoidance

4.5. Materials and methods

4.5.1. Collection details

Animals used in this project were collected from multiple sample sites throughout Europe with the assistance of the Functional Ecology Research Group, Hull and members of the Stazione Zoologica Anton Dohrn, Ischia (Naples, Italy) between July 2009 and November 2011. Table 4.1 summarises the sampling sites used.

P. dumerilii collected in the UK were sampled during low tide on the rocky shore in Bristol. Specimens were transported back to the laboratory in 1 cm depth damp coral sand in small containers to prevent desiccation. Boxes were placed in insulated coolers with appropriate quantities of ice to lower the temperature to between 8 and 12 °C.

Conversely, *P. dumerilii* collected in Italy were collected by hand from various macroalgae (*Dictyota* spp., *Halopteris scoparia*, *Cladophora prolifera* and *Corallina* spp.) by snorkelers or SCUBA divers at:

1. 1 - 2 m depth at the Castello Aragonese d'Ischia (Naples, Italy) vent site S3 (see Figure 4.1).
2. 1 - 2 m depth at the control site San Pietro (approximately 4 km from the vent sites).
3. At the control site S. Anna islets in the Cartaromana Bay (approximately 600 m from the vent sites).
4. At the control site Forio (approximately 12 km from the vent sites).

Seven to thirty individuals were sampled from each location, shipped to the UK (live animals did not appear to be distressed upon arrival in the UK) and preserved in 95% ethanol or in DMSO (dimethyl sulfoxide), frozen at -80 °C and processed. *P. dumerilii* collected from the control site Forio and CO₂ vent site S3 (see Figure 4.1) were subject to behavioural bioassays (in the UK) detailed below prior to preservation.

Table 4.1. Table to show *P. dumerilii* sample sites around Europe, including distance from Ischia (Naples, Italy) CO₂ vent system Details include number of specimens of *P. dumerilii* successfully processed for cytochrome *c* oxidase subunit I (COI) and the Internal Transcribed Spacer (ITS) sequencing.

Sample site	Latitude/Longitude	Acidified (A)/ Control (C)	Collection date	Distance from vents	<i>P. dumerilii</i>		
					Specimens	COI	ITS
Castello S3	40°43'51.18''N 13°57'47.45''E	A	07/07/2010	--	28	12	2
Forio, Ischia	40°44'25.08''N 13°51'41.54''E	C	20/05/2012	12 km	13	7	0
S. Anna, Ischia	40°43'35.76''N 13°57'36.95''E	C	21/11/2011	600 m	28	14	3
S. Pietro, Ischia	40°44'47.59''N 13°56'39.86''E	C	17/11/2011	4 km	27	17	2
Nisida, Gulf of Naples	40° 46'32.60''N 14°9'45.52''E	C	14/11/2011	15 km	29	15	3
S. Caterina, Ionian Sea	40°7'50.86''N 17°59'39.11''E	C	15/11/2011	>600 km	11	9	3
Bristol Channel	51°12'50.48''N 3°7'28.84''W	C	28/07/2011	>2000km	15	15	4

4.5.2 Behavioural assays

Feeding response

P. dumerilii from the control site Forio (n = 14) were transferred from culture tanks to small crystallising dishes (approximately 67 mm diameter) containing pH 8.2 seawater (to 1 cm depth). Individuals were starved for 48 hours prior to investigations. Organisms were then presented with a visible food source (in this case minced spinach leaves). Following a 10 second observation period, the presence/absence of feeding was noted. An animal was said to exhibit a feeding response when it actively moved toward the food source and visible feeding behaviour, i.e. extension of the jaw, took place. Each animal was tested in pH 8.2 and then 7.8.

The above was repeated with *P. dumerilii* from the CO₂ vent of Ischia (n = 8). Each animal was tested first in pH 7.8 and then 8.2.

Escape and feeding response in 'presence' of predator (*Rhithropanopeus harrisi*)

Two small *Rhithropanopeus harrisi*, estuarine mud crab, were placed in a glass beaker containing 30 ml seawater an hour prior to investigations to produce a sample of crab conditioned water.

As above, *P. dumerilii* from the control site Forio (n=14) were transferred from culture tanks to small crystallising dishes containing pH 8.2 seawater. Individuals were exposed to 0.25 ml of crab conditioned water. Following a 10 second observation period, the presence/absence of an escape response was noted. An animal was said to exhibit an escape response when it actively swam away from the site where crab conditioned water was injected. A further 5 minutes later, animals were presented with a visible food source, as in the previous investigation, to test feeding response in a simulated predator presence. The presence/absence of feeding was noted. Each test was carried out in pH 8.2 and then 7.8.

The above was repeated with *P. dumerilii* from the CO₂ vent of Ischia (n = 8). Each animal was tested first in pH 7.8 and then 8.2.

4.5.3. Sequence generation

P. dumerilii samples were subject to DNA extraction using the hotshot method described in Montero-Pau et al. (2008). Two gene regions were amplified using polymerase chain reaction (PCR): the mitochondrial cytochrome *c* oxidase subunit I (COI, 658-710 bp) and the nuclear Internal Transcribed Spacer (ITS, alignment of 495 bp). Forward and reverse primers outlined by Folmer et al. (1994) and Nygren et al. (2009) (Table 4.2) were used. PCRs were performed in total volumes of 20 μ l and 25 μ l for COI and ITS, respectively. See Table 4.3 for PCR volume mixtures.

Table 4.2. Forward (F) and Reverse (R) primers used for amplification of the two markers and original references.

Marker, name of primer	Sequence	Reference
COI F: LCO-1490 R: HCO-2198	GGTCAACAAATCATAAAGATATTGG TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. 1994 Folmer et al. 1994
ITS F: ITS18SFPoly R: ITS58SRPoly	GAGGAAGTAAAAGTCGTAACA GTTCAATGTGTCCTGCAATTC	Nygren et al. 2009 Nygren et al. 2009

Table 4.3. Polymerase Chain Reaction (PCR) volume mixtures (μ l) for cytochrome *c* oxidase subunit I (COI) and Internal Transcribed Spacer (ITS).

	MgCl ₂	dNTPs	Primer				<i>Taq</i> Polymerase	PCR Buffer	ddH ₂ O	DNA extract
			HCO	LCO	ITS - F	ITS - R				
COI	1.25	2.5	0.5	0.5	--	--	0.1	2.5	8.65	4.0
ITS	1.5	2.5	--	--	0.5	0.5	0.4	2.5	14.1	3.0

Samples were subject to the following PCR temperature profile: initial denaturation at 94 °C for 3 min followed by 35 cycles where denaturing was set at 94 °C for 30 s,

annealing at 52 °C for 40 s and extension at 72 °C for 1 min 10s. Finally, samples were subjected to 72 °C for 10 min.

PCR products were then visualised by agarose gel electrophoresis and purified by Macrogen Europe prior to sequencing. Sequences were generated at Macrogen Europe and edited with CodonCode Aligner.

