Reproduction in a changing ocean: the effects of ocean acidification and other environmental stressors on the sea urchin *Paracentrotus lividus* and the Polychaete *Ficopomatus enigmaticus*

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Abstract

Atmospheric CO_2 enrichment is a key factor contributing to global climate change. Major consequences of climate change include increasing sea surface temperature and decreasing seawater pH (ocean acidification) – both of which are predicted to lead to an increase in ocean hypoxic events. Early ontogenetic stages of invertebrates have shown sensitivity to these environmental changes. Previous research has focused on short-term exposure to these environmental factors; however, the long-term and multi-generational effects of ocean acidification on organismal reproduction and development have received little attention. The aim of this thesis is to address this shortcoming for reproduction and early embryonic development of the economically- and ecologically-important sea urchin, *Paracentrotus lividus* (Lamark, 1816) and the reef-forming invasive polychaete, *Ficopomatus enigmaticus* (Fauvel, 1923).

Fertilisation success and early embryogenesis of *P. lividus* were negatively impacted under conditions of increased pCO_2, at ambient temperature and ambient +2°C after 12 months exposure. Sperm motility – determined by computer assisted sperm analysis (CASA) – showed a significant increase in average swimming speed measured as curvilinear velocity (VCL) at increased pCO_2 levels after 6 months but by 12 months VCL values had decreased. There was no overall significant effect of pCO_2 on VCL but there was a significant reduction in fertilisation success under hypoxic conditions. Increasing pCO_2 levels appeared to buffer the effects of hypoxia, however, with significantly lower fertilisation success observed only under hypoxic conditions at ambient pCO_2.

Multi-generational experiments examined the effects of increased pCO_2 on *F.enigmaticus*. Both percentage sperm motility and sperm VCL were similar between experimental treatments, but there was a significant difference between generations 0 and 2. In contrast, fertilisation success was negatively affected by both pH and generation, with a significant reduction seen with pH at all generations and a significant reduction seen between generation 0 and generation 2. Oocyte diameter was also significantly affected by pH and generation, with a significant increase in egg diameter seen at generation 1 in all pH treatments except pH 7.95, which shows a significant increase and generation 2, which suggests differing levels of maternal investment.

This research suggests that in the long term, ocean acidification, temperature and hypoxia may seriously impact the reproduction and development of two important

marine invertebrate species. This research highlights the need for further long-term investigation involving multiple stressors to provide a more comprehensive understanding of the long-term effects arising from changing oceanic chemistry.

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1.1 Introduction

1.1.1 The global oceans and climate change

Atmospheric carbon dioxide mixing ratios (parts per million by volume) have risen from 280ppm in pre-industrial times (1750-1800) to the present day values of 380ppm-390ppm (Denman et al., 2007). This increase in CO_2 is primarily as a result of burning fossil fuels, deforestation and the production of cement (Worrell et al., 2001; IPCC, 2007). Almost half (48%) of the anthropogenically derived CO_2 produced to date has been absorbed by the oceans (Sabine et al., 2004). Owing to increasing global levels of CO_2 there have already been numerous changes in oceanic processes including heat budget, ocean circulation, tropical storms, storage and transfer of CO_2, climate and ocean acidification (Reid et al., 2009). The oceans function as a biological pump; this term describes a variety of separate processes, many of which revolve around carbon and its storage (Riebesell et al., 2007). The biological carbon pump is crucial in maintaining atmospheric CO_2 s. This is achieved by the fixation of CO_2 by phytoplankton. This biomass then sinks and is oxidised thereby helping to maintain a vertical gradient of dissolved inorganic carbon with the higher values found at depth (Hofmann and Schellnhuber, 2009). The long-term effects of increasing temperature are not yet known. The oceans have acted to buffer the effects of increasing levels of CO_2; however the oceans ability to store excess CO_2 is not limitless and the consequences of decreased absorption rates may lead to major effects for organisms and ecosystems as well as climate change itself, by increasing the rate at which climate change is occurring (Reid et al., 2009).

1.1.1 Ocean acidification

Ocean acidification (OA) can be defined as the change in ocean chemistry driven by the oceanic uptake of chemical inputs to the atmosphere, including carbon nitrogen and sulphur compounds (Guinotte and Fabry, 2008). For 650,000 years prior to the Industrial Revolution, atmospheric CO_2 fluctuated between 180ppmv and 300ppmv. Since the Industrial Revolution (during the last 250 years), however, approximately one third of the CO_2 produced has been absorbed by the oceans and during this time CO_2 levels have risen by almost 40% to 380ppmv (Sabine et al., 2004; Fabry et al.,2008). Levels of CO_2 have already seen increases with the average atmospheric CO_2 for 2012 being recorded at 393ppm (http://www.esrl.noaa.gov/gmd/ccgg/trends/). This rapid increase in atmospheric CO_2 has been previously attributed to increased industrialisation, fossil fuel consumption and modern agriculture (Raven et al., 2005; Doney et al., 2009). Atmospheric CO_2 levels are still rising at an estimated rate of 0.5% a year, which is more than 100 times faster than has been recorded in the 650,000 years prior to the Industrial Revolution (Raven et al., 2005; IPCC, 2007; Fabry et al., 2008; Portner, 2008; Turley, 2008; Doney et al., 2009). By 2100 it is estimated that there will be an increase in CO_2 to between 800ppmv and 1000ppmv (Calderia and Wickett 2003; 2005; Orr et al 2005; Raven et al., 2005; IPCC, 2007).

Currently ~38,000Gt (gigatonnes) of carbon is stored in the world's oceans which is estimated to be 95% of the global carbon budget, with the atmosphere storing approximately 700Gt and the terrestrial biosphere storing approximately 200Gt (Raven et al., 2005;Turley, 2008). For its ability to sequester carbon, the marine environment is referred to as a reservoir for CO_2. Approximately 6Gt of CO_2 are produced every year, 2Gt (net of CO_2 per annum) of which is absorbed by the oceans. Without the reservoir effect of the marine environment mitigating the anthropogenic sources, the atmospheric CO_2 would be 55% higher than it is currently (Sabine et al., 2004; Fabry et al., 2008b; Turley, 2008; Reid et al., 2009)

The ocean's surface waters are naturally alkaline with a pH of c. 8.1 to 8.2 which can vary by +/- 0.3 units depending on the geographical location, temperature variations and areas with upwelling's of CO_2 rich water. (Raven et al., 2005; IPCC, 2007). CO_2 is naturally important in defining the pH of surface waters. Any acidity entering the ocean is often buffered by the natural alkalinity of sea water; this is referred to as a carbonate buffer. Beyond natural buffering capacity, CO_2 acts to increase the acidity of sea water. The current decline in the pH of the global surface oceans is an

important consequence of surface water and atmosphere interchange, due to the increasing concentrations of anthropogenically generated atmospheric CO_2 (Calderia and Wickett, 2003; Raven et al., 2005; McDonald et al., 2009; Wohlers et., 2009).

The increasing levels of atmospheric CO_2 are leading to an increase in oceanic CO_2 via thermodynamic equilibrium. When CO_2 enters the ocean system there is a series of reactions which occur, as shown below:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+ \rightleftharpoons CO_3^{2-} + 2H^+ \quad (2)$$

Equation 1.1; the above reaction controls ocean pH and therefore acidity. An increase in CO_2 causes the equation equilibrium to shift to the right favouring bicarbonate and releasing more hydrogen ions (H+) in to the sea water (Brown et al., 1995; Bates and Peters, 2007).

Atmospheric carbon diffuses into the oceans and forms carbonic acid (H_2CO_3); this weak acid then rapidly dissociates to produce hydrogen and dissolved inorganic carbon (DIC). There are three main forms of inorganic carbon in seawater; two of these are electrically charged forms, bicarbonate (hydrogen carbonate) and carbonate, and the third is aqueous CO_2. Once the carbonic acid dissociates, it loses protons and forms bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) (Raven et al., 2005; Widdicombe and Spicer, 2008; Doney et al., 2009). When CO_2 is added to sea water it increases the dissolved CO_2, bicarbonate and hydrogen ion concentrations. When the surface pH of sea water is around 8.2 , approximately 90% of the dissolved inorganic carbon is present as bicarbonate, with only 9% as carbonate and 1% as dissolved CO_2 (Raven et al., 2005; Milero et al., 2006; Doney et al., 2009). Biological processes, including photosynthesis by marine algae and the production of calcium carbonate by organisms, rely on all three forms of DIC. With decreasing pH there is a decrease in saturation states of aragonite and calcite, two crystalline forms of biogenic calcium carbonate , that are also important for calcifying organisms (Havenhand and Schlegel, 2009). These processes concerning DIC and hydrogen play an important role in the ability of the ocean to act as a carbon sink. Organisms are also important to the ocean carbon cycle as once they are consumed or die they release carbon back to the environment. This carbon can remain in the surface water, be released back in to the environment or can fall as particulate calcium carbonate to the deep ocean. This process of the carbonate moving

from the surface waters to the deep ocean is known as a biological pump (see section 1.1), and allows for the surface water to absorb further anthropogenic CO_2 (Raven et al., 2005)

It is hypothesised that increasing levels of oceanic CO_2 and H^+ ions will have detrimental effects on marine organisms (Fabry et al., 2008; Hoffmann et al., 2010, Kroeker et al., 2010; Byrne et al., 2011a). Marine organisms regulate intra-cellular pH by the metabolic inter-conversion of acids and bases, active ion transport and the passive chemical buffering of intra- and extra-cellular fluids (Pörtner et al., 2004; McNeil and Matear, 2006). Imbalances of these factors can lead to dissolution of exoskeletal components such as calcareous shells, reduce protein synthesis and activity and act to suppress metabolism (McNeil and Matear, 2006). This increase in hydrogen ions (H^+) causes a decrease in pH (= -Log10 [H^+]). So far the increased atmospheric CO_2 emissions have caused the surface pH of the oceans to decrease by 0.1 units, representing an approximate 25-30% increase in hydrogen ions (Figure 1.1).

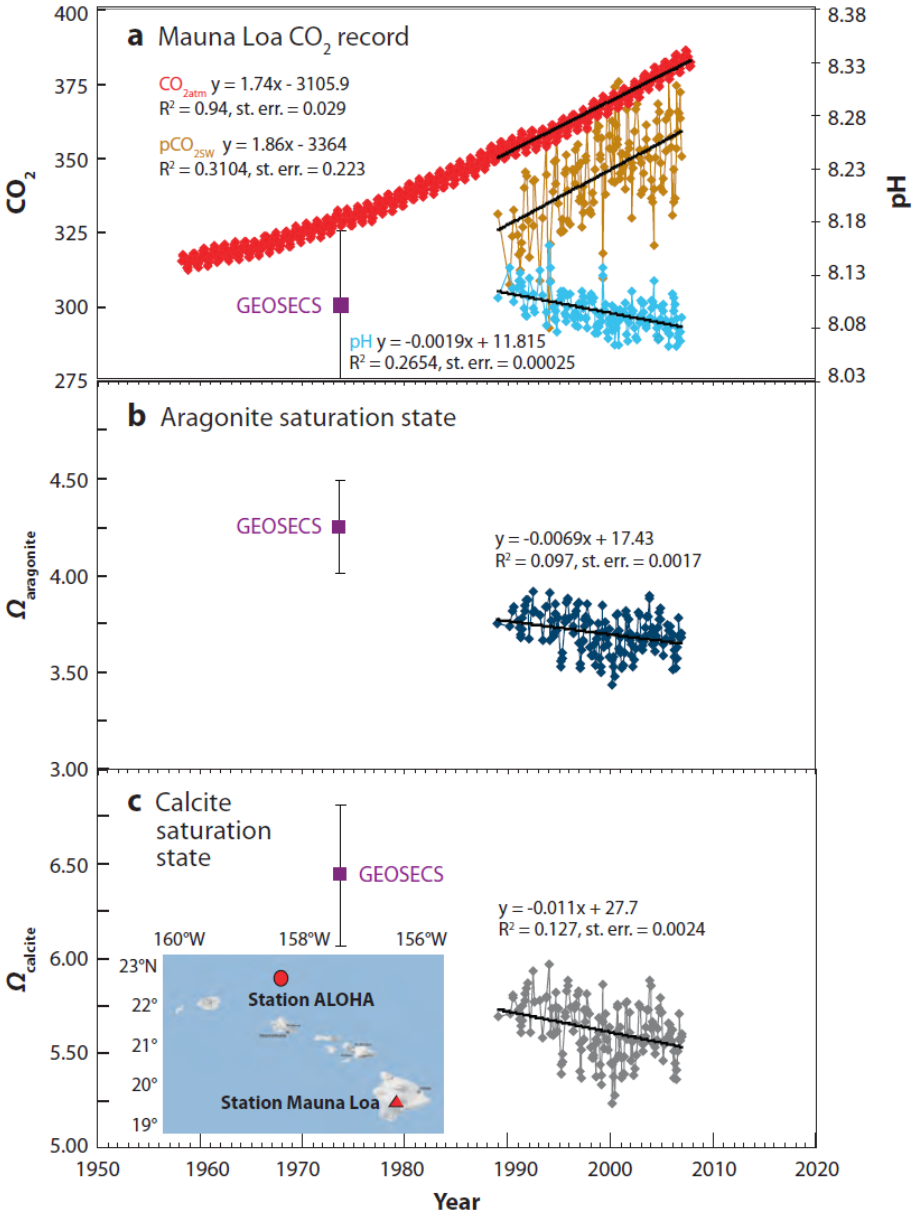

Figure 1.1 Taken from Doney et al, (2009) showing a time series of *a*) atmospheric CO_2 at Mauna Loa (ppmv), surface ocean pH (*cyan*) and pCO_2 (µatm) *b*) aragonite saturation (dark blue) and *c*) calcite saturation (gray) at oceanic station ALOHA (Doney et al., 2009).