4.5.4. Sequence analysis

COI sequences were aligned using the CrustalW algorithm in BioEdit (Hall, 1999). ITS sequences were aligned using the software Muscle as implemented in MEGA 5 (Tamura et al., 2011). The final COI alignment had a length of 568 bp whilst the final ITS alignment had a length of 608 bp. Selected outgroups available in GenBank were used to root the COI tree. Appropriate outgroups were not available in GenBank (no closely related sequences were available) to root the ITS tree.

MEGA 5.0 (Tamura et al., 2011) was used to produce trees using Maximum Likelihood as the optimality criterion under a general time reversible model with gamma distribution of substitution rates (five discrete steps) and a proportion of invariable sites (GTR+I+G model). 1000 bootstrap replicates were used to calculate branch support. Trees were then edited in FigTree v. 1.3.1 (Rambaut 2006-2009).

4.5.5. Data analysis

Data collected was analysed using SigmaPlot 12.0. Tests for normality and equality were used to justify the use of parametric and non-parametric deductive tests. The following statistical analyses were carried out on the respective data sets:

Feeding response: Paired t-test

Escape response: Paired t-test

Feeding response in 'presence' of predator: Paired t-test

4.6. Results

4.6.1. Phylogenetic analyses

P. dumerilli were obtained from multiple sites throughout Europe, including the CO₂ vents of Ischia (Naples, Italy). DNA extraction and COI analyses were carried out to investigate the levels of relatedness and phylogeographic patterns of these populations. The phylogenetic trees produced (Figure 4.2) for *P. dumerilli* show multiple distinct evolutionary lineages with low genetic diversity between them. The COI tree (Figure 4.2A) shows that 10 of the 12 sequenced individuals from the acidified site, in addition to a single individual from a nearby control site, form one genetic lineage. Meanwhile, one individual from the acidified site belongs to a clade comprising individuals from control sites. One individual from the acidified site forms its own genetic lineage. The two individuals from the acidified site form a genetic lineage distinct from all other populations in the ITS tree (Figure 4.2B).

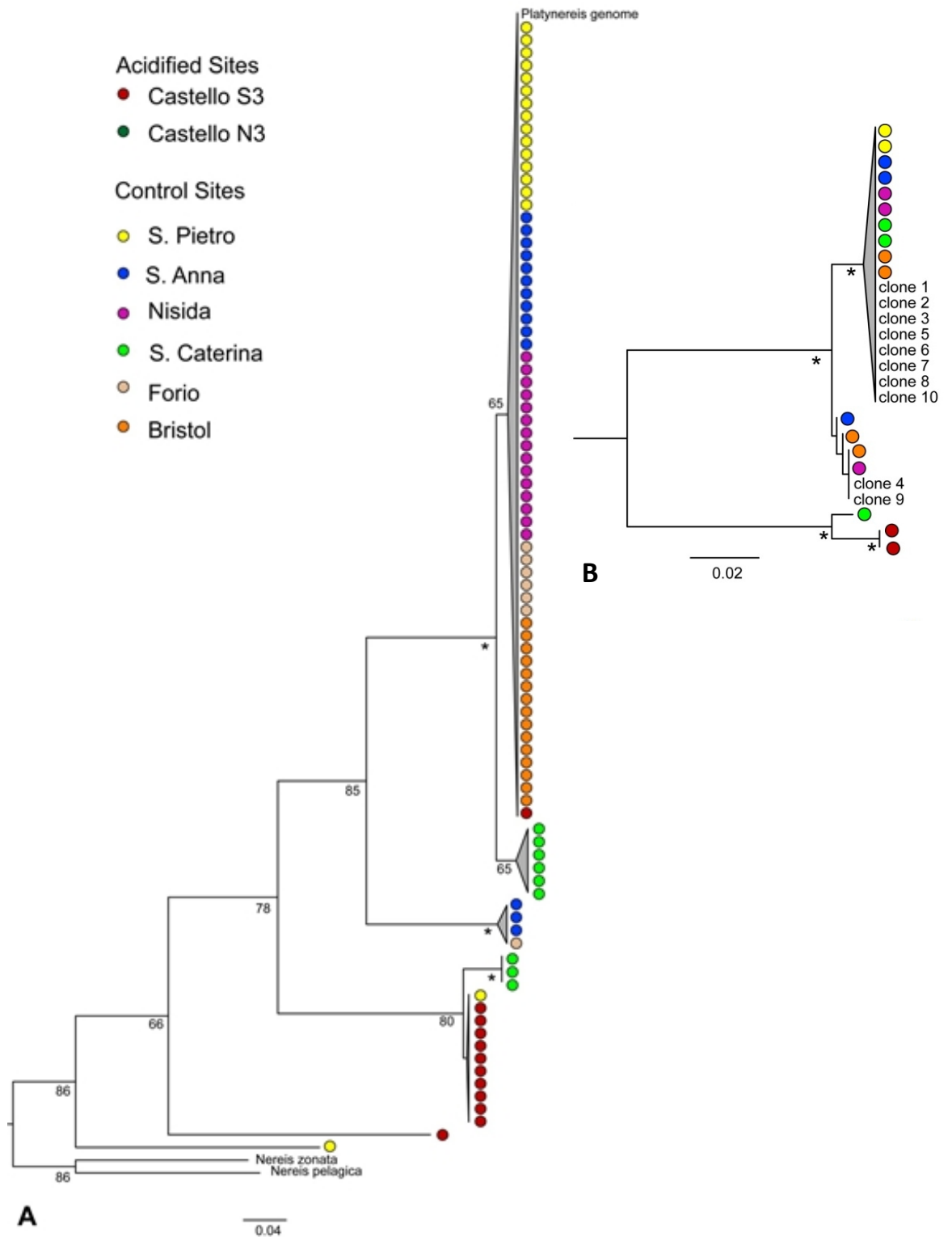


Figure 4.2. Phylogenetic trees (Calosi et al., 2013) utilising cytochrome *c* oxidase subunit I (COI) and Internal Transcribed Spacer (ITS) sequence data. Bootstrap percentages (1,000 pseudoreplicates) indicate branch support with asterisk denoting bootstrap values >98%. (A) COI tree for *P. dumerilii*. Genbank accession numbers for outgroups: *Nereis zonata*: HQ024403; *Nereis pelagica*:GU672554; (B) ITS tree for *P. dumerilii*. GenBank accession numbers for “clone 1” through “clone 10” refer to Hui et al., 2007.

4.6.2. Behavioural assays

Feeding response

Animals were isolated and starved for 48 hours prior to experimentation; individuals were then presented with a food source (spinach) in pH treatment 8.2 and 7.8. The presence/absence of feeding was noted. Figure 4.3 below shows the average (%) feeding response to spinach in pH treatment 8.2 and 7.8.

When animals were obtained from Forio data passed a Shapiro-Wilk test for normality ($P > 0.05$). There was a statistically significant reduction in mean feeding response at pH treatment 7.8 (mean = 2.571 ± 1.158 S.D.) (t-test, $t = 11.500$, $df = 13$, $P < 0.001$) indicating a lower response to food in control site worms at vent pH conditions.

Likewise when animals were obtained from the CO₂ vent of Ischia data passed a Shapiro-Wilk test for normality ($P > 0.05$). There was a statistically significant reduction in mean feeding response at pH treatment 7.8 (mean = 4.250 ± 1.581 S.D.) (t-test, $t = 3.870$, $df = 7$, $P < 0.01$) indicating a lower response to food in vent site worms at vent pH conditions.

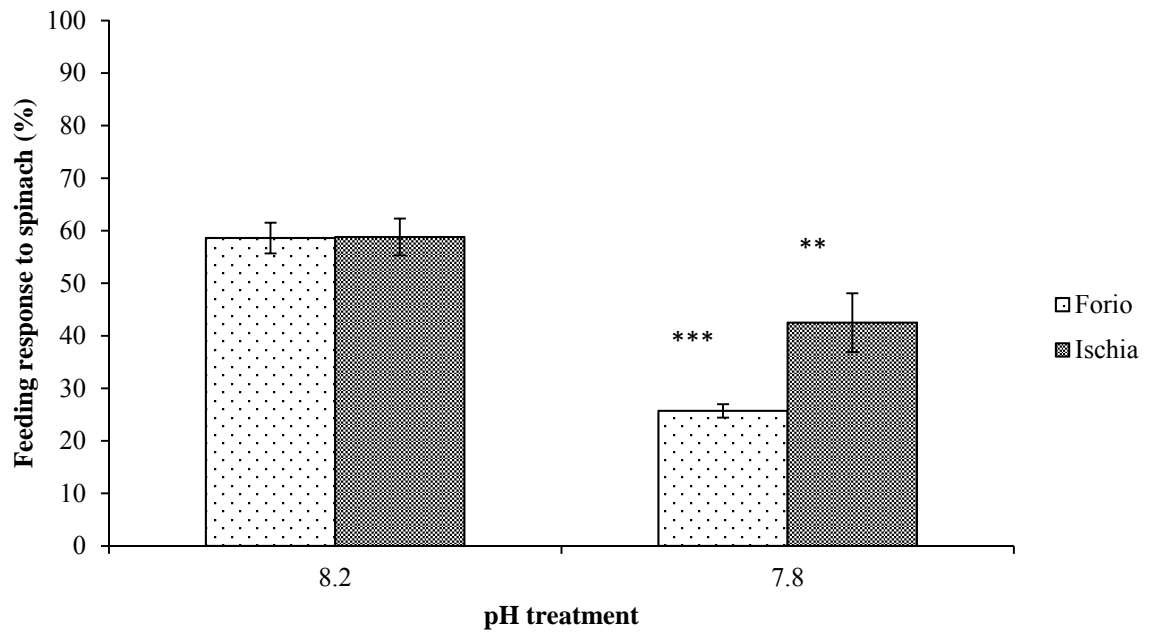


Figure 4.3. Mean feeding response (\pm SE) of *P. dumerilii* from the control site, Forio and the CO₂ vent of Ischia to spinach in two pH treatments, 8.2 and 7.8, n = 14. Significant difference, *** ($P < 0.001$).

Escape response

Animals were isolated prior to experimentation; crab conditioned water (odour) was injected into the water column in pH treatment 8.2 and 7.8. The presence/absence of an escape response was noted. Figure 4.4 below shows the average (%) escape response to crab conditioned water in pH treatment 8.2 and 7.8.

When animals were obtained from Forio data passed a Shapiro-Wilk test for normality ($P > 0.05$). There was a statistically significant reduction in mean escape response at pH treatment 7.8 (mean = 3.786 ± 1.251 S.D.) (t-test, $t = 2.679$, $df = 13$, $P < 0.05$) indicating a lower response to predator odour in control site worms at vent pH conditions.

Likewise when animals were obtained from the CO₂ vent of Ischia data passed a Shapiro-Wilk test for normality ($P > 0.05$). There was no statistically significant difference in mean escape response between pH treatment 8.2 (mean = 6.125 ± 1.642 S.D.) and 7.8 (mean = 5.750 ± 1.832 S.D.) (t-test, $t = 0.424$, $df = 8$, $P > 0.05$) indicating detection of a predator (and subsequent escape response) is not improved in vent site worms at control pH conditions.

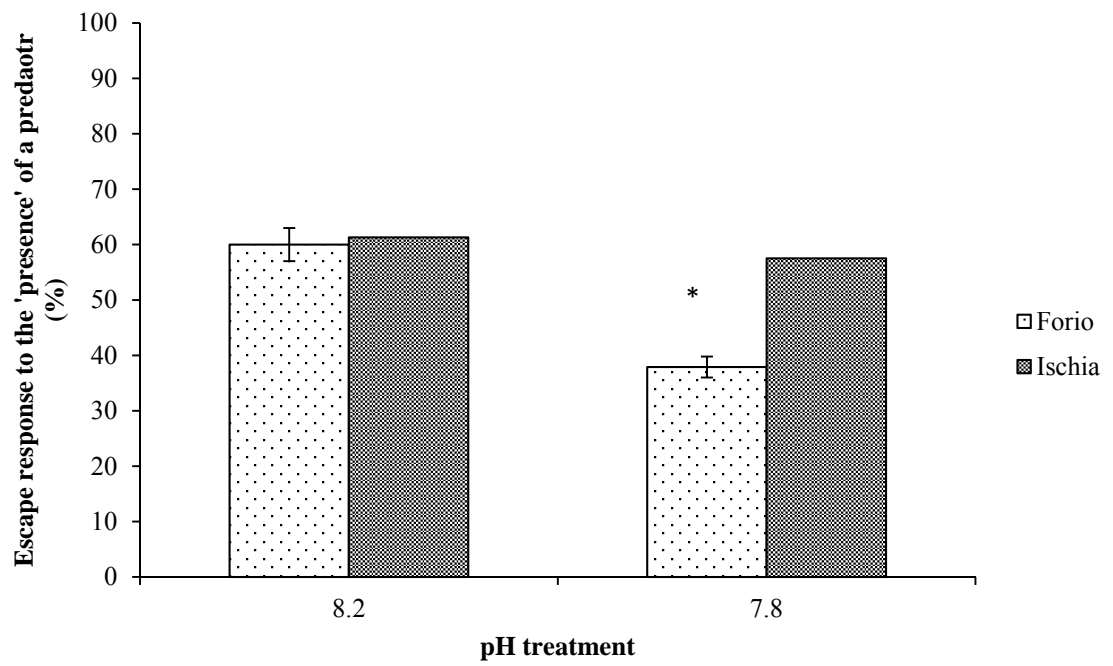


Figure 4.4. Mean escape response (\pm SE) of *P. dumerilii* from the control site, Forio to the ‘presence’ of a predator (*R. harrisii* odour) in two pH treatments, 8.2 and 7.8, n = 14. Significant difference, * ($P < 0.05$).