It is thought that the current rate of pH reduction is one of the fastest to have been experienced, as previous OA events happened over much longer timescales, i.e. several thousand years (Zachos et al., 2005; Turley et al., 2008). A further decrease of 0.3-0.5 pH units is predicted by the year 2100 (Caldeira and Wickett 2003; 2005). This predicted drop is equivalent to a 150% increase in H^+ ions and each decrease of a full pH unit is equal to a 10 fold increase in acidity. The increasing hydrogen ion concentration acts to decrease the carbonate ion concentration which alters the saturation states of biologically important calcium carbonate building materials. Many marine organisms, including sea urchins, molluscs and crustaceans, require carbonate

ions to form their calcareous skeleton or shells, a process known as calcification. Carbonate ion concentration within the ocean largely determines if there is precipitation or dissolution of calcite and aragonite, which are the two natural polymorphs of calcium carbonate ($CaCO_3$) (Orr et al., 2005). Assuming that the predicted pH unit drop is realised the carbonate ion concentration will be reduced by 50%. The current rate of OA is likely to have dramatic biological effects on the ocean system throughout the 21st century, with an expected increase of 0.5% CO_2 concentration per year (Guniotte and Fabry, 2008). Calcifyng organisms which have a greater dependence on arganoite for shell building, e.g. corals and pterapods, may be at greater risk that those using calcite, because aragonite saturation is predicted to decrease faster with increasing pCO_2 concentration (Feely et al., 2004; Fabry et al., 2008; Doney et al., 2009)

1.1.2 The importance of effects of OA on reproduction and development

Ocean acidification is one of the major threats to the marine ecosystem; however the effect it may have on the marine environment and the organisms therein is largely unknown. For many marine invertebrates, their gametes are directly exposed to the external environment through broadcast spawning. This could potentially impair the capacity to repair cellular damage, which is vital for gametes which are single cells and an embryo's which are rapidly dividing collections of cells (Adiyodi, 1985).

It has been widely hypothesised that the reproductive and developmental stages of marine invertebrate's life cycles are especially sensitive to increasing levels of OA. The processes incorporated in the terms of reproduction and development encompasses a wide variety of reproductive endpoints including gametogenesis, embryogenesis, sperm motility, egg development, fertilisation success, and larval development (Adiyodi, 1985). Fertilisation success has been used in a wide range of previous studies concerning OA effects, and there are a variety of methodologies employed. Broadcast spawning, seen in many echinoderms and polychaetes, involves the release of gametes in to the marine environment which may have a negative impact on the gametes as they can be sensitive to changes in environmental conditions including pH (Havenhand et al., 2008; Byrne 2011). It is possible that organisms relying on this method of fertilisation may be more severely affected by changing oceanic chemistry than those utilising other methods. Organisms reproduce only if there is sufficient energy available to do so (Yoneda and Wright, 2005; Petes et al., 2008), and under stressful conditions, where

there is little surplus energy, the organisms may postpone reproduction until a more favourable time (Yoneda and Wright, 2005), or may produce more, smaller progeny (Schreck et al., 2001). Some organisms e.g. sea anemones have the ability to utilise both sexual and asexual reproduction, and it is possible that in times of environmental stress they may choose to reproduce asexually, which is energetically less expensive (Hand and Uhlinger, 1992). However, if asexual reproduction is continued for prolonged periods there may be consequences for genetic variability, leaving the population as a whole vulnerable (Hand and Uhlinger, 1992). Internal fertilisation may help to buffer the effects of increasing OA on reproductive success as the gametes are not directly exposed to the environment but egg development and juveniles will still be exposed and these life stages are also particularly sensitive to environmental change (Fabry et al., 2008; Kurihara et al., 2008)

To date 78 studies have been carried out on the effects of ocean acidification on the reproduction and development of marine invertebrates, with 67 species being used. Echinoderms are the most studied making up 45% of the published studies, followed by Mollusca which account for 27% of all studies (Figure 1.1). The majority of previous work has utilised growth and development as endpoints (45) (Figurer 1.2), and this is followed by survival and mortality of larvae (30). There have also been a large number of studies on the effects of OA on larval abnormalities (29) and fertilisation success (23). Only two previous studies (Kurihara and Ishimatsu, 2008; Fitzer et al., 2012) have examined multi-generational effects of OA and these both focus on copepods. There are an increasing number of studies on combined stressors, including temperature, salinity, metal contamination and UV irradiation (Table 1.2). These multiple stressor studies are relatively recent, appearing since 2009 and with 47 % having been published since 2012. There are also very few studies on long term (30 days and over) exposure to environmental stressors (17). Of these, only four have looked at the effects of parental exposure/acclimation on subsequent offspring (Dupont et al., 2012; Parker et al., 2012; Uthicke et al.2012; McConville et al., 2013).

Table 1.1: Showing the reproductive endpoints and phyla examined throughout OA research

Phyla	Growth and development	Survival and mortality	Morphometrics/ abnormalities	Fertilisation success	Development/ Growth rate	Hatching rate	Calcification	Gamete production	Sperm motility	Metabolism/ respiration	Egg fecundity/ size	Limb regeneration	Gene expression	Other	Percentage of studies using the phyla (%)
Annelida	✓	✓	✓	✓					✓						1
Arthropoda	✓	✓	✓		✓	✓	✓	✓			✓			✓	1
Cnidaria	✓	✓		✓	✓				✓	✓				✓	8
Echinodermata	✓	✓	✓	✓	✓		✓	✓	✓	✓	✓	✓	✓	✓	17
Mollusca	✓	✓	✓	✓	✓	✓	✓							✓	27
Nemertea			✓	✓											45

Table 1.2 Showing the effects of OA (significant reduction -SR, no significant effect - NSE, Significant increase – SI, significant effect - SE) on various reproductive endpoints, across a range of species.

Species	PH adjust method	pH/ pCO_2 (ppm/ µatm)	Combined stressor	Duration	Endpoints and General effects	Reference
Annelida						
Pomatoceros lamarckii	CO_2	7.20, 7.40 7.45, 7.60 7.75, 7.8 8.0, 8.18	Metal contamination	7 days	• Sperm VCL - SR • Sperm VSL – NSE • Fertilisation – SR • Larval survival – SR • Larval size - NSE • Asymmetry - SI	Lewis et al., 2012
Arthropoda						
Amphibalanus amphitrite	CO_2	7.4 8.2	n/a	120 hours	• Cyprid size - NSE • Survival – NSE • Egg production - NSE	McDonald et al., 2009
Acartia erythraea	CO_2	6.84, 7.40 8.1, 6.86 7.02, 8.20	n/a	24 hours	• Hatching rate – SR at 10,000ppm • Nauplius mortality – SI after 5000ppm	Kurihara et al., 2004b
Acartia steueri	CO_2	6.8, 7.0 7.4, 7.6	n/a	4- 6 days	• Egg production - SR	Kurihara et al., 2004a

		7.8, 8.0				
Acartia tsuensis	CO_2	7.3 8.2	n/a	3 generations	Multigenerational • Survival – NSE • Egg production – NSE • Hatching rate – NSE • Development - NSE	Kurihara and Ishimatsu, 2008
Calanus finmarchicus	CO_2	6.95 8.2	n/a	72 hours	• Egg production – NSE • Hatching – SR	Mayor et al., 2007
calanus glacialis	CO_2	6.9 7.6 8.2	n/a	9 days	• Egg production – SR • Hatching – SR	Wedymann et al., 2012
Centropages typicus	CO_2	380, 480 620, 750 9830	n/a	4 days	• Egg production – NSE • Hatching success - NSE	McConville et al., 2013
Echinogammarus marinus	CO_2	7.5 8.0	Salinity	18 days	• Hatching – NSE • Development time – SR • O_2 uptake - NSE	Egilsdorrit et al., 2009
Elminius modestus	CO_2	7.7 8.0	Temperature	30 days	• Growth rate – SR • Calcium content – NSE • Survival - NSE	Findlay et al., 2010b

Homarus gammarus	CO_2	8.1 8.4	n/a	28 days	• Larval growth – NSE • Larval mass - SR • Mineral content - SR	Arnold et al., 2009
Pandalus borealis	CO_2	7.6 8.1	n/a	35 days	• Survival – NSE • Accumulated mortality - SI (day 35) • Development - SR	Bechmann et al., 2011
Petrolisthes cinctipes	CO_2	7.9 7.6	n/a	10 days	• Hatching – NSE • Survival – NSE • Cardiac performance – NSE • Yolk consumption – NSE • Larval activity – NSE • Morphometrics - NSE	Caballos-Osuna et al., 2013
Semibalanus balanoides	CO_2	346 922	n/a	104 days	• Developmental rate – SR	Findlay et al., 2009
Semibalanus balanoides	CO_2	7.3 7.7 8.1	Temperature	20 days	• Survival – NSE • Developmental rate – SR • Growth – SR • Mineral content - NSE	Findlay et al., 2010a
Semibalanus balanoides	CO_2	7.7 8.0	Temperature	30 days	• Growth rate – NSE • Calcium content – NSE • Survival - NSE	Findlay et al., 2010b
Temora longicornis	CO_2	380, 480	n/a	4 days	• Egg production – NSE • Hatching success - NSE	McConville et al., 2013

		620,750 9830				
Tisbe battagliai	CO_2	7.67, 7.82 7.95,8.06	n/a	Multi-generational	• Growth – SR • Cuticle oxygen – SR	Fitzer et al., 2012
Cnidaria						
Acropora digitifera	CO_2	8.05 7.57 7.33	n/a	7 days	• Survivorship – NSE • Oxygen consumption – NSE • Metamorphosis - SR	Nakamura et al., 2011
Acropora digitifera	CO_2	300, 400 1000ppm	n/a	n/a	• Sperm motility - SR	Nakamura and Morita, 2012
Acropora millepora	CO_2	8.12 7.98	Temperature	7 days	• Fertilisation success- NSE • Survival - NSE • Metamorphosis - NSE	Chua et al., 2013a
Acropora palmata	CO_2	400 560 800µatm	n/a	11 days	• Fertilisation success – SR • Settlement – SR • Growth - SR	Albright et al., 2010
Acropora tenuis	CO_2	8.17	Temperature	7 days	• Fertilisation success- NSE	Chua et al., 2013b

		8.06			• Survival - NSE • Metamorphosis - NSE	
Pocillopora damicornis	CO_2	7.98 7.76	Temperature	5 days	• Protein content – NSE • Respiration – NSE • Survivorship – SR • Symbiodinium densities - NSE	Cumbo et al., 2013
Porites astreoides	HCL	7.95, 7.88 7.80	n/a	7 days	• Aragonite saturation state – NSE • Growth rate - SR	Albright et al., 2008
Porites astreoides	CO_2	380, 560 800µatm	n/a	48 hours	• Larval metabolism – SR • Settlement assay – NSE • Juvenile growth - SR	Albright and Langdon, 2011
Echinodermata						
Arbacia dufresnei	CO_2	7.40, 7.69 7.95	n/a	5 days	• Developmental delay- SI • Abnormalities - NSE	Catarino et al., 2012
Amphiura filiformis	CO_2	6.8, 7.3 7.7, 8.0	n/a	40 days	• Regeneration rate – SI • Calcification – SI • Metabolism – SI • Survival - NSE	Wood et al., 2008
Amphiura filiformis	CO_2	6.8, 7.3 7.7, 8.0	n/a	40 days	• Regeneration rate – SI • Calcification – SI • Metabolism – SI • Survival - NSE	Wood et al., 2009

Centrostephanus rodgersii	CO_2	7.6, 7.8 7.9, 8.2	Temperature	n/a	• Fertilisation – NSE	Byrne et al., 2010b
Centrostephanus rodgersii	CO_2	7.6, 7.8 8.01	n/a	5 day	• Normal Development – SR • Growth – SR	Doo et al., 2011
Centrostephanus rodgersii	CO_2	7.6, 7.8 8.1	Temperature	24 hours	• Cleavage – SR • Gastrulation – NSE	Foo et al., 2012
Crossaster papposus	CO_2	7.7 8.1	n/a	38 days	• Growth rate – SI • Survival – NSE • Skeletogenesis - NSE	Dupont et al., 2010a
Dendraster excentricus	CO_2	380 1000ppm	n/a		• Morphometric – SE • Swimming performance - NSE	Chan et al., 2011
Evechinus Chloroticus	CO_2	7.6, 8.0 7.7, 8.1 7.7, 8.1 7.8, 8.2	n/a	9-13 days	• Survival – NSE - NSE until pH 6.0 • Size – SE • Calcification – SR • Skeletal integrity – SR	Clark et al., 2009
Echinometra mathaei	CO_2	6.8, 7.0 7.4, 7.6 7.8, 8.0	n/a	3 days	• Fertilisation success – SR • Growth rate – SR • Size – SR	Kurihara et al., 2004a