Feeding response in 'presence' of predator (odour)

Animals were isolated and starved for 48 hours prior to experimentation; crab conditioned water (odour) was injected into the water column. Following a 5 minute period, individuals were presented with a food source (spinach) and the presence/absence of feeding was noted. Tests were carried out in pH treatment 8.2 and 7.8. Figure 4.5 below shows the average (%) feeding response in the presence of a predator (crab conditioned water, i.e. odour) in pH treatment 8.2 and 7.8.

When animals were obtained from Forio data passed a Shapiro-Wilk test for normality ($P > 0.05$). There was no statistically significant difference in mean feeding response in the 'presence' of a predator between pH treatment 8.2 (mean = 5.429 ± 2.623 S.D.) and 7.8 (mean = 4.143 ± 2.107 S.D.) (t-test, $t = 1.172$, $df = 13$, $P > 0.05$) indicating feeding in the presence of a predator is not adversely affected in control site worms at vent pH conditions.

Likewise when animals were obtained from the CO₂ vent of Ischia data passed a Shapiro-Wilk test for normality ($P > 0.05$). There was no statistically significant difference in mean feeding response in the 'presence' of a predator between pH treatment 8.2 (mean = 4.250 ± 2.315 S.D.) and 7.8 (mean = 3.625 ± 3.114 S.D.) (t-test, $t = 0.478$, $df = 8$, $P > 0.05$) indicating no increase in response to food in the 'presence' of a predator in vent site worms at control pH conditions.

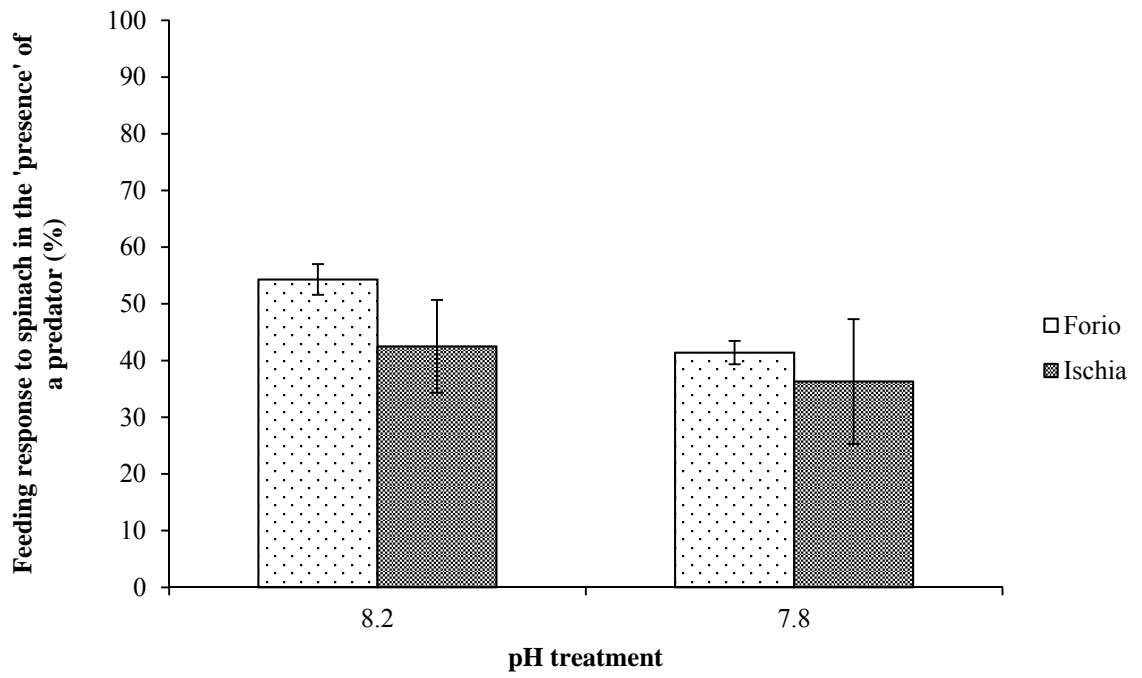


Figure 4.5. Mean feeding response (\pm SE) of *P. dumerilii* from the control site, Forio to spinach in the ‘presence’ of a predator (*R. harrisii* odour) in two pH treatments, 8.2 and 7.8, n = 14.

4.7. Discussion

This study suggests that the marine ectotherm *P. dumerilii* are able to physiologically adapt to chronic and elevated levels of $p\text{CO}_2$. The behavioural responses of organisms are significantly reduced (in all but one behavioural trial, possibly owing to a low number of replicates) when animals maintained (and obtained) at ‘normal’ pH are subject to low pH conditions. In contrast, those maintained (and obtained) at ‘low’ pH show no significant differences between pH conditions. It should be noted that adaptation may not be ubiquitous amongst species within the marine environment, even those with similar ecologies inhabiting the same or similar environment.

4.7.1. Acclimatisation and adaptation

Marine invertebrate studies predict species will (1) tolerate change due to their existing phenotypic plasticity; (2) adapt genetically; (3) migrate or (4) undergo extinction or extirpation (Peck, 2005; Sultan, 2007; Przeslawski et al., 2008; Visser, 2008; Wethey & Woodin, 2008). These responses will influence the outcome for species populations (Bryne, 2011). These different strategies (i.e. acclimatisation and adaptation) entail very different genetic, ecological and conservation implications. It is important, therefore, to clearly discriminate between these strategies for use within this discussion. Their costs and benefits will be considered in order to better predict how marine life will respond to $p\text{CO}_2$ conditions anticipated in the future.

4.7.2. Persistence and potential for acclimatisation and adaptation in a changing ocean

Predictive ecological information is crucial to the scientific community and will provide important insight to managers and conservationists worldwide. Such information can then be used to mitigate and adapt to likely changes to key marine resources and biodiversity over the coming decades.

It should be noted that oceanic changes, such as increasing acidity, will not and have not occurred rapidly. The marine environment has been changing gradually for decades, with some regions changing more than others (IPCC, 2007). It is possible that some

species and populations may have experienced some degree of phenotypic and genetic change already (Byrne, 2011). The oceans are, however, changing at a much faster pace than in the geological past and we cannot say with certainty if adaptive genetic change can occur at a rate that will avoid extirpation and species extinctions.

Freshwater and terrestrial invertebrates have been shown to resist environment stressors (Bridle & Vines, 2006; Derry & Arnott, 2007) providing hope that marine species may be equally capable of adaptive evolution to climate change stressors. Work carried out by Calosi et al. (2013) provides evidence that the polychaete *P. dumerilii*, obtained from the same CO₂ vent used within this investigation, have been able to physiologically adapt to chronic and elevated levels of *p*CO₂. When transplanted to pH 8.2 organisms from the vent suffered respiratory distress and vice versa (Calosi et al., 2013). Notably, this adaptation may have occurred over a relatively short geological time as the CO₂ vents of Ischia have existed for a mere 1,850 years (Lombardi et al., 2011). Copepods inhabiting lakes acidified to pH 6.0 over a period of 6 to 8 years due to SO₂ are capable of rapid genetic-based adaptation (Derry & Arnott, 2007). A number of other studies have pointed to the potential for marine metazoans to adapt to elevated *p*CO₂ conditions (Pistevos et al., 2011; Sunday et al., 2011; Foo et al., 2012; Schlegel et al., 2012).

The first response to environmental stressors, phenotypic plasticity, comes at a cost (Hoffmann, 1995; Dewitt et al., 1998). Plastic responses are generally associated with the reallocation of the available energy budget away from growth and reproduction. When these costs become too great, phenotypic selection for those better able to cope with vent conditions is a more favourable, less 'expensive' strategy. Local adaptation can reduce energy costs of regulation, maintenance and allow organisms to persist at a local level. Where adaptation occurs at the expense of genetic diversity, this may ultimately lead to a decrease in the performance of other traits, for example, life history traits.

COI analysis obtained within this investigation suggests *P. dumerilii* populations sampled are a complex of cryptic species due to the presence of multiple genetic lineages. Cryptic species are a common occurrence in polychaete species (Schulze, 2006; Audzijonyte et al., 2008; Barroso et al., 2010; Pires et al., 2010; Nygren et al., 2011). *P. dumerilii* possess pelagic larvae, and as such may be expected to display a relatively high degree of genetic homogeneity over small geographic scales. Molecular

analyses obtained within this investigation, however, suggest otherwise. Populations at and around the CO₂ vent systems possess high levels of genetic variation. These results indicate strains of *P. dumerilii* are physiologically (and genetically) adapted to life in a high *p*CO₂ environment. Vent strains are genetically distinct from all others tested. It is possible that such genetic variation has occurred as a result of rapid evolution at the, relatively recent, CO₂ vents or via speciation at another CO₂ vent system. In addition to the previously referenced studies (Derry & Arnott, 2007; Pistevos et al., 2011; Sunday et al., 2011; Foo et al., 2012; Schlegel et al., 2012), evidence for rapid evolution along a continuous environmental gradient has been shown to occur in the Trinidadian guppies (Reznick & Ghalambor, 2002; Torres-Dowdall et al., 2012).

The apparent segregation of individuals between the acidified sites and nearby control sites suggests modest exchange between individuals from these respective sites. The reproductive capability of animals inhabiting the high *p*CO₂ habitat is yet to be examined. Equally so is the potential for reproduction between animals from the acidified sites and nearby control sites.

Behavioural biosassays within this investigation support the COI analyses discussed above. When animals were obtained from control seawater (pH 8.2), both feeding and escape responses were reduced in acidified seawater (pH 7.8) treatments. Conversely, feeding in the presence of a predator did not appear to be significantly different between pH treatments. These findings support the theory that acclimatisation to adverse pH conditions within a lifetime is not possible.

Interestingly, when animals were obtained from acidified seawater escape response and feeding in the presence of a predator were not significantly different between pH treatments and worms did not appear 'distressed' as in the study by Calosi et al. (2013). Following a lifetime in low pH conditions, short term exposure to control seawater did not significantly improve an organism's ability to detect a potential threat. These results, implying animals are adapted to the low pH environment from which they are sampled; provide further evidence for the potential of adaptation. Feeding response was significantly reduced in the acidified seawater treatment. This result was unexpected and unaligned with the aforementioned findings. It should be noted that whilst significantly reduced at pH treatment 7.8, feeding in these organisms was still twice that of those organisms in the same treatment obtained from control pH conditions. It would

appear therefore that these organisms are more capable of successfully locating food in adverse pH conditions, likely due to their origin in low pH conditions. Replicates were low in the bioassays discussed. To provide more conclusive evidence I would suggest further replicates in the future. These preliminary findings do, however, appear to support other analyses.

The ability of marine ectotherms (and marine organisms in general) to persist in a rapidly changing ocean (Calosi et al., 2013) will ultimately depend upon taxa ability for rapid physiological adaptation. Adaptation may occur via genetic assimilation of emerging phenotypes (Byrne, 2011). It should be noted that there will likely be significant differences between species and life history stages in tolerance to ocean change stressors such as ocean acidification (Byrne, 2011).

Short-lived species with fast generation times are more likely to be capable of evolutionary adaptation to climate change stressors than slow-developing species (Fabry et al., 2009). It is possible therefore that *P. dumerilii* and *Alitta succinea* may have a greater capacity for adaptation than larger individuals owing to their short life cycle and consequently generation time. Indeed the findings within this investigation suggest that *P. dumerilii* have been able to adapt over a relatively short geologic time scale. Whilst ocean acidification is expected to worsen occur over an even shorter period these results provide some hope for resilience to a harsh high $p\text{CO}_2$ environment of the future.

Chapter 5:
General discussion

5.1. Effects of environmental change

Environmental variation over space or time can have both positive and negative effects on biodiversity and ecosystem function in direct relation to the rate, magnitude, duration and spatial scale of environmental change (Knoll et al., 2007). Species, populations and genotypes evolve through exploitation of novel habitats, or specialization in response to environmental variation (temperature, habitat complexity, oxygen concentration, light, etc.) and biological interactions (trophic, competitive, or mutualistic) (Barry et al., 2010).

Evidence for biodiversity shifts in response to environmental change exists in fossil records (Mayhew et al., 2008). The effect of CO₂ levels on extinction rates in marine genera was greater than that of temperature (Mayhew et al., 2008). Whilst marine life recovered following each extinction, recovery required millions of years (Mayhew et al., 2008). It is possible this could be attributed to slow rates of evolutionary diversification or (persistently) unfavourable conditions, or both (Knoll et al., 2007). It is predicted that the ongoing large and rapid changes in oceanic pH and carbonate saturation will drive environmental changes unseen in recent evolutionary history of marine organisms (Barry et al., 2010). Such rapid changes will pose an evolutionary challenge for organisms to acclimatise and adapt (Barry et al., 2010).

Ocean acidification driven extinction will, no doubt, alter the function of marine ecosystems (Orr et al., 2005). Changes to the relative abundance and activities of a species will have similar effects (Orr et al., 2005). It is important to remember that species may be affected by ocean acidification directly or indirectly. Restructuring of marine communities is possible if there are significant shifts in the abundance of 'losers' and 'winners' (Barry et al., 2010).

5.2. Potential for nereidid polychaete survival in a changing aquatic environment

Acidified seawater, as predicted for the year 2100, has been shown to negatively impact the nereidid polychaetes *Platynereis dumerilii* and *Alitta succinea* at a number of stages in the life cycle.