Echinometra mathaei	CO_2 and HCl	6.8, 7.0 7.4, 7.6 7.8, 8.0	n/a	3 days	• Fertilisation - SR • Cleavage - SR • Pluteus larvae - SR	Kurihara and Shirayama, 2004
Echinometra mathaei	CO_2	7.5, 7.7 7.9, 8.1	n/a	7 weeks parent acclimation and 48 hours	• Egg size - NSE • Male gamete production – SR • Hatching - NSE • Normal larvae – SR • Larval size – SR • Arm symmetry - SR	Uticke et al., 2012
Heliocidaris erythrogramma	CO_2	7.7 8.1	n/a	24 hours	• Sperm motility - SR • Sperm velocity - SR • Fertilisation - SR • Normal development - SR	Havenhand et al., 2008
Heliocidaris erythrogramma	CO_2	7.6, 7.8 7.9, 8.2	Temperature	19-20hours	• Fertilisation – NSE • Normal development- NSE	Byrne et al., 2009
Heliocidaris erythrogramma	CO_2	7.6, 7.8 8.2	Temperature	n/a	• Fertilisation – NSE	Byrne et al., 2010a
Heliocidaris erythrogramma	CO_2	7.6, 7.8 7.9, 8.2	Temperature	n/a	• Fertilisation – NSE	Byrne et al., 2010b

Heliocidaris erythrogramma	CO_2	7.6, 7.8 8.2	Temperature	21 hours and 5 days	• Abnormalities – SI	Byrne et al., 2011a
Heilocidaris erythrogramma	CO_2	7.6, 7.8 8.1	n/a	n/a	• Sperm motility - SR • Sperm VCL - NSE • Fertilisation - NSE	Schlegel et al., 2012
Heliocidaris tuberculata	CO_2	7.6, 7.8 7.9, 8.2	Temperature	n/a	• Fertilisation – NSE	Byrne et al., 2010b
Hemicentrotus pulcherrimus	CO_2	6.8, 7.0 7.4, 7.6 7.8, 8.0	n/a	3 days	• Fertilisation success – SR • Growth rate – SR • Size – SR	Kurihara et al., 2004a
Hemicentrotus pulcherrimus	CO_2 and HCl	6.8, 7.0 7.4, 7.6 7.8, 8.0	n/a	3 days	• Fertilisation - SR • Cleavage - SR • Pluteus larvae - SR	Kurihara and Shirayama, 2004
Holothuria	CO_2	6.6, 7.3 7.6, 7.7 7.8, 8.0	n/a	n/a	• Sperm flagella motility - SR	Morita et al., 2010
Lytechinus pictus	CO_2	7.78 7.87	n/a	142 hours	• Size – SR	O'Donnell et al., 2010

		7.93			• Down regulation in gene expression in genes central to energy metabolism and bio-mineralisation – SR Up regulation in some genes concerned with ion regulation and acid-base balance pathways- SI	
Meridiastra calcar	CO_2	288 568	Temperature	3 and 5 days	• Cleavage – NSE • Hatching – SR • Larval mortality – NSE • Percentage normal larvae – NSE	Nguyen et al., 2012
Odontaster validus	CO_2	7.0, 7.6 7.8 8.1	n/a	58 days	• Fertilisation success – SR • Survival – SR • Developmental rate – SR • Abnormal development – SI • Morphometrics - SR	Gonzalez-Bernat et al., 2013
Ophiothrix fragilis	CO_2	7.7, 7.9 8.1	n/a	8 Days	• Survival – SR • Normal development – SR • Size - SR	Dupont et al., 2008
Paracentrotus lividus	CO_2	7.0, 7.25 7.5, 7.7 7.9, 8.1	n/a	3 days	• Fertilisation – NSE • Survival –NSE • Larval Length – SR • Symmetry index – SR • Gene expression - SE	Martin et al., 2011

Paracentrotus lividus	CO_2	6.8, 7.0 7.2, 7.4 7.6, 7.8 8.0	n/a	72 hours	• Fertilisation - SR • Cleavage - SR • Rod size – SR • Abnormal pluteus – SI	Moulin et al., 2011
Patiriella regularis	CO_2	7.6, 7.8 7.9, 8.2	Temperature	n/a	• Fertilisation – NSE	Byrne et al., 2010b
Patiriella regularis	CO_2	7.0, 7.6 7.8, 8.15	Temperature	28 days	• Fertilisation - NSE • Embryo development 4 h - NSE • Embryo development 15 h – SR • Embryo development 24 h – SR • Mortality - SI • Normal larvae – NSE • Larval density – SR • Larval length - SR	Byrne et al., 2013
Psammechinus miliaris	CO_2	7.67, 7.82 7.95, 8.02	Temperature	n/a	• Sperm motility - SI • Sperm VCL - SI	Caldwell et al., 2011
Pseudechinus huttoni,	CO_2	7.6, 8.0 7.7, 8.1 7.7, 8.1	n/a	9-13 days	• Survival – NSE until pH 6.5 • Size – NSE • Calcification – SR • Skeletal integrity - SR	Clark et al., 2009

		7.8, 8.2				
Sterechinus neumayeri,	CO_2	7.6, 8.0 7.7, 8.1 7.7, 8.1 7.8, 8.2	n/a	7 days	• Survival – NSE until pH 6.0 • Size – SR • Calcification – NSE • Skeletal integrity - NSE	Clark et al., 2009
Sterechinus neumayeri	CO_2	7.0, 7.3 7.7, 8.01	n/a	n/a	• Fertilisation – SR at low sperm density • Cleavage – NSE • Abnormal embryos - NSE	Ericson et al, 2010
Sterechinus neumayeri	CO_2	7.5, 7.7 8.0	Temperature	70 hours	• Fertilisation success - SR • Embryonic development - SR	Ericson et al., 2012
Sterechinus neumayeri	CO_2	8.0, 7.8 7.6	Temperature	21 days	• Larval morphometrics – SE • Normal development - NSE	Byrne et al., 2013 b
Sterechinus neumayeri	CO_2	410, 510 730	n/a	30 days	• Developmental delay - NSE • Larval length – SR • Pleuteus length - SR	Yu et al., 2013
Strongylocentrotus droebachiensis	CO_2	7.7 8.1	n/a	4 and 16 months	4 Months • Egg fecundity - SR • Egg size - NSE • Settlement, - NSE	Dupont et al., 2012

					• Larval daily mortality - SI 16 months • Egg fecundity - NSE • Egg size - NSE • Settlement, - NSE • Larval daily mortality - NSE	
Strongylocentrotus franciscanus	CO_2	380, 540 970ppm	n/a	4 days	• Down regulation of *hsp70* after heat shock (25°C)	O'Donnell et al., 2009
Strongylocentrotus franciscanus	CO_2	400, 800 1800ppm	n/a	n/a	• Fertilisation – SR	Reuter et al., 2011
Strongylocentrotus franciscanus	CO_2	7.9, 8.3	n/a	7 days and 60 hours	• Larval size – SR	Sunday et al., 2011
Strongylocentrotus purpuratus	CO_2	380, 540 1020 ppm	n/a	70 hours	• Down regulation of gene expression - SE	Todgham, and Hofmann, 2009
Strongylocentrotus purpuratus	CO_2	7.60, 8.02	site	5 days	• Size – SR • Larval respiration - NSE	Kelly et al., 2013
Tripneustes gratilla.	CO_2	7.6, 7.8 7.9, 8.2	Temperature	n/a	• Fertilisation – NSE	Byrne et al., 2010b

Tripneustes gratilla.	CO_2	7.6, 8.0 7.7, 8.1 7.7, 8.1 7.8, 8.2	n/a	4 days	• Survival – NSE until pH 7.0 • Size – SR • Calcification – SR • Skeletal integrity – NSE	Clark et al., 2009
Tripneustes gratilla	CO_2	7.6, 7.8 8.15	Temperature	5 days	• Normal larvae – SR • Arm asymmetry – NSE • Larval growth - SR	Sheppard Brennand et al.,2010
Mollusca						
Argopecten irradians	CO_2	7.50, 7.85, 8.07	n/a	21 days	• Survival – SR • Survivorship - SR • Metamorphosis – SR • Length - SR	Talmage and Gobler, 2009
Bembicium nanum	CO_2	7.6, 8.2	Temperature UV radiation	72 hours	• Mortality – SI • Development rate - SI	Davis et al., 2013
Crassostrea angulate	CO2	7.4, 7.6 7.9, 8.1	Temperature and salinity	5 days	Ambient temp and salinity • Survivorship - NSE • Shell growth - NSE	Thiyagarajan and Ko, 2012
Crassostrea ariakensis	CO2	7.76, 7.91 8.06, 8.16	n/a	30 and 32 days	• Shell area – NSE • Calcification – NSE	Miller et al., 2009

Crassostrea gigas	CO2	7.4 8.2	n/a	2, 3, 8, 24 and 48 hours	Larval development • Shell length – SR after 24 and 48 hours • Shell mineralisation - SR after 24 and 48 hours	Kurihara et al., 2007
Crassostrea gigas	CO2	7.8, 8.15	n/a	n/a	• Fertilisation - NSE	Havenhand and Schlegel, 2009
Crassostrea gigas	CO2	375, 600 750, 1000	Temperature	24 hours	• Fertilisation – SR • Development – SR • Abnormality - SI • Larval size – SR	Parker et al., 2010
Crassostrea gigas	CO2	Ambient (0.0) (-0.4), (-0.7)	n/a	144 hours	• Fertilisation – SR • Hatching rate – SR • Mortality – SI • Abnormal D shaped larvae – SI • Larval Growth – SR	Barros et al., 2013
Crassostrea virginica,	CO2	7.76, 7.91 8.06, 8.16	n/a	30 and 32 days	• Shell area – SR • Calcification – SR	Miller et al., 2009
Crassostrea virginica	CO2	7.48, 7.83, 8.08	n/a	21 days	• Survival – SR • Survivorship – NSE • Metamorphosis – SR • Length - SR	Talmage and Gobler, 2009

Crassostrea virginica	CO2	400 700-800 µatm	Salinity	11 weeks	• Mortality – SI • Shell mass - NSE • Body mass - NSE • Shell hardness – NSE • CA activity – NSE	Dickinson et al., 2012
Dolabrifera brazieri	CO2	7.6, 8.2	Temperature UV radiation	72 hours	• Mortality – SI • Development rate – NSE	Davis et al., 2013
Haliotis coccoradiata	CO2	7.6, 7.8 7.9, 8.2	Temperature	n/a	• Fertilisation – NSE	Byrne et al., 2010b
Haliotis coccoradiata	CO2	7.6, 7.8 8.2	Temperature	21 hours and 5 days	• Percentage calcification – SR • Abnormalities – SI • Absence of shell - SI	Byrne et al., 2011a
Haliotis kamtschatkana	CO2	1800, 800 400ppm	n/a	8 days	• Survival – SR • Larval shell deformities - SI	Crim et al., 2011
Limacina helicina	CO2	230, 350 750, 1100	Temperature	29 days	• Mortality – SI • Shell degradation – SD • Shell growth – SR	Lischka et al., 2011
Littorina obtusata	CO2	7.6	n/a	30 days	• Embryo viability – SR	Ellis et al., 2009

		8.1			• Embryo activity - SR • Hatching success – SR • Development time - SI • Larval hear rate - NSE • Shell length – SR • Shell height – SI	
Mercenaria mercenaria,	CO2	7.49, 7.84, 8.02	n/a	21 days	• Survival – SR • Survivorship – SR • Metamorphosis – SR • Length - SR	Talmage and Gobler, 2009
Mytilus californianus	CO2	380, 540 970ppm	n/a	8 days	• Shell strength - SR • Shell area - SR • Shell thickness - SR	Gaylord et al., 2011
Mytilus edulis	CO2	7.6 8.1	n/a	64 days	• Fertilisation – NSE • Percentage deformed larvae - NSE • Shell size – SR • Feeding - NSE	Bechmann et al., 2011
Mytilus galloprovincialis	CO2	7.4 8.1	n/a	2, 4, 24, 54, 120, and 144 hours	• Percentage distribution – NSE • Percentage d veliger larvae after 54 hours – SD • Developmental delay at 120 and 144 hours • Abnormal development – SI • Shell length – SR	Kurihara et al., 2008

					• Shell height - SR	
Mytilus trossulus	CO2	7.9, 8.3	n/a	7 days and 60 hours	• Larval size - SR	Sunday et al., 2011
Ostrea lurida	CO2	7.8, 7.9 8.0	n/a	52 days	• Larval shell growth – SR • Shell area – SR • Growth rate – SR	Hettinger et al., 2012
Saccostrea glomerata	HCl	375, 600 750, 1000 ppm	Temperature	48 hours	• Fertilisation – SR • Percentage D veliger - SR • Size – SR • Abnormalities - SI	Parker et al., 2009
Saccostrea glomerata	CO2	375, 600 750, 1000	Temperature	24 hours	• Fertilisation – SR • Development – SR • Abnormality - SI • Larval size – SR	Parker et al., 2010
Saccostrea glomerata	CO2	7.89 8.19	Oyster source	Adult exposure and 19 days	• Survival – SR • Rate of development – SR • Shell length - SR	Parker et al., 2012
Sepia officinalis	CO2	7.6 7.9 8.1	Temperature	17 and 27 days	• Egg weight – SI • Weight at hatching – NSE • 110mAG accumulation in eggs – SI • 109Cd accumulation in eggs – SD • 65 Zn accumulation in eggs - SE	Lacoue-Labarthe et al., 2009

Nemertea						
Parborlasia corrugatus	CO_2	7.0, 7.3 7.7, 8.01	n/a	8 days	• Fertilisation – NSE • Cleavage – SR • Abnormal larvae – SI	Ericson et al, 2010

1.2 Reproductive end points

The effect of ocean acidification on differing reproductive endpoints has been examined in much of the previous literature (Table 1.2). To date, results have been varied, with species and life history stages differences. For example, the copepod *Calanus finmarchicus* showed a reduction in egg development under acidified conditions of pH 6.95, with only 4% of eggs yielding nauplii after 72 hours, however there was no negative effect seen at later developmental stages (Mayor et al., 2007). Similarly, *Littorina obtusata* after being exposed to acidified conditions (pH7.67) for 6 days experienced decreased egg viability to 94% and after 7 days it reduced further to 88% (Ellis et al., 2009). The latter study utilised a more ecologically relevant pH which may explain the reduction in viability. It was also found that *Littorina obtusata* produced fewer hatchlings under acidified conditions (n=449) when compared to control treatments (n=575) (Ellis et al., 2009). There has been a variety of reproductive endpoints used in ocean acidification (Table 1.2) and eco-toxicological studies, and there has been much ambiguity as to what constitutes a particular endpoint. To date, several endpoints have been considered and these include sperm motility (1.4.1), fertilisation success (1.4.2), and larval development (1.4.3).