5.2.1. Survival, development and reproductive output

When cultured in low pH *P. dumerilii* were less likely to survive and successfully reach maturity. Where successful maturation occurred, heteronereids were significantly fewer in number than the control culture. When obtained from low pH cultures mature females released significantly fewer eggs whilst male swimming ability (an essential reproductive behaviour) was impaired. These results (addressing acclimatisation) paint a rather bleak picture for the species and it is interesting to note the potential impacts that acidification may have upon fertilisation and larval development (see Chapter 2). Conversely, Chapter 3 implies the potential for acclimatisation further reinforcing how complex a topic ocean acidification is and the need for further research. It was not possible to conduct fertilisation and larval success experiments with *P. dumerilii* within this investigation due to asynchronous development and low numbers of mating pairs. A closely related species, *A. succinea*, with a similar life history was used. Time permitting it would be best to perform all experiments for each species. Experiments utilising both species show similar outcomes and as such it is likely that experiments conducted with one species only would yield similar results for the other.

As with *P. dumerilii*, *A. succinea* egg production and male swimming ability was significantly reduced when cultured in low pH. Where mature males and females occurred, mating pairs were formed and eggs and sperm combined in different seawater treatments. Fertilisation was significantly reduced in low pH treatments irrespective of the culture mating pairs originated from. Furthermore, fertilisation success was significantly reduced when males were cultured at low pH. Similarly, larval success was significantly reduced in low pH treatments irrespective of the culture mating pairs originated from. Larval success was significantly reduced when females were cultured at low pH. Similar findings have been noted in Havenhand et al. (2008). Results

obtained here suggest males cultured in low pH are the limiting factor in one of the first stages of reproduction, fertilising the egg (see Chapter 2). This could be attributed to a general reduction in fitness; previous results suggest swimming ability is impaired at low pH, or due to a reduction in gamete quality, i.e. poor quality sperm. The female then appears to be the limiting factor in development; it is likely that it is now the quality of the egg that dictates the potential for successful development from fertilised egg to larvae.

5.2.2. Chemoreception mediated behaviours

Whilst the previous points all address negative impacts on stages of development, i.e. gamete production and beyond; acute and prolonged exposure to acidified seawater has also been shown to reduce the response to a variety of feeding and predator cues in *P. dumerilii* (see Chapter 3). The effects of this will be potentially severe, increasing foraging times needed to compensate failed attempts to locate will increase energy output leaving individuals exposed and at greater risk of predation, predation by predators they can no longer detect. Consumption of food was found to be significantly less in those animals cultured at low pH, suggesting individuals are no longer able to detect and successfully locate a food source. It should be noted in these experiments wet weight was used and as such it is likely there was some degree of error in amounts of food weighed and distributed between tanks. These results should be interpreted with caution due to such variation.

This study indicates pH-induced disruption to chemoresponsiveness in polychaetes and in turn, potentially other taxa. Effects are likely to differ between species dependent upon their tolerance to pH changes. Moreover, chemical signals differ in their structures and the effects of pH upon such structures will differ. Such widespread, variable and unpredictable changes will undoubtedly have the potential to re-order aquatic community dynamics and structure.

Further studies should be carried out utilising a range of taxa to determine the potential effects ocean acidification may have upon essential life processes such as feeding and predator avoidance, throughout the entire aquatic ecosystem. Many studies to date have been short-term in nature and are limited in both space and time; as such they may not capture important processes (e.g. acclimatisation and adaptation, multispecies biological

interactions, chronic low-level impacts). Time to acclimatise varies between species and has been shown to be approximately 6 weeks in fishes (Deigweiher et al., 2008) and a matter of hours in coccolithophores (Barcelos e Ramos et al., 2010). Long term studies utilising long-lived species, e.g. fish, are still problematic as these cultures and experiments are unlikely to allow adequate time for multiple generations and therefore potential adaptation to occur. Short-lived species, such as *P. dumerilii*, are ideal for such purposes due to their short generation time; multiple generations can be cultured within the laboratory allowing for the possibility of potential adaptation in long term studies.

It is clear from the results obtained and discussed above, that ocean acidification negatively impacts *P. dumerilii* and *A. succinea* at various stages of the life cycle. Whilst each impact is deleterious in isolation, the combined effects will pose a great challenge to these species if they are to overcome the stresses associated with ocean acidification. Effects on different life stages can amount to significant impacts on population success (Barry et al., 2010). In a study by Findlay et al. (2010) exposure to low pH was predicted to reduce the survival of early life stage barnacles along the south-west coast of the United Kingdom by 25%, potentially reducing the local population abundance. According to this study, *P. dumerilii* and *A. succinea* are not capable of acclimatisation in one lifetime. The breeding of multiple generations required for adaptation was out of the scope of this investigation.

5.2.3. Potential for adaptation

The naturally occurring CO₂ vents of Ischia (Naples, Italy) provide a naturally acidified marine environment and have been the focus of a number of ocean acidification in recent years (Rodolfo-Metalpa et al., 2011; Hall-Spencer et al., 2008; Calosi et al., 2013). The presence of *P. dumerilii* around and within these vents (present for thousands of years) suggests potential adaptation to low pH within this species. *P. dumerilii* obtained from these vents and various locations throughout Europe were sequenced to produce COI and ITS phylogenetic trees. Vent organisms were genetically distinct from others forming their own genetic lineage; only one control organism was present in this lineage (see Chapter 4).

Cryptic species are a common occurrence in polychaetes and it is often difficult to distinguish between them as they are morphologically similar. It is possible that

organisms sampled at the CO₂ vent are indeed a cryptic species. They are, however, likely adapted to this environment. As discussed in Chapter 5, when transplanted to pH 8.2 organisms from the vent suffered respiratory distress and vice versa (Calosi et al., 2013). Furthermore, individuals showed some signs of adaptation in feeding trials conducted within this investigation. There was no statistically significant reduction in the detection of food and predators as in control cultures. These findings provide further evidence for the potential for adaptation in organisms sampled at the vent and further work is needed to understand how these animals are able to adapt to or tolerate these conditions.

5.3. Effects of ocean acidification on organisms

Ocean acidification studies to date have shown that some organisms may be more robust and tolerant of low pH than others (Doney et al., 2009). Taxa such as cyanobacteria currently carbon-limited may be amongst ‘winners’ in a high CO₂ ocean of the future (Barry et al., 2010). A number of taxa, including coral and molluscs, have been shown to exhibit reduced calcification in acidified conditions (Michaelidis et al., 2005; Kuffner et al., 2008; Doney et al., 2009). Interestingly, in the same conditions, increased calcification rates have been observed in a small number of taxa (Ries et al., 2009). It is expected that increases in calcification rates would require energetic trade-offs thereby reducing overall performance (Wood et al., 2008). Such variable responses to ocean acidification highlight the complexity of predicting the impact of ocean acidification.

Even taxa tolerant of low pH will experience changes to the physiological ‘cost of living’ required for basic biological functions (Seibel and Walsh, 2003). High CO₂ waters are expected to disrupt the acid-base status of many marine organisms (Barry et al., 2010). Such disruption will reduce respiratory efficiency, enzyme activity and metabolic depression (Seibel and Walsh, 2003). Assuming a constant total energy budget, Barry et al. (2007) have predicted the change in the ‘cost of living’ expected as a result of pH stress (Figure 5.1). Taxa affected by physiological stress are expected to experience reduced growth, reproductive output and survival (Barry et al., 2010). A number of these impacts have been demonstrated within this investigation using *P. dumerilii* and *A. succinea*. As previously discussed, impaired performance and survival of individuals will have negative impacts at the population level (Barry et al., 2010).

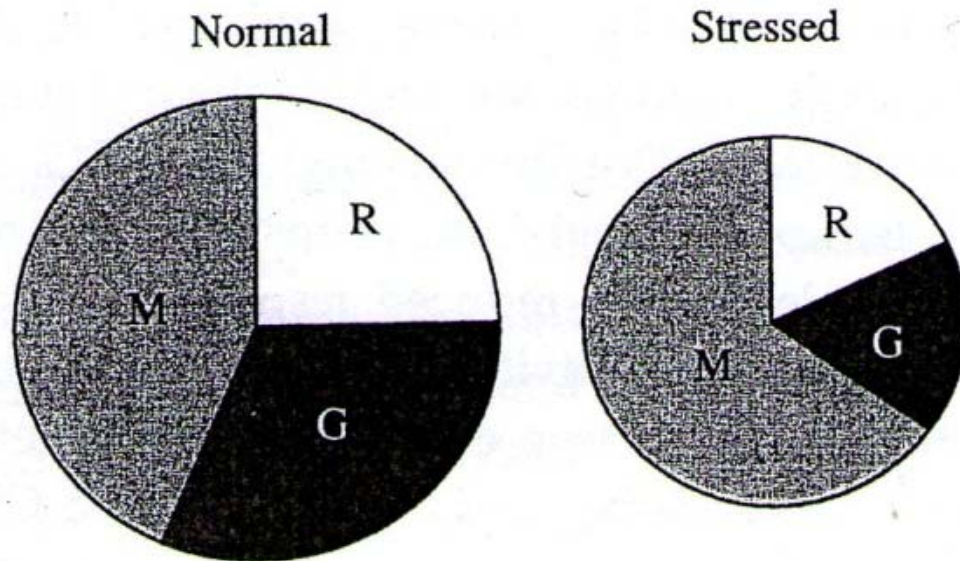


Figure 5.1. Hypothetical energy budget for normal and stressed organisms; M = maintenance, R = reproduction, G = growth. (Barry et al., 2010).

Sensitivity of marine organisms to ocean acidification is expected to be dictated largely by fundamental physiological adaptations (Barry et al., 2010). For example, large organisms such as fishes, crustaceans and cephalopods have a natural capacity for gas exchange and as such may be pre-adapted to many of the stresses related to ocean acidification (Melzner et al., 2009). These organisms are capable of intense aerobic activity which generates metabolic CO₂ (Barry et al., 2010). This physiological challenge, already faced, may provide these organisms with a greater scope for tolerance to high CO₂ waters.

Similarly, taxa inhabiting areas with variable or low pH may possess adaptations that allow them to tolerate or thrive in low pH environments (Goffredi and Barry, 2002). *A. succinea* is a brackish water species exposed to fluctuating salinity. It is possible that they may be tolerant of fluctuating pH. Broadcast spawners, such as *P. dumerilii* and *A. succinea*, are assumed to be more susceptible to acidification in the early life stages as gametes are released into the water column (Havenhand et al., 2008). Reduced fertilisation and larval success have been demonstrated within this investigation (see

Chapter 2) thereby reinforcing this prediction. It can be reasoned, therefore, that ocean acidification may have varying effects at different stages of the life cycle. Future studies should take this into account.

Making predictions is even more complex when taking into account that ocean acidification is not expected to occur uniformly across the globe (Doney et al., 2009). Certain areas, such as cold water corals of the southern ocean where CO₂ and CaCO₃ is increased, are likely to experience more rapid water chemistry changes than others (Sabine et al., 2004).

5.4. Future research

5.4.1. Investigating the effects of ocean acidification on whole communities

The majority of studies to date, including this one, investigate the effects of ocean acidification upon a single species in isolation (Hoffman et al., 2010). Whilst these investigations may provide us with some insight into the fate of marine life in acidified waters, there also a number of limitations. These studies are not able to provide a clear picture as to how ocean acidification will affect communities and ecosystems as a whole (Kroecker et al., 2011).

In natural systems, *P. dumerilii* and *A. succinea* are single species in a larger community interacting with a number of other species to form a complex web. As previously discussed, the effects of ocean acidification can be both direct and indirect. Nereidid polychaetes are preyed upon by fish and birds. A reduction in polychaetes as a result of ocean acidification could ultimately mean less prey available to predators thereby impacting higher trophic levels.

P. dumerilii are found on the rocky shore of Hinkley Point, Bristol amongst a variety of algal species, e.g. *Corallina officianlis* and *Ulva lactuca*, which they use as a suitable habitat. Studies suggest that some algal species may be relatively tolerant to low pH (Roleda et al., 2012). Should these findings translate to the natural environment it is possible that *P. dumerilii* may not suffer acidification induced habitat loss as greatly as other species. At the same time predator species may reduce in abundance thereby increasing the likelihood of survival in *P. dumerilii*. *A. succinea* collected from the

harbour walls of Cardiff Bay, Wales, live amongst established mussel beds. These mussel communities provide *A. succinea* with an important habitat and food source. A number of studies have reported reduced growth (Berge et al., 2006), metabolic rate (Michaelidis et al., 2005), protein degradation and immune response (Bibby et al., 2008) when *Mytilus* species are exposed to acidified waters. A reduction in habitat providing species such as *Mytilus* will add further pressure to inhabitants, e.g. *A. succinea* when already faced with the challenges of acclimatizing/adapting to low pH conditions predicted for the future. These varied findings highlight the importance for investigating the community as a whole.

A reduction in nereidid polychaetes could lead to an increase in more robust benthic invertebrates; such changes would result in a reduction in biodiversity thereby creating a less complex community. Similar shifts in biodiversity have been observed at naturally occurring CO₂ vents, Ischia (Naples, Italy). Here, species richness is reduced and species composition changed (Hall-Spencer et al., 2008). A number of studies utilising these areas of acidified water compare habitat structure with nearby non-venting areas (Hall-Spencer et al., 2008). Diversity and structural complexity of reef habitats appear reduced with seagrass and macro algal growth being noted in CO₂ venting areas around Papua New Guinea (Fabricus et al., 2011).

5.4.2. A mesocosm approach

Whilst efforts are made, laboratory based experiments cannot account for all factors that would normally occur in the aquatic environment. Mesocosms are an experimental tool which replicates a whole part of a community whilst still allowing the experimenter adequate control and manipulation when necessary. The main aim of the mesocosm approach is to compromise on constraints present in laboratory based studies.