1.2.1 Sperm motility

If the sperm lose the ability to locate eggs in the marine environment, for broadcast spawning species possessing motile sperm, the reproductive ability will be severely limited (Morita et al., 2010). Motility is essential to establish successful fertilisation. Sperm flagellum motility is regulated by an elevation of intracellular sperm pH. If the external pH is low it is likely to cause acidification of the body fluids as well as changes in ion balances within marine organisms (Christen et al 1983; Lee et al., 1983; Nakajima et al., 2005; Raven et al., 2005; Morita et al., 2010). There have been few studies to determine the effects that increasing OA has on sperm motility, and it has been studied under several different classifications in the previous literature. These include percentage motility, sperm swimming speed and flagella motility (Havenhand et al., 2008; Havenhand and Schlegel 2009; Morita et al., 2009; Caldwell et al., 2011; Lewis et al., 2012).

Much of the published literature to date has reported a reduction in sperm motility with increasing OA conditions over a range of species including; sea urchins, sea cucumbers, and corals (Havenhand et al., 2008; Havenhand and Schlegel 2009; Morita et al., 2009). Havenhand et al., (2008) observed a significant reduction in sperm motility at pH 7.7 which had a direct impact on fertilisation success in the sea urchin *Heliocidaris erythrogramma*. Havenhand et al., (2008) noted that both sperm swimming speed and sperm motility were significantly affected. Fertilisation kinetics models utilising these data predicted a 24.9% decrease in fertilisation success under future increased OA conditions and this corresponded closely to the 20.4-25.9% observed decrease in fertilisation success during the study. This study highlighted the significant effect that sperm motility can have on fertilisation success. Equally Morita et al., (2009) found that there was a significant decrease in flagellar motility in two broadcast spawning marine organisms, the coral *Acropora digitifera* and the sea cucumber *Holothurian spp*. *Acropora digitifera* highlighted that a small decrease in pH (0.2 to 0.4 pH units) showed a significant reduction in flagellar motility to 49% at pH 7.8 and after this it decreased to 20%, whilst the *Holothurian spp* showed that as pH drops below 7.8 there was a maximum of 30% motility. In contrast, studies utilising the oyster *Crassostrea gigas* found no significant effect of OA on sperm motility and sperm swimming speed (pH 8.15= 92.1±4.8μms^{-1}, pH 7.8= 94.3± 5.5μms^{-1}) (Havenhand and Schlegel 2009).

In more recent studies (Caldwell et al., 2011), the sea urchin *Psammechinus miliaris* showed that under near future levels of predicted OA (pH 7.95) there was a significant increase in both percentage motility and sperm swimming speed and this increase was consistent across temperature treatments (14, 17 and 20°C). Sperm motility and swimming speed remained higher than the control in all decreased pH treatments (pH 7.95, 7.82 and 7.67) at both 14 and 17 °C; however at 20 °C pH 7.82 showed a reduction in both sperm motility and sperm VCL. Conversely Lewis et al., (2012) found that when the polychaete *Pomatoceros lamarckii* was subjected to pH 8.1-7.4 and a more extreme pH condition (pH 7.2) there was a significant reduction in sperm swimming speed but with a peak at pH 7.75. The percentage of sperm retaining some extent of motility also showed a reduction with reducing pH but this was not a linear relationship as there were increases at pH 7.7 and 7.45. In contrast, Frommel el at., (2010) found that OA did not significantly affect average swimming speed or percentage motility in the Baltic code *Gadus morhua*; it was however noted that both

differed significantly between males. A recent study by Schlegel et al. (2012) also noted a significant decrease in sperm motility (%) under elevated CO_2 (pH 7.8, and 7.6) conditions, but found that there was no significant effect of CO_2 on average sperm swimming speed ($\mu m/s^{-1}$) ($p=0.710$). This particular study looked at variations between males and found that the response of different males differed significantly for both sperm motility (%) and swimming speed (µm/s).

It has previously been suggested that activation pH plays an integral role in sperm motility with the connection between intracellular sperm pH and sperm activation and longevity being well studied (Christen et al., 1983b; Caldwell et al., 2011). It has previously been suggested that activation pH varies between species with early studies by Christen et al., (1983b) reporting an activation pH of 7.6 in the sea urchin *Strongylocentrotus purpuratus*. Similarly, Pacey et al. (1994) reported that there was an activation pH of 7.6 in the polychaete *Arenicola marina*. However Nakajima et al, (2005) reported the activation pH of the starfish *Asterina pectinifera* was higher than the previously mentioned species with the activation pH being reported as pH 7.8. It is possible that these variations in activation pH could explain some of the reported variation in sperm motility and swimming speed seen between studies.

Activation pH alone does not explain all of the differences seen between studies. There has been a wide variety of methodologies used for sperm motility analysis, some of these variations may help to explain the other differences seen. One of the principal variations is sperm densities used, with the majority of studies choosing to use high sperm densities (10^6-10^7sperm ml^{-1}) (Morita et al., 2009; Caldwell et al., 2011; Lewis et al., 2012) however some studies have chosen to use lower sperm densities (10^3-10^4 sperm μl^{-1}) (Havenhand and Schlegel, 2009). It is possible that variations seen in sperm motility could be attributed to these variations in sperm densities, as it has been hypothesised that high sperm concentrations may have an effect on sperm motility due to an increase in the frequency of collisions (Wilson-Leedy and Ingermann, 2006).

It is also possible that variations seen could be attributed to the duration of the motility analysis. There is little unification across sperm motility methodologies with tracking times ranging from two seconds (Schlegel et al., 2012) to 30 minutes (Caldwell et al., 2011). Two of the reported studies above utilised the CASA (computer assisted sperm analysis) sperm tracking system which allowed for tracking for 30 minutes. The results from the studies using the CASA sperm tracking system differed from other

sperm motility studies which utilised other experimental techniques including different tracking times. For example Caldwell et al. (2011) saw a significant increase in sperm motility at a pH of 7.95 in the sea urchin *Psammechinus miliaris* which differs from previous studies by Havenhand et al, (2008) and Havenhand and Schlegel, (2009) which reported a reduction in sperm motility at reduced pH in the sea urchin *Heliocidaris erythrogramma* and the oyster *Crassostrea gigas*. The latter studies tracked sperm only for two seconds and tracking was started immediately. It is therefore possible that sperm were not completely activated. In contrast Caldwell et al, (2011) started tracking 1 minute after activation and continued at 10, 20 and 30 minutes, found an increase in sperm swimming speed.

It is apparent from previous literature that it is important to have a unified methodology for sperm motility analysis. The majority of variations seen throughout the results can be attributed to variations in methodology and differences in activation pHs of the sperm. The aforementioned studies have concentrated only on short term exposure of gametes and it is unclear what the long term impacts of decreased pH will be. There is a necessity for further long term and multigenerational exposure to try and determine the long term impacts on sperm viability and consequently fertilisation success and larval development.

1.2.2 Fertilisation success

Fertilisation can be defined as the production of a diploid zygote through the amalgamation of two sexually different haploid gametes. Fertilisation can be split in to two main categories: 1) internal where the sperm is transferred directly to the female from the male and fertilisation occurs within the female; and 2) external fertilisation, where the gametes are released directly in to the environment and fertilisation occurs in the external environment (Caldwell, 2009). There are examples of marine invertebrates that utilise either of these strategies through internal copulation and broadcast spawning. These variations in reproductive strategies cause difficulties when trying to distinguish how best to assess fertilisation success. It has been suggested that fertilisation experiments should be standardised to allow for easy comparison across studies. Marshall, (2006) described three recommendations to standardise fertilisation experiments: 1) sperm egg contact time being 1 hour; 2) sperm from multiple males are used in each assay to reduce variation between experimental runs; and, 3) the use of

serial sperm dilutions to create a fertilisation curve, and maximum fertilisation concentrations used for experiments. These guidelines, however, do not take into account many external factors such as temperature. It also suggests that the highest sperm concentrations should be used; however, it is possible that this could affect results as the experiments would represent highly unnatural sperm densities. It is also apparent that these guidelines do not standardise how fertilisation success is defined. The majority of the studies concerning fertilisation success in broadcast spawning marine invertebrates have measured fertilisation in two ways: 1) elevation of the fertilisation envelope/membrane (Kurihara et al., 2004; Kurihara and Shirayama, 2004; Moulin et al., 2011 Ericson et al., 2012) ; or, 2) beginnings of polar body formation and cleavage and therefore early embryonic development (Kurihara et al., 2007; Havenhand et al., 2008; Havenhand and Schlegel, 2009; Parker et al., 2009; Parker et al., 2010; Moulin et al., 2011; Lewis et al., 2012; Schlegel et al., 2012). Both methods are valid but they represent different fertilisation events and have different sensitivities to eco-toxicants. It is therefore important to differentiate between the stages (Byrne, 2012). A number of studies have used both stages to represent the same endpoint of fertilisation success (Byrne et al., 2009; Byrne et al., 2010a; Byrne et al., 2010b; Ericson et al., 2010; Martin et al., 2011; Reuter et al., 2011; Gonzalez-Bernat et al., 2012). By utilising both elevation of the fertilisation membrane and cleavage, the results achieved will not accurately represent either stage and make comparison between studies difficult. It would be beneficial for future research to use both elevation of the fertilisation envelope membrane and first polar body cleavage but not to represent a single endpoint as fertilisation success can be seen as elevation of the fertilisation envelope whereas early polar body cleavage can be used to look at the effects of OA on early embryonic development.

It is also possible that differing percentages of fertilisation success could be as a result of differential compatibility of male and female gametes or naturally variable gamete quality (Kurihara and Isimatsu, 2008). It is difficult to assess the impact of individual gamete quality on fertilisation success, however, as the majority of fertilisation studies rely on pooled gametes. The use of this methodology has been justified previously as being representative of a population of spawners. Recent studies (Sunday et al., 2011; Foo et al., 2012; Schlegel et al., 2012) have found, however, that parental mating significantly affects the outcome of fertilisation success. By using single dam-sire interactions it is possible to determine individual response to ocean

acidification. When pooled gametes are utilised it can cause a skew in results, as certain samples are stronger than others and when mixed will out compete weaker sperm samples; this in turn may create unrealistic levels of fertilisation success. By using single dam-sire interactions it is possible to see the effects that fertilisation success will have on an individual. This is important as it has been clearly shown that individuals react differently to increased acidification and thermal stress, and group spawning studies negate individual effects as individuals all contribute differently to the next generation (Foo et al., 2011; Schlegel et al., 2012).

There are many factors affecting fertilisation success in broadcast spawning marine invertebrates including gamete dilution, sperm-egg contact time, adult aggregation, and sperm longevity. All of these factors have roles in determining fertilisation success (Pennington, 1985; Levitan et al., 1991).

One of the major discrepancies in experimental protocols for assessing fertilisation success is the use of different sperm densities. Sperm densities have previously been well documented as playing an important role in fertilisation success and kinetics (Pennington, 1985; Levitan et al., 1991). There is little consistency between studies for sperm concentration or sperm: egg ratios. Sperm densities used in OA studies have ranged from 10 sperm ml^{-1} to 10^9 sperm ml^{-1}(Byrne et al., 2010). Previously, it has been shown that sperm density has a significant impact on fertilisation success regardless of treatment pH or temperature. Studies by Byrne et al., (2010a) utilised a variety of sperm densities, and concluded that fertilisation success is not significantly impacted by decreased pH but it is negatively impacted by reduced sperm concentration. Similarly, Havenhand and Schlegel et al., (2009) showed no significant difference in fertilisation success of the oyster *Crassostrea gigas* at varying CO_2 treatments (pH 8.15= 63.4%; pH7.8= 64.1%). This study also reported that there was no significant difference between the sperm concentration at which maximum fertilisation (S_{max}) occurred (10864 and 10941 sperm μl^{-1} in pH 8.15 and pH 7.8, respectively). In contrast, studies concerning *H.erythrogramma* found that sperm density had a significant effect on fertilisation success, with lower sperm densities (01.1) showing a significant reduction in fertilisation success (Byrne et al., 2010a)

Adult aggregation is another important factor determining fertilisation success because gametes dilute rapidly when released to the environment. Previous studies have shown that fertilisation success decreases significantly when sperm travels more

than 10 cm from the source, meaning that if there a sparse community of spawning adults the chances of fertilisation are reduced (Pennington, 1985; Levitan et al., 1991). In broadcast spawning events larger aggregations allow for greater numbers of individuals to contribute to the next generation, which allows for greater genetic diversity. In OA studies there has been much variation in the number of adults that gametes are collected from. Recent studies have collected gametes from large numbers of individuals, for example 18 males and 18 females (Schlegel et al., 2012); however, many studies have used single males and females in fertilisation success experiments (Kurihara et al., 2007; Havenhand and Schlegel, 2009; Ericson et al., 2010; Reuter et al., 2011; Ericson et al., 2012). This is not ideal as there is high natural variability in sperm performance, and not all gametes are compatible and gamete compatibility dictates fertilisation success (Palumbi, 1999; Boundry et al., 2002; Levitan and Ferrell, 2006; Byrne, 2012), and there is high variability between individuals (Foo et al., 2012; Schlegel et al., 2012)

Sperm longevity also plays a vital role in fertilisation success in broadcast spawning marine invertebrates, as sperm may have to stay active for long periods of time for successful fertilisation to occur. Williams and Bentley (2002) found that sperm longevity had a marked impact on its viability in the polychaetes *Arenicola marina* and *Nereis virens* and the asteroid *Asterias rubens*. Similar results have been found in the sea urchin *Strongylocentrotus franciscanus* (Levitan et al., 1991). Studies concerning the effects of OA on fertilisation success use varied time scales for egg: sperm contact time. Many allow fertilisation to occur for a relatively short time period (12 – 15 minutes) and the eggs are rinsed to remove excess sperm (Kurihara et al., 2007; Kurihara et al., 2008; Byrne et al., 2009; Byrne et al., 2010a; Byrne et al., 2010b; Lewis et al., 2012). Some have used far shorter time periods (30 seconds; Reuter et al.,2011) while others did not rinse excess sperm from eggs (Kurihara et al., 2004; Parker et al., 2010). Previously egg-sperm contact time has been shown to significantly affect fertilisation success with greater fertilisation occurring with increasing time (Levitan et al., 1991). By removing excess sperm from experiments after a set time period it is possible that the true fertilisation success is not being seen, as fertilisation may have continued past the point of sperm removal which means the percentage fertilisation success may not provide an accurate result.

Acidification methodology has also differed between studies. Two major variations of acidification have been used within OA experiments 1) the addition of

gaseous CO_2 and 2) the addition of HCl. The addition of CO_2 has been used more frequently (For a review see Byrne, 2012, and see Table 1.2) than HCl (Kikkawa et al., 2004; Kurihara and Shirayama, 2004; Parker et al., 2009). Although the vast majority of studies achieve acidified conditions through CO_2 injection (see table 1.2), an early study carried out by Kurihara and Shirayama (2004) on two sea urchins (*Hemicentrotus pulcherrimus* and *Echinometra mathaei*) acidified sea water using two methodologies; 1) bubbling with CO_2, or; 2) the addition of 1N HCI. A significant decrease in fertilisation success was seen for both species under both acidification methods. The reduction in fertilisation success showed a more linear relationship in *H. pulcherrimus* with a dramatic decrease occurring at pH 6.8 (<10%) under CO_2 acidification. There was still a significant result, but not as dramatic a decrease as using HCI (pH 6.8, 77% fertilisation success). There was a significant difference between acidification methodologies seen in *E. mathaei*. There have also been comparative studies between the two methodologies (Kikkawa et al., 2004; Kurihara and Shirayama, 2004) and these studies found that there was a less severe effect on reproduction and development with the addition of HCl. In contrast, Parker et al. (2009) choose to use HCl addition, after an unpublished study found no significant difference in results between the two methods; however, later studies by the same author used only CO_2 bubbling to manipulate sea water pH (Parker et al., 2010; Parker et al., 2012).

A considerable body of literature has been published on the effects of ocean acidification on fertilisation success (Table 1.2), and as seen for sperm motility literature (section 1.4.1) the response of fertilisation success to OA appears to be largely species specific with contrasting results found throughout. For example, with literature related to echinoderms 61% of studies show a negative effect of OA, 5% show a positive response, and 34% show no measurable response (Dupont et al., 2010b; Dupont et al., 2012). Early studies by Kurihara et al, (2004), showed a significant reduction in fertilisation success of the sea urchins *H. pulcherrimus* and *E. mathaei* at the lowest pHs (10,000ppm; pH 6.8) measured (*H. pulcherrimus* 44% ; *E. mathaei* 7.6%). However a more recent study by Uticke et al. (2012) found that with increased levels of CO_2 there was no significant effect on egg size or hatching but there was a significant reduction in male gamete production. Between these particular studies discrepancies in results can be attributed to differing levels of acidification with Uticke et al. (2012), utilising more ecologically relevant pH values than Kurihara et al, (2004) (see Table 1.2). Similarly, Havenhand et al, (2008) used *Heliocidaris erythrogramma*, and found that with

increasing CO_2, there was a 30.4% reduction in fertilisation success at pH7.7; the study also estimated fertilisation success reduction of 25% through use of fertilisation kinetics models, which was in keeping with the observed data. In contrast to the previous study, Byrne et al., (2009) showed no significant effects of decreased pH on fertilisation success in the same sea urchin species. In contrast to Havenhand et al, (2008) Byrne et al, (2009) utilised 4 experimental pH treatments (pH 7.6, 7.8, 7.9 and 8.2) whereas the initial study utilised only 2 (pH 7.7, and 8.1), which overlapped with the study conducted by Byrne et al, (2009). There was a discrepancy in sperm densities used by Byrne et al, (2009) utilising 10^3 sperm ml^{-1} and Havenhand et al, (2008) using 10^5 sperm ml^{-1}. However, the main difference between the two studies is the use of single dam –sire crosses (Havenhand et al., 2008) and pooled gametes (Byrne et al., 2009), and as mentioned above, there is a significant difference between the two methodologies. There were also differences in the stage used as fertilisations success with Havenhand et al., (2008) using 2-cell cleavage and Byrne et al., (2009) using both elevation of the fertilisation envelope and cleavage. There have also been discrepancies in results for the sea urchin *Paracentrotus lividus*. For example Moulin et al. (2011) showed a significant reduction in fertilisation success under OA scenarios. Conversely, Martin et al. (2011) found that there was no effect of decreasing pH on fertilisation success. There are several differences between the studies in terms of methodologies with Moulin et al, (2011) treating elevation of the fertilisation envelope and cleavage as separate endpoints as opposed to Martin et al., (2011) which used both to score fertilisation success. This difference is similar to that seen between Havenhand et al. (2008) and Byrne et al, (2009) and highlights the need for a more standardised fertilisation success protocol. It also supports the idea that fertilisation success and cleavage should not be used to represent the same endpoint as they show different things, with fertilisation success showing the elevation of the vitelline envelope, and cleavage success showing early embryonic development, and are differently affected by OA. By combining the two endpoints, the studies have found that there is no significant effect of CO_2 on fertilisations success (Byrne et al., 2009; Martin et al., 2011). However when viewed separately there was a significant effect (Havenhand et al., 2008; Moulin et al., 2011).

1.2.3 Larval development

Larval growth and development is by far the most studied area of reproductive and developmental studies within the field of ocean acidification. This particular

developmental stage encompasses a wide range of reproductive endpoints including growth, larval morphometrics and survival. Through the course of larval development research a large variety of species from a wide range of habitats have been used and contrasting results found (see table 1.2). During marine invertebrate development, the larval stages of an organism often have different modes of life (e.g. planktonic versus benthic) than the adult stages (Pörtner, 2008), and it is suggested that different life stages are able to cope differently with a changing environment. It has been largely predicted that early developmental stages of an organism's life cycle are the most sensitive to environmental changes such as CO_2 (Dupont and Thorndyke, 2008). Larval stages are vital to marine organisms; larval development is reliant on normal development from embryos. If there is an increased incidence of larval abnormalities, this could be significantly impacted and lead to a reduction in recruitment success. There is a varied response in larval response to increased OA conditions leading to the hypothesis that the effects of OA are going to be species specific.

A seen above for sperm motility (section 1.2.1) and fertilisation success (section 1.2.2), there are contradictory results pertaining to the positive or negative effects of increased OA conditions. For example a significant reduction in larval survival rate was seen in the hard clams *Mercenaria mercenaria* , the bay scallop *Argopecten irradians,* and the eastern oyster *Crassostrea virginica* (Talmage and Gobler, 2009). Conversely the barnacle *Semibalanus balanoides* exhibited no significant impact of OA on larval survival (Findlay et al., 2010). Research concerning the effects of OA on growth varied, with the majority showing a reduction in growth (Kurihara et al., 2004; Clark et al., 2009; Findlay et al., 2009; Parker et al., 2009; Talmage and Gobler, 2009) and others reporting an increase in growth after increased OA exposure (Kurihara et al., 2007; Kurihara and Ishimatsu, 2008; Miller et al., 2009; Dupont et al., 2010a). As mentioned previously it has been suggested that calcifying organisms will be especially affected by increased CO_2 and this is supported by the majority of studies (Kurihara et al., 2007; Clark et al., 2009; Miller et al., 2009).

1.2.4 Larval mortality

Survival or mortality has been reported from several studies in a variety of species. Changes to individual survival could significantly affect species survival and recruitment. The vast majority of OA studies concerned with larval survival or mortality

have found that with increasing *p* CO_2 there is a significant reduction in larval survival. Dupont et al., (2008) saw that survival rates were significantly decreased in the brittle star *Ophiothrix fragilis* when exposed to increased *p*CO_2. It was reported that there was a <0.1% survival in treatment pH conditions which at pH 7.95 equates to a mortality rate of 35+/- 10.5% d^{-1}, and at pH 7.7 mortality rate was increased further to 50.4 +/- 10.5% d^{-1} . Significant increases in mortality at decreased pH treatments were first observed after 7 days at pH 7.95 and after 5 days for pH 7.7 Similarly studies by Crim et al., (2011) saw a 40% reduction in the survival of the Abalone *Haliotis kamtschatkana* when exposed to increased *p* CO_2 (800ppm and 1800ppm). Davis et al., (2013) also showed a significant reduction in survival in the Opisthobranch's *Dolabrifera brazieri, Bembibium nanum* at decreased pH ($p<0.0001$) and this was further exacerbated at lower temperatures. In contrast Clark et al., (2009) looked at the effects of increased *p*CO_2 on the survival of 4 sea urchin species, a tropical species, *Tripneustes gratilla*, two temperate species *Pseudechinus huttoni*, and *Evechinus chloroticus* and a polar species *Sterechinus neumayeri*. In all species, pH had a significant effect on larval survival which presented as a decrease. However in all treatments significant reductions were only seen at pH 7.0 and below. The effects of pH were seen first in *T. gratilla* which showed a significant reduction in survival at pH 7.0 and below ($p<0.00001$). *S. neumayeri* and *E. Chloroticus* showed the greatest resilience to decreasing pH with a significant reduction only being seen at pH 6.0 (*S. neumayeri* , $p<0.00001$; *E. Chloroticus*, $p<0.00001$) after 22 days and 13 days exposure respectively. Similarly Nguyen et al., (2012) saw no significant difference in survival of the sea star *Meridiastra calcar* when exposed to increased *p*CO_2; however there was a significant effect of temperature.

Although the majority of variations in larval mortality can be attributed to species specific responses it is possible that some variations could be attributed to differing experimental methodology with some studies immersing the larvae in acidified conditions from fertilisation (Kurihara et al., 2007; Byrne et al., 2011a) and others only after development has already begun (Dupont et al., 2008). Previously, increases in mortality have also been suggested as a knock on effect caused by developmental delay and increased time to metamorphosis (Byrne, 2012) It is important for future research to become more long term and encompass more than one endpoint to see how reproductive endpoints link to one another

1.2.5 Larval Growth

Similar to the results seen in larval mortality, the results for effects of OA on growth and size vary greatly dependant on species. Many of the species studied previously showed a reduction in growth rate. For example Martin et al. (2011) showed a slowed larval growth rate in the sea urchin *P. lividus*, when exposed to decreased pH. Similarly Gaylord et al., (2011) showed that when the larvae of *M. californianus* are exposed to increased pCO_2 (970ppm) there is a reduction in shell area. After 5 days shell area was 5% smaller when compared to control and after 8 days there was a 5% reduction. Hettinger et al., (2013) also reported that decreased pH significantly affected larval shell growth rate at 9 days post larval release. Larvae reared at the lowest pH conditions exhibited a 15% reduction in shell growth rate when compared to the control. This decrease in size at increased pCO_2 was seen through to the settlement stage where larvae reared in pH 7.8 were 7% smaller, and further to 7 days post settlement where pCO_2 significantly reduced growth exhibited as a 41% decrease in shell growth rates. Barros et al., (2013) showed a reduction in the shell length and height of D-shaped veliger's in the oyster *C. gigas* when exposed to decreased pH conditions (Length $p<0.001$; Height $p<0.001$). However growth rate was similar in each treatment. Similarly Byrne et al., (2013) showed that after 3 days exposure to decreased pH treatments there was no significant effect of pH on larval growth in the sea star *Patiriella regularis*. There was however a significant impact of temperature ($p=0.0002$). In contrast to previous studies Dupont et al, (2010) showed that when the sea star *Crossaster papposus* was exposed to decreased pH conditions there was an increase in larval and juvenile growth and this increase did not significantly impact larval survival or skelotogenesis.

The majority of studies concerned with larval growth have seen a reduction when exposed to decreased pH, with species showing varying sensitivities. For example Clark et al. (2009) reported varying sensitivities of echinopleutus skeletal growth when exposed to varying pCO_2 treatments in a variety of echinoderm species (see Table 1.2) Variations seen within and between studies highlight the variations in tolerance thresholds of different organisms when exposed to ocean acidification. The reduction in size seen throughout the studies may lead to problems associated with survival by increasing time to metamorphosis and increasing the risk of predation.

1.2.6 Larval morphometrics and abnormalities

One of the most studied areas of larval growth and development is larval abnormality. This is a particularly important endpoint as changes seen in early development can have serious ramifications on future developmental stages. Therefore if there is an increase in larval abnormalities it may have knock on consequences for further development and populations.

Many of the previous studies concerned with larval morphometrics have seen that with decreasing pH there is a significant increase in larval abnormalities and asymmetry in a variety of species. Early studies by Kurihara et al., (2008) showed that when the larvae of the mussel *Mytilus galloprovincialis* were subjected to reduced seawater pH there was a significant effect on larval development at 120 and 144 hours. At the control pH there was less than 1% larval abnormality seen, however under decreased pH (pH7.42) nearly all (>99%) had abnormal morphology. The abnormal morphologies exhibited included indentation of the shell margin protrusion of the mantle from the shell, convexation of the hinge, and a combination of the latter 2 conditions. Similarly studies concerning the oyster *Saccostrea glomerata* showed that when exposed to increased pCO_2 the percentage of abnormal D veligers increased, with the greatest amount exhibited at 1000ppm when compared to ambient (375ppm). There was also a significant effect of temperature which led to increases in abnormal development. The study found that the as experimental time increases the less abnormal veligers developed with the exception of 1000ppm where the amounts of abnormal veligers were close to 100% (Parker et al., 2009). Similarly studies by Lewis et al., (2012) utilised the tube worm *Pomatoceros lamarckii*. It was found that asymmetry in larval stages became significantly more variable with decreasing pH ($p=0.002$). There was also a significant increase in the mean asymmetry index for 7 day old larvae at the lower pH treatments (pH 7.6 and 7.4).

Echinoderms are one of the most studied phyla in terms of effects of OA on larval development. Studies also encompass a wide range of species from a variety of habitats including temperate, tropical and polar species. Studies using the temperate brittle star *Ophiothrix fragilis* showed that a large proportion of the larvae raised under decreased pH (pH 7.9 and 7.7) treatments were either asymmetric or abnormally developed. The frequency of abnormal larvae through time followed a normal distribution with the highest proportion of abnormal larvae being observed after 3.7 ±0.09 days at pH 7.7 and after 4.92±0.07 days at pH 7.9. However abnormalities were

completely absent in control larvae. After 2 days a significant proportion of larvae showed asymmetry at reduced pHs, with percentage asymmetry at pH 7.9 being 25 % and 32% at a pH of 7.7 (Dupont et al., 2008). Similarly studies concerning the Antarctic sea star *Odontaster validus* saw reductions in developmental rate and differences in larval morphology with larvae appearing less developed in treatment pH conditions (pH 7.6). There was also the presence of larval abnormality, specifically in the form of a poorly formed oral hood and smaller stomach and with the left and right enterocoels showing small extensions both anteriorly and posteriorly at pH 7.0 (Gonzalez-Bernat et al., 2013). Similarly studies by Doo et al, (2011) looked at the effects of decreasing pH on the sea urchin *Centrostephanus rodgersii*. The study showed that with increasing pCO_2 there was a decrease in percentage normal prism larvae at pH 7.8 and 7.6 (p=0.001). After 3 days arrested and abnormal prism stage larvae were present in acidified treatments and at day 5 larval arm length was significantly lower in pH 7.6 (p=0.006). In contrast however there was no significant effect on pH on the percentage larvae with arm length asymmetry among the three pH treatments (p= 0.052)

Whilst the majority of species have shown a significant decrease in larval asymmetry and a significant increase in larval abnormalities with decreasing pH, certain species have shown a degree of resistance to the effects of OA. For example studies by Martin et al. (2011) utilising the sea urchin *Paracentrotus lividus* showed that symmetry index was high regardless of pH treatment with a mean ratio of the left to right overall length close to 1. There was, however, a significant effect of pH on symmetry index. However the lower symmetry index values were seen only at the lower pH treatments used (pH 7.25 and pH 7.0). Similarly studies by Moulin et al. (2011) also concerned with the sea urchin *P.lividus* reported that symmetry index for body rod was significantly lower at decreased pH treatments and there was also a significant effect of pH on larval form. The percentage of pluteus larvae showing abnormality was also increased with decreased pH but this was only significant at pH 7.0.

In contrast to much previous research some species have shown a high tolerance to decreasing pH. For example in the sea urchin *Tripneustes gratilla* larval abnormalities were reported due to increased temperatures but pH was found to have no significant effect (Temperature, p=<0.003; pH p=0.04). There was a reduction in larval symmetry at pH 7.8 at 27ºC. However, this reduction is thought to have occurred as this temperature was at the thermal limit of the species. Overall larval arm asymmetry was

not significantly affected by pCO_2 (Sheppard- Brennand et al., 2010). Similarly studies concerned with the sea star *Meridiastra calca*r saw no significant effect of pH on percentage normal larvae after 5 days development ($p>0.05$). However temperature was seen to have a significant negative effect on percentage normal larvae (Nguyen et al., 2012).

A large proportion of studies have considered the effects of OA on larval morphometrics with variations in results being seen. However the majority of results indicate an increase in larval abnormality with decreasing pH. Variations in results can not only be attributed to species specific response but also to variations in methodologies. Similar to methodologies for larval mortality experiments there are variations in experimental protocols in terms of juvenile submersion with some studies incubating from fertilisation (Kurihara et al., 2008; Byrne et al., 2009; Parker et al., 2010; Byrne et al., 2011a) and others from later developmental stages (Dupont et al., 2008; Ericson et al., 2010; Crim et al., 2011; Moulin et al., 2011). It is likely that increases in larval abnormality will have consequences for future development and survival.

Previous studies concerned with larval growth and development have considered a wide variety of endpoints and found a wide variety of results. It is clear from these results that response to the increasing oceanic pCO_2 is likely to be varied and species specific. It is important for future research to increase the variety of species used to gauge the whole ecosystem response to changing oceanic pH. It is also important to think about larval development in terms of longer studies and parental acclimation to see if these factors significantly impact larval growth and development. Another area of research which deserves attention is the effect that increased larval abnormalities will have when grown through to maturity.

1.2.7 Ocean acidification and multiple stressors

As mentioned in section (1.1) the acidification of the world's oceans is not likely to happen as a singular event. Along with increasing partial pressures of carbon dioxide there is also an estimated increase in average global temperature of between 1.1-6.4°C by the year 2100 (IPCC, 2007). Temperature is not the only stressor that is set to increase with increasing pCO_2, it has been predicted that there will be an increase in salinity (Salma et al., 2012), and pollutants (Lewis et al., 2012). It has also been

hypothesised that with increasing pCO_2 and temperature the marine environment will suffer from reduced oxygen solubility, and this in turn will lead to an increase in the frequency of hypoxic events (Portner and langenbuch, 2005; Portner, 2008; Oschlies et al., 2008; Hofmann and Schellnhuber, 2009).

The interacting effects of temperature and OA have received the most attention of all combined stressors in previous literature (see Table 1.2), as temperature is known to be a key factor in many stages of organism development. Previous studies concerning the combined effects of increasing temperature and pCO_2 have been carried out on a variety of invertebrates including echinoderms, (Byrne et al., 2009; Byrne et al., 2010a; Byrne et al., 2010b; Sheppard-Bernard et al., 2010; Caldwell et al., 2011; Wood et al., 2011;Ericson et al., 2012; Nguyen et al., 2012), molluscs (Parker et al., 2009; Byrne et al., 2010b; Talmage and Gobler 2012; Thiyagarajan and Ko, 2012), including cephalopods (Lacoue-Labarthe et al., 2009) and gastropods (Byrne et al., 2011a; Lischka et al., 2011), and crustaceans (Findlay et al, 2010a; 2010b; 2010 c; Walther et al., 2010; Pansch et al., 2012),

There have been varying results shown for the effects of increasing pCO_2 and temperature, for example Byrne et al. (2009) found that there was a significant effect of temperature on fertilisation success, and development in *the sea urchin Heliocidaris erythrogramm*, with no significant effect of pH and no significant interacting effect between pH and temperature. However later studies by the same (Byrne et al., 2010a; Byrne et al., 2010b) authors using the same species showed no significant effect of temperature or pH and similar to previous studies no significant interacting effect. Further to these experiments further work with echinoderms showed no significant effect on the species *Centrostephanus rodgersii, Heliocidaris erythrogramma, Heliocidaris tuberculata, Patiriella regularis* and *Tripneustes gratilla.* The results achieved in these studies suggest that echinoderm reproduction will be robust to near future pCO_2 and temperature conditions. In contrast the abalone *Haliotis coccoradiata* showed that increased temperature and decreased pH had a significant effect on percentage normal juveniles. (Temperature – $p<0.001$; pH– $p<0.001$) and there was a significant interaction between the two factors (– $p<0.005$). Spine number also showed a significant effect when subjected to decreased pH and increased temperature (temp – $p<0.001$; pH– $p<0.001$) with juveniles reared at +2°C pH 7.6 and 7.8 having more spines than those reared in ambient temperature at equivalent pH values. A significant interaction (–=$p<0.001$) between the two factors also occurred (Byrne et al., 2011a).

Similarly studies concerning the oyster *Saccostrea glomerata* found a reduction in fertilisation success under increased pCO_2 and temperature conditions, with fertilisation being significantly reduced at suboptimal temperature and increased pCO_2. The lowest rate of fertilisation success occurred at 18ºC and 1000ppm with a mean of 39% cleavage. There was also a significant effect of the two factors on embryonic development and larval abnormality, with larval abnormalities increasing with pCO_2 and temperature (between 600-1000ppm and 18-30°C) with lowest abnormality happening at ambient pH under optimal temperature. These increased abnormalities were combined with a decrease in size (Parker et al., 2009). Later studies also showed a significant interaction of pH, temperature and species on fertilisation success, percentage development and larval abnormalities in the oysters *Saccostrea glomerata*, and *Crassostrea gigas*, as well as a significant effect of temperature and pCO_2 (Parker et al., 2010).

Effects of increased temperature and pCO_2 have also had varied effects on polar species, with the pterapod *Limacina helicina*, showing that neither factor had a significant effect on shell diameter and no significant interaction occurring between the two factors. There was however a significant effect of temperature and pCO_2 on mortality. There were no significant interacting effects but temperature had a stronger effect than pH (Lischka et al., 2011). A more recent study by Ericson et al. (2012) using the Antarctic echinoid *Sterechinus neumayeri* showed that fertilisation success was significantly reduced with increasing temperature and pCO_2 (Temperature = $P<0.05$; pH $p<0.001$) and a significant interaction occurred between both factors ($p< 0.005$). Embryonic development showed that temperature had no significant effect on cleavage success but did significantly negatively affect the blastula stage ($p< 0.001$). pH showed a significant effect on cleavage but not on development to the blastula stage; however at +3°C there was a 10-11% decrease in normal embryos across all pH treatments

This combined and interacting effect of temperature and pCO_2 could have further ramifications on organisms. Previous studies have shown that in response to thermal stress there is often an up regulation in the transcription of heat shock proteins which are molecular chaperones involved in cellular defence, and have previously been used as an indication of an organism's ability to tolerate environmental stress (O'Donnell et al. 2009). Studies carried out by O'Donnell et al. (2009) examined the effect of increasing pCO_2 and temperature on the expression of hsp70. It was found that

when *Strongylocentrotus francisanus* larvae were raised under increased pCO_2 there was a change in the expression profile of hsp70. Larvae from all treatment pCO_2 conditions showed an up regulation of hsp70 in response to 1 hour of exposure to temperature increases; however the expression levels of hsp70 and peak expression differed. There was a significant effect of both temperature and pCO_2 (Temperature $p<0.0001$; pCO_2 $p= 0.0037$). This study shows that increased pCO_2 has a significant effect on the expression of hsp70 in the urchin *S.francisanus* and this suggests that in a changing ocean the ability of organisms to cope with thermal stress may be decreased.

Temperature is by far the most studied synergistic factor associated with increasing OA. However recent studies have begun to consider the effects of three abiotic factors in multiple stressor experiments. Thiyagarajan and Ko, (2012) have considered the effect of pCO_2, salinity and temperature, on larval growth in the Portuguese oyster *crassostrea angulate*. As an individual factor pCO_2 did not have a significant effect on survival ($p >0.05$) or larval shell growth ($p =0.41$), however when combined with low salinity there was a significant reduction a pH 7.4 ($p<0.05$). When this increase in pCO_2 and decrease in salinity was coupled with an increase in temperature there was no significant effect of temperature or pH on larval shell growth: however larvae raised at low salinity had significantly larger shells than those raised in ambient salinity. There was also a combined effect of salinity and temperature with larvae that were raised at low salinity and higher temperature showed increase growth ($>8mm^2$). Davis et al. (2013) looked at the combined effects of temperature, pCO_2 and UV radiation on mortality and developmental rate in *Bembicium nanum* and *Dolabrifera brazier*. Both species showed mortality was driven by temperature and pH, with mortality being greatest at lower temperatures (22°C) and in acidified water (pH 7.6), and there was a significant interacting effect between the two factors (*B.nanum*, P= 0.0263; *D.brazier*, P= 0.0471). There was however no effect of UV radiation on mortality. There was however a significant interaction of temperature and pH on developmental rate in both species (*B.nanum*, P= 0.0007; *D.brazier*, P= 0.0169), Full spectrum light also influenced developmental rate with development being slower when present for *B.nanum* (p=0.0125). These two studies indicate the need for further work on a greater range of combined multiple stressors as they show interactions not previously seen.

From previous studies it is clear that there needs to be an increase in the number of studies concerned with combined multiple factors and their effects on larval

development. There needs to be diversification of the factors studied and the species that are used because as mentioned previously ocean acidification is a phenomenon not likely to happen in isolation.

1.2.8 Long term and multigenerational studies

There is an increasing need to see the long term and multigenerational effects that changing oceanic chemistry has on reproduction and development of marine invertebrates (Ross et al., 2011). The majority of OA studies concerned with reproduction and development have looked at the short term effects that OA will have on reproductive endpoints. In terms of fertilisation success past studies have utilised differing levels of gamete incubation prior to fertilisation success with several studies not placing gametes in to experimental conditions until fertilisation (Albright et al., 2010; Parker et al., 2010; Moulin et al., 2011). Of the studies that have incubated gametes prior to fertilisation the longest acclimation period was 30 minutes, and the sperm was not acclimated at all (Havenhand et al., 2008). However there are an increasing number of studies concerned with longer term exposure to decreased oceanic pH, and parental exposure.

As mentioned above there have been relatively few long term exposure studies. Of these the majority have seen a negative effect of long term exposure on reproductive and developmental processes. For example Yu et al., (2013) saw that during a 30 day development period, larval growth of the sea urchin *Sterechinus neumayeri* showed a significant reduction. After 7 days the spicule of urchins grown under increased pCO_2 (730 μatm) showed a significant decrease and between days 15 and 30 unfed larvae at 730 μatm were significantly smaller than unfed control urchins. Hettinger et al., (2012) also saw a significant effect of decreasing pH on the oyster *Ostrea lurida* during a 52 day exposure period. The oysters were cultured through platonic larval period to metamorphosis and in to early juvenile life. When larvae were reared at pH 7.8 there was a decrease in larval shell growth of 15 % and in shell area of 7%. This decrease continued, as data attained from one week post settlement exhibited a 41% decrease in shell growth rate. This study suggested a persistent carry over effect from larvae to juvenile. This persistent carry over effect has also been suggested in studies concerned with parental exposure.

Recent studies concerned with the effects of OA on marine organisms have taken

an approach of parental exposure. Parker et al. (2012) found that when parents of the oyster *S glomerata* were exposed to increased pCO_2 (856ppm) for 6 months prior to spawning there was a significant effect on their progeny. After 19 days of exposure, larval survival from pCO_2 conditioned adults showed a significant reduction. There was also a significant effect on larval development with significant reduction being seen from adult exposure and increased pCO_2 conditions. Uticke et al., (2012) found that when adult sea urchins *Echinometra mathaei* was exposed to increased pCO_2 for 7 weeks gonad conditioning prior to spawning there was a significant reduction in males spawning ability, but not in females spawning ability. There was also a significant decrease in larval survival and percentage of normal larvae produced from pre conditioned sea urchins. There was also an increase in arm asymmetry at increased pCO_2. In contrast to previous literature Dupont et al., (2012) found variations in the response of the sea urchin *Strongylocentrotus droebachiensis* when exposed for 4 months and 16 months prior to spawning at increased pCO_2 (1200uatm). After 4 months exposure a significant decrease in female fecundity (4.5 fold when compared to control) and a decrease in larval settlement success was recorded, with 5-9 times fewer larvae reaching the juvenile stage. However in contrast after 16 months exposure there was no significant effect of increased pCO_2 on larval or juvenile survival.

From the aforementioned studies it is clear that long term exposure is likely to significantly impact larval reproduction and development. The studies by Dupont et al., (2012) do suggest that organisms may be able to adapt if given long enough. It does however highlight the need for further research and increased species variety.

The previous two multi-generational studies have looked at copepods and found contrasting results. Early studies by Kurihara and Ishimatsu (2008) saw that there was no significant effect of increasing pCO_2 conditions (+2000ppm) on survival ($p>0.05$), development, egg production ($p>0.05$), and hatching rate ($p>0.05$), in the copepod *Acartia tsuensis*. In contrast more recent studies by Fitzer et al. (2012) saw a significant decreased in nauplii number at pH 7.82 ($p=<0.001$), however there was no significant decrease seen at pH 7.67 ($p=0.229$). There was also a significant reduction in growth when compared to the control (pH 7.82 $p=0.008$; pH 7.67 $p-<0.001$) in the copepod *Tisbe battagliai*. There are differences between studies, for example the species generation time (*A. tsuensis* – 9 days, *T. battagliai* – 14 days) as well as the pH's used. Kurihara and Ishimatsu (2008) used only a control and treatment pH, whereas Fitzer et al, (2012) looked at a control and three pH treatments. It is possible that the reason for

differing results is due to the treatments used as Fitzer et al., (2012) saw contrasting increases and decreases between pH conditions, however as Kurihara and Ishimatsu (2008) only used one treatment (+2000ppm).

The contrasting results seen throughout long term, parental acclimation and multigenerational studies supports the need for further research in to the effects that OA is likely to have over long time periods and multiple generations, as well as in a greater range of species. Previous long term and mutigenerational studies have generally concentrated on larval survival and growth and have left a gap in knowledge for reproductive process such as sperm motility and fertilisation success which are fundamental aspects of reproduction. The current thesis aims to fill some of the knowledge gaps left by previous studies by looking at long term parental exposure and the effects it may have on sea urchins as well as the multigenerational effects on an important polychaete species.

1.3 Marine organisms and acidification

Benthic marine organisms are a popular choice of study animal when researching OA effects. They are ecologically very important and make up 98% of all marine species, and they have living relatives in all but 1 of the 29 non symbiotic animal phyla (Widdicombe and Spicer, 2008). These organisms are chosen as test species due to their diversity and this is important as it is widely accepted that OA will affect all organisms differently, i.e. it is species specific, in that tolerance to a changing environment is going to change dependant on the organisms ability to adapt, and some organisms will have a higher adaptive capacity than others. Ocean acidification is likely to cause a diverse range of problems, and reduce marine biodiversity in a number of ways, including the loss of organisms that show sensitivity to specific environmental conditions (Widdicombe and Spicer, 2008; Dupont and Thorndyke, 2009). There has been much research considering impacts on organisms and biological processes.

Echinoderms are one of the most studied taxa in terms of ocean acidification research in terms of reproduction and development (see Figure 1.1). The early developmental stages of Echinoderms, especially sea urchins, have been seen to be particularly vulnerable to decreasing pH (see table 1.2). *Paracentrotus lividus* is a well characterised and well documented model species with a broad geographic distribution

in the Mediterranean Sea and along the Atlantic coast from southern Morocco up to the west coast of Ireland (Byrne et al., 1990; Martin et al., 2011; Moulin et al., 2011). This equates to an Atlantic thermal range of 9-24 ^{o}C .However due to wide geographical distribution of *P. lividus* the thermal and pH tolerances are likely to vary between different populations depending on habitat, for example those inhabiting rock pools may be better equipped to deal with wide variations in pH and temperature than those from sub-tidal habitats. Animals collect from Irish populations for this experiment have an optimum breeding temperature of between 11-16^{o}C, although the possible synergistic effects of pH and temperature remain to be investigated. *P.lividus* reproduction has been previously well documented and the gametes used extensively in embryological research. They are also an important species both economically, in sea urchin fisheries, and ecologically as a key stone ecosystem engineer. *P. lividus* is an important grazer species, and one of the dominant grazer in many shallow benthic communities. *P.lividus* has previously been described as a model organism and used in many studies concerned with embryonic development (Carata et al., 2012). The well documented lifecycle of *P.lividus* as well as its role as keystone species in many benthic marine communities make it an ideal model species for use in the current studies.

As a group polychaetes have previously received little attention in terms of ocean acidification research, especially in the field of reproduction and development. Polychaetes represent a key group of ecologically important marine invertebrates with non-calcifying larvae (Lewis et al., 2012). *Ficopomatus enigmaticus* is a reef building serpulid tubeworm with a broad distribution throughout temperate and subtropical waters in the northern and southern hemisphere (Obenat and Pezzani, 1994, Obenat et al., 2006). Reefs of *F.enigmaticus* have been reported with sizes up to 7 metres in diameter and 0.5 metres in height and are comprised of thousands of individuals and calcareous tubes (Obenat et al., 2006). F.enigmaticus is an invasive species as well as a problematic fouling species. It is also an ecosystem engineer and has been seen previously to strongly modify estuarine ecosystems by increasing species richness in structurally simple environments as well as modifying bed load sediment transport and patterns of water flow (Obenat et al., 2006). The reproductive biology of serpulid polychaetes has been previously well documented (Obenat and Pezzani, 1994; Kupriyanova et al., 2001; Obenat et al., 2006). In general sexes are separate with some reports of hermaphroditic populations (Straughan, 1972; Dixon, 1981). Gametes are maintained free in the coelom until they reach maturity. Fertilisation is external and

gametes are expelled through ducts or ruptures in the body wall (Obenat et al., 2006). *F .enigmaticus* have previously shown to be resilient to abiotic factors including salinities (from 6 to 35; Straughan, 1972; Fornós et al., 1997) and temperatures (Fornós et al., 1997). When coupled with its ability to have short generation times (3 months; Current study) and its well documented reproductive cycles it provides an excellent model species. As *F .enigmaticus* is a polychaete, it also represents a previously understudied taxa with only one previously study looking at the effects of ocean acidification on reproduction and development (Lewis et al., 2012).

1.4 Conclusion and hypotheses for this study

The effects that ocean acidification has on the reproductive processes of marine invertebrates is far from clear. From the current review it is clear that marine invertebrate's exhibit variations in response to climate change in terms of variations in ocean chemistry and temperatures dependant on species and reproductive endpoint (see table 1.2). Previous research has left knowledge gaps in terms of the long term and multigenerational effects that these stressors will have on reproductive and developmental processes. There are also relatively few studies regarding combined multiple stressors. The most studied has been the effects of increasing temperature but all previous studies have been concerned with only short term exposure.

The present thesis aims to fill previous knowledge gaps by examining the long term combined effect that decreasing pH and increasing temperature has on reproduction and development in a keystone echinoderm species, as well as addressing the effects of parental acclimation. The present thesis is the first to consider long term exposure to combine multiple stressors. It is also one of the first studies to look at the effects of long term exposure and how it affects larval development. The current thesis is also the first to look at the multigenerational effects of decreased pH on sperm motility and fertilisation success in an ecosystem engineering polychaete.

Understanding the long-term and multigenerational effects that decreasing pH will have on reproductive and developmental processes may help to explain how benthic marine communities will be affected in the future under decreasing pH levels predicted for the year 2100. This may help to explain the effect that a changing ocean

will have not only on juveniles but also on adult organisms as well as the impacts on an ecosystem level.

The hypotheses for the current thesis are;

1. long term exposure to decreased oceanic pH will negatively impact sperm motility and fertilisation success
2. Fertilisation success and sperm motility will be significantly negatively affected when subjected to long term exposure of the combined multiple stressors of increasing oceanic pCO_2 and increasing temperature.
3. If a hypoxic event were to occur after long term exposure to increased pCO_2 conditions it will cause a significant reduction in both fertilisation success and sperm motility
4. Larval development, of larva derived from acclimated adults, will be significantly impacted by decreasing oceanic pH and increasing temperature, manifested as an increase in larval asymmetry and a reduction in growth.
5. Multi-generational exposure to decreasing oceanic pH will inhibit sperm motility and fertilisation success.

Chapter 2: The effect of OA and temperature on reproductive capacity of the sea urchin Paracentrotus lividus over a 12-month exposure period

2.1 Introduction

Early life history stages of echinoderms are especially sensitive to environmental change. Development of the echinopleutus larva is negatively impacted by ocean acidification (OA) consistent with this trend (Parker et al., 2009; Dupont and Thorndyke 2009; Gonzalez-Bernat et al., 2013). Echinoderms generally employ broadcast spawning followed by external fertilisation, which has inherent risks as the gametes are exposed directly to the environment. The effects of OA on gametes appear to be varied. Negative (Havenhand et al., 2008; Havenhand and Schlegel, 2009; Frommel et al., 2010; Morita et al., 2010; Lewis et al., 2012), positive (Caldwell et al., 2011) and no (Schlegel et al., 2012) effects of OA on sperm swimming speed have all been found. Conversely eggs appear robust to near future ocean acidification scenarios. Wood et al. (2008) reported no significant effect on eggs size or structure when exposed to increased pCO_2. Previous research has also shown the robust nature of eggs to decreased pH conditions through the routine use of low pH (pH 5.0) solutions to strip the jelly coat prior to fertilisation assays (Byrne, 2011). It has also been suggested, however, that pH have may have a negative effect on sperm-egg binding compatibility (Palumbi, 1999; Levitan and Ferrell, 2006; Schlegel et al., 2012).

During past OA research there has been a large variety of phyla used and this has highlighted the species specific responses to increasing levels of OA. One of the most highly studied phyla is Echinodermata (See chapter one Table 1.2, and Figure 1.1), which include sea urchins. Sea urchins are marine calcifiers and have varied life stages including a marine planktonic life stage. As well as being ecologically important some species of sea urchin, such as *Paracentrotus lividus*, are also economically important as

a food source. Previous research has shown various responses to increasing OA on a variety of factors including calcification, reproduction, and metabolism.

Table 2. 1: Duration of mid and long term studies concerned with the effect of increasing OA on marine invertebrates.

Duration	Reference
7 weeks	Michaelidis et al., 2005
7 weeks	Shirayama and Thornton, 2005
7 weeks	Dashfield et al., 2008
15 and 30 weeks	Kurihara et al., 2008
40 days	Wood et al., 2008
28 days	Arnold et al., 2009
30 days	Ellis et al., 2009
1 month and 1 year	Rodolfo-Metalpa et al., 2010
6 months	Hernroth et al., 2011
4 and 16 months	Dupont et al,. 2012
178 days	Form and Rieebsell, 2012
52 days post settlement	Hettinger et al., 2012
5 weeks adult exposure and experiments at 9 and 19 days	Parker et al., 2012
7 weeks	Uthicke et al., 2012
70 days	Navarro et al., 2012

To date there have been very few mid-term (8-30 days) and long-term (30 days and above) studies on the effects of OA on marine invertebrates (see Table 2.1) and of these only three consider the possible effect of parental exposure on reproduction and development (Dupont et al., 2012; Parker et al., 2012; Uthicke et al 2012) . Most of the aforementioned studies have concentrated on larval growth, development and survival, with contrasting results compared to short-term studies. For example, Dupont et al. (2012) reported a 4.5-fold decrease in fecundity in the sea urchin *Strongylocentrotus droebachiensis* after 4 months exposure as well as a negative impact of adult exposure (4 months) on larval settlement and survival to the juvenile stage. Conversely, there was no significant negative effect on the aforementioned factors after 16 months exposure.

Conversely, a 7-week exposure to increased pCO_2 did not have a significant effect on egg fecundity of *Echinometra mathaei* , though there was a negative impact on sperm production (Uthicke et al., 2012).

The majority of previous research has centred on short term (6 days and below) exposure to environmental stressors. This short term exposure has often meant that adults or gametes are placed directly in to pH adjusted water which has not allowed an acclimation period prior to experimentation. The majority of short term studies has utilised an exposure period of less than two weeks (Dupont et al., 2010b; Dupont et al., 2012). This short exposure time has not taken in to account a stress response due to their rapidly changing environment, and results of short-term studies may not reflect true effects of increasing OA.

The studies reported here aim to determine the effects that an increase in both pCO_2 and temperature will have on the reproductive and developmental capacity of *P.lividus* (sperm motility, and fertilisation and cleavage success), after 3, 6 and 12 months of exposure. *P. lividus*, is an important grazing species with a wide distribution between the Mediterranean and the North East Atlantic between Southern Morocco and Ireland. It inhabits rocky scores (intertidal and sub-tidal) and sea grass meadows. It acts as bio-turbator and is an important part of the food chain, as part of the grazing community as well as prey source. This species is not only ecologically important, but also economically important in sea urchin fisheries. It has well defined reproductive endpoints which have been studied in detail (Byrne et al., 1990; Martin et al., 2011; Moulin et al., 2011.

2.2 Methods

2.2.1 Animal husbandry

In order assess the effects of long-term parental exposure to elevated temperature and pCO_2 on developmental stability, larvae and adult *Paracentrotus lividus* (supplied by Dunmanus Seafoods Ltd, Durrus, Bantry, Co. Cork, Ireland), were exposed for 3, 6 and 12 months to mean temperature and pCO_2 conditions predicted to occur by the end of this century (Caldeira and Wickett, 2003; Orr et al., 2005). These were the five nominal treatments were:

Table 2.2: Referenceing the different pH conditions used.

Nominal treatment	***p*CO₂ level (µatm)**
Ambient	380 µatm and ambient seasonal temperature cycle
Elevated pCO_2	750 µatm and ambient seasonal temperature cycle
Extreme elevated pCO2	1000 µatm and ambient seasonal temperature cycle
Elevated temperature	380 µatm and 2°C added to the ambient seasonal temperature cycle
Combined	Combined

Within each of these nominal treatments urchins were haphazardly assigned to one of four tanks (tank volume 1 m^3). Within each of these separate tanks urchins were further divided into three baskets (30 cm x 20 cm x 20 cm) with original stocking densities of six urchins per basket (18 per tank). pCO_2 and temperature were maintained separately for each tank. Ambient pCO_2 treatments were maintained by bubbling untreated air through the water in each tank. Elevated pCO_2 treatments were maintained by enriching the air with CO_2 before bubbling (after Findlay et al., 2008). CO_2 levels (ppm) of both the untreated and CO_2 enriched air were monitored using a CO_2 Analyser and pH was monitored 3 times a week (LI-820, Li- Cor , Lincoln, USA) and converted through calculation to pCO_2 (µatm). The temperature of each tank was maintained using aquarium heaters (Aqua One, 150W, Kong's (UK) Limited, Romsey, UK.) and chillers (BOYU L-350) which were used to heat or cool the water as necessary. To prevent pCO_2 and temperature gradients forming within the tanks, the water was circulated using pumps (Aquael 1000 filter, Aquael, Warszawa, Poland). Natural seasonal variation in temperature and photoperiod is known to impact on gametogenesis and spawning condition. Consequently these cycles were recreated in the laboratory by monthly adjustments in temperature appropriate to replicate the mean ambient monthly

seasonal temperature of Plymouth Sound (as work carried out at Plymouth Marine Laboratory). Photoperiod was also adjusted monthly by changing the length of time the lighting was on each day, using T8 triphosphor fluorescent tubes, (which are designed to meet saltwater aquarium lighting requirements) to match natural seasonal changes in day length. Each tank received a one-third by volume water change every three weeks or if nitrate levels, which were monitored weekly using a nutrient auto analyser (Branne and Luebbe Ltd. AAIII) (Brewer and Riley, 1965) exceeded 25 mg L^{-1}. Urchins were fed *ad libitum* for 48 h once every week with fresh macroalgae (*Ulva lactuca* and *Laminaria* sp; approx. 500 g per basket) collected from Plymouth Sound. Following feeding the remaining macro-algae and any faecal pellets were removed to prevent nitrate build up.

2.2.2 Carbonate chemistry

Sea water temperature, salinity (using a WTW LF197 multi-meter with a tetra con 325 electrode) and pH (using a Metrohm 826 pH meter and Metrohm 6.0228.000 pH electrode) were measured 3 times a week, and pH measurements were measured to 0.01 of a pH unit. Total alkalinity was measured once a week. 100ml of water per microcosm were stored in borosilicate glass bottles preserved with 100µl $HgCl_2$ for total alkalinity analysis, following analytical procedures by Dickson et al. (2007). Total alkalinity (TA) was determined using the open cell potentiometric titration method on 12 ml sample volumes using an automated titrator (Apollo SciTech Alkalinity Titrator Model AS-ALK2). Calibration was made using Certified Reference Materials (Dickson, Batch 119). Total alkalinity (TA) was corrected with calibration factor and for additional mercuric chloride. Carbonate system parameters that were not directly measured were calculated from temperature, salinity, pH and TA using CO2SYS (Pierrot et al., 2006), employing constants from Mehrbach et al. (1973) refitted to by Dickson and Milero (1987) and the KSO dissociation constant from Dickson (1990) (Table 2).

2.2.3 Spawning induction

Over all time points and treatments, 203 sea urchins were induced to spawn through intra-coelomic injection of 0.5 M 0.5-1.0ml KCl. Sperm was collected dry (i.e. undiluted) and stored on ice for no more than 1 hour. Females were left to express eggs in to 200ml of pCO_2 adjusted filtered sea water (FSW) (0.22 µm) for 1 hour after which time the females were removed. Gametes were collected from three males and three

females where possible at each treatment; eggs from females were spawned into relevant pCO_2 adjusted sea water. Gametes were not mixed as this may have had a negative effect on overall gamete quality.

2.2.4 Sperm motility

Sperm samples were taken from at least two males for all treatments and all time points. Sperm densities were determined by haemocytometer and adjusted to 10^6 sperm ml^{-1}. Sperm were held at 9°C (3 and 12 month ambient), 11°C (3 and 12 month ambient +2°C), 15°C (6 months ambient), and 17°C (6 months ambient +2 °C). Subsamples were taken at 10-min intervals (from 0 to 60 min) and immediately transferred to a glass slide. Sperm motility was determined as percentage motility and swimming speed (curvilinear velocity, VCL) was measured by computer-assisted sperm analysis (CASA) at 20^{o}C according to Caldwell et al. (2011). In brief, CASA sperm tracking was performed by a Microptic sperm class analyser® (Microm UK), which incorporated a Nikon Eclipse 50*i* negative phase contrast microscope (200x magnification) fitted with a Peltier cooled stage. Image capture was by a Basler A602F camera at a rate of 100 frames s^{-1}. Multiple fields were captured each for duration of 0.5 seconds until a minimum of 200 sperm were scored per treatment.

2.2.5 Fertilisation success

Egg densities were determined by counting 3 x 50 µL aliquots of eggs from 2-3 females. Between 450 and 500 eggs were added to individual wells of 6-well multiplates containing 10ml of FSW and mixed with sperm from 2-3 males at a range of concentrations: 2. 5 x 10^3, 10^4, 10^5 and 10^6 sperm ml^{-1}. Fertilisation assays were conducted at varying CO_2 treatments. Temperature treatments varied depending on time of year as natural/ seasonal variations were used throughout the experiments (380µatm and 750 µatm, and 1000µatm, at 9°C (3 and 12 month ambient), 11°C (3 and 12 month ambient +2°C), 15°C (6 months ambient), 17°C (6 months ambient +2 °C). Fertilisation success was determined after 2 hours (3 and 12 months) and 1 hour (6 months) as presence of the fertilisation membrane. Cleavage success was assessed after 4 hours (3 and 12 months) and 2 hours (6 months) as the presence of two to four cell cleavage stages (see figure 2.1). Fertilisation success and cleavage success were treated as separate reproductive endpoints as they represent different stages of development.

2.2.6 Statistical analysis

Motility data from sperm with a head area lower than 5 μm² and exceeding 35 μm² were discounted from analysis to eliminate false negatives attributable to sperm clumping. A test for normality (Kolmogorov-Smirnov) was carried out and data logged using a natural log, when not normally distributed. A two-way ANOVA was conducted on the log VCL data to determine significant factors and interactions. For percentage sperm motility data were arcsine transformed prior to statistical analysis and a test for normality (Kolmogorov-Smirnov) was carried out. A two-way ANOVA was conducted using time as a covariate. Two-way ANOVAs were carried out separately for each time-point due to variations in temperature.

Fertilisation success and cleavage success data were arcsine transformed, a test for normality was carried out (Kolmogorov-Smirnov) and a two-way ANOVA was performed using sperm density as a covariate. Further statistical analysis of the effects of sperm density on fertilisation success under elevated temperature and pCO_2 were performed on normally distributed data (Kolmogorov-Smirnov) using a one way ANOVA.

A univariate analysis was carried out for fertilisation success, cleavage success and sperm VCL measurements over all treatments to determine the impact of long-term exposure. Each treatment was classed as a single event and assigned a number and a univariate analysis was performed to determine where significant interactions occurred.

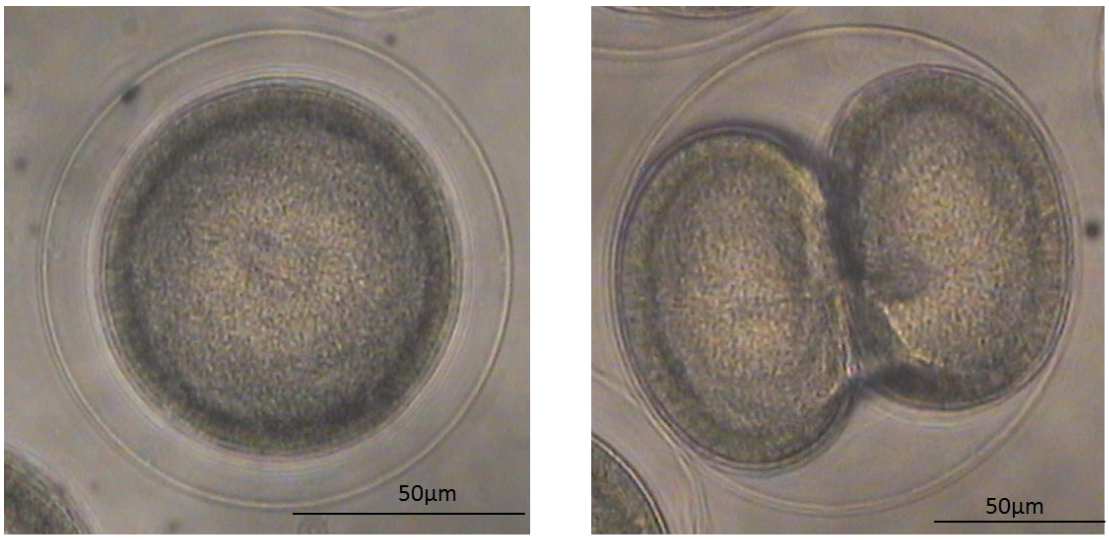

Figure 2.1: Elevation of the fertilisation envelope (a) and the 2-cell stage of cleavage (b).

2.3 Results

Long-term exposure to elevated pCO_2 and temperature both individually and in combination showed complex and interactive effects on sperm motility and fertilisation success. In general, sperm motility was significantly greater under increased pCO_2. In contrast, fertilisation and cleavage success showed in general a significant reduction under increased pCO_2, as well as significant interacting effects between pCO_2 and temperature.

2.2.7 Sperm motility

After three months exposure to increased pCO_2, both the pCO_2 (p=0.004) and time (p=<0.005) had a significant effect on sperm VCL (see figure 2.2), with the value at1000 uatm being significantly higher than ambient (p=<0.005). Similarly after 6 months exposure there was a significant effect of both pCO_2 (p=<0.005) and time (p=<0.005) on sperm VCL, with the value for 380 µatm ambient significantly lower than 750 µatm (p=0.001), 1000 µatm (p=<0.005) and 750 µatm plus 2°C (p=0.036. After 12 months exposure to increased pCO_2, pH was no longer a significant factor (p=0.132), but temperature (p=0.021) and time (p=<0.005) had a significant impact on sperm VCL. Sperm motility was not affected significantly by CO_2 (p==0.485), temperature (p=0.643) or time (p=0.118) (see figure 2.3). After 6 months of exposure there was a significant impact of pCO_2 (p=<0.005), time (p=0.013) as well as a significant interaction between pCO_2 and temperature (p= 0.002). Under increasing acidification