Outdoor mesocosms have been used to study the effects of ocean acidification upon coral reefs in Hawaii (Kuffner et al., 2007). Seawater was sourced from the nearby reef and the only experimental manipulation was pH adjustment, all external variables (e.g. solar radiation, temperature, seawater chemistry) were natural and uncompromised. Following seven weeks of exposure to acidified seawater, recruitment and growth rates of crustose coralline algae were negatively impacted (Kuffner et al., 2007). Similarly,

Jokiel et al. (2008) reported supporting findings following a laboratory based mesocosm in which crustose coralline algae were exposed to acidified conditions.

Whilst *P. dumerilii* reside within a slightly more complex ecosystem than *A. succinea*, it would be possible to perform mesocosm based studies with the latter species. *A. succinea* live alongside a small number of species, amongst these mussels (*Mytilus edulis*), barnacles (*Semibalanus balanoides*) and mud crabs (*Rhithropanopeus harrisi*). This could be easily replicated within the laboratory to assess ocean acidification effects upon *A. succinea* using a mesocosm based approach.

5.4.3. Naturally occurring CO₂ vents provide areas in which to study the long term effects of ocean acidification on marine organisms and ecosystems

In recent years the mesocosm based approach has become increasingly popular in the study of ocean acidification. But perhaps the most obvious and reliable mechanism for study is to utilise naturally occurring CO₂ vents (as in Chapter 4). An increasing number of studies utilising venting areas have emerged in recent years (Hall-Spencer et al., 2008; Rodolfo-Metalpa et al., 2011; Calosi et al., 2013).

As previously discussed, most ocean acidification studies expose organisms to low pH over a short period of time. Such experiments do not allow adequate time for acclimatisation and adaptation to occur. As such, vent sites allow researchers to examine communities and species which have been exposed to acidified conditions (to varying degrees) over generations, and often thousands of years (Hall-Spencer et al., 2008).

Rodolfo-Metalpa et al. (2011) have used the CO₂ vents of Ischia (Naples, Italy) as ready-made laboratories, transplanting test species to be monitored over a period of months. Similar experiments have been carried out by Calosi et al. (2013) in which it has been shown that *P. dumerilii* from control pH suffer metabolic depression when transplanted to the vent site. Similar results were seen when *P. dumerilii* from the vent site were transplanted to control pH. Furthermore, as discussed above, *P. dumerilii* have been shown to be genetically distinct from other *P. dumerilii* populations. These results suggest adaptation and further studies should be carried out to investigate how these organisms are able to tolerate unfavourable conditions.

It is highly likely that further studies will be carried out utilising these vent sites due to the valuable information these habitats provide. With pH expected to drop a further 0.3 units by 2100 there is great urgency to understand the implications of these changes. Naturally occurring CO₂ vent sites will allow scientists to acquire reliable information quickly potentially affecting future policy.

5.4.4. Understanding the mechanisms of ‘signal disruption’

Haye et al., (2012) have suggested that chemical reception in the marine environment may be disrupted by ocean acidification in four ways:

1. Changes to the charge distribution of odour molecules disrupt receptor-ligand interactions.
2. Changes to the charge distribution of the odour receptors disrupt receptor-ligand interactions.
3. Physical damage to the sensory organs.
4. Reduction in motivation associated with increased metabolic load (to maintain acid-base balance in low pH conditions).

Each of the points above has been discussed in detail in Chapter 3. Research into this area is still in its infancy and the varying hypotheses above highlight the need for further research into how acidified waters will impact chemical communication.

Many studies to date have relied upon behavioural observations, as is the case in this investigation. Electrophysiological techniques may be used to quantify the biological response to chemical signals instead of relying upon behavioural observations only. Electrophysiology measures the electrical activity in neurons following stimulation; this technique may be used to determine potential reduction in activity following exposure to acidification. Such studies are currently underway within the research group using *A. succinea*. Molecular dyeing and radiolabelling techniques may be useful tools to investigate receptor-ligand binding activity (Hardege et al., 2013, under review). Labelled signal molecules can be used to visualise receptor-ligand binding and the extent to which it occurs in acidified vs. control conditions.

There is still a great deal of research to be carried out before we can truly understand the implications of ocean acidification. Naturally occurring CO₂ vent sites are providing us with new, exciting insight into this topic and will continue to do so. The development of mesocosms and techniques discussed will prove to be equally important in the coming years should we hope to inform and impact upon policy.

6.0. Conclusions

This study has shown that when subject to pH stress, the nereidid polychaete *Platynereis dumerilii* exhibits reduced survival and fails to successfully reach the mature heteronereis stage. When mature, *P. dumerilii* and *Alitta succinea* females produce fewer eggs, similarly males fail to fully respond to reproductive cues. Fertilisation and larval success are significantly reduced in *A. succinea*. These results suggest *P. dumerilii* and *A. succinea* are not capable of acclimatisation within one lifetime.

In addition to impacting development and reproductive fitness, exposure to low pH negatively affects detection of food and predators in *P. dumerilii*. Such findings could have serious implications for the individual, population and species as a whole.

P. dumerilii sampled and sequenced from a naturally occurring CO₂ vent are genetically distinct from other known populations of *P. dumerilii* throughout Europe. This could be indicative of a pH adapted cryptic species. Behavioural assays carried out show no significant differences between pH treatments. Further research is now needed to identify how these individuals have adapted and are able to tolerate low pH.

It is clear that future levels of ocean acidification will affect many organisms, both directly and indirectly. Chemoreception in the complex aquatic environment is crucial and further research is urgently needed to understand the effects ocean acidification will have. Future studies should be carried out over adequate time periods to allow for acclimatisation and/or adaptation. Where possible, mesocosm and in-situ approaches will provide greater, reliable insights to the effects acidified waters will have on a natural system.

Summary

1. The number of individuals to survive and successfully reach maturity was reduced when *Platynereis dumerilii* were cultured in acidified seawater (pH 7.8).
2. Egg production was significantly reduced when *P. dumerilii* and *Alitta succinea* were cultured in acidified seawater.
3. The physical ability of male *P. dumerilii* and *A. succinea* to perform reproductive swimming behaviour was impaired when cultured in acidified seawater.
4. The fertilisation success of *A. succinea* was significantly reduced in acidified treatments (regardless of culture conditions) and when males were cultured in acidified seawater.
5. The larval success of *A. succinea* was significantly reduced in acidified treatments (regardless of culture conditions) and when females were cultured in acidified seawater.
6. Acute and long-term exposure to acidified seawater impaired the ability of *P. dumerilii* to respond to a variety of feeding and predator stimulants.
7. Long-term exposure to acidified seawater impaired the detection and thereby consumption of food in *P. dumerilii*.
8. *P. dumerilii* obtained and sequenced from the CO₂ vent of Ischia (Naples, Italy) formed a genetic lineage distinct from others in Europe (COI and ITS analysis).
9. *P. dumerilii* obtained from the CO₂ vent of Ischia showed some signs of adaptation to pH stress in feeding trials.

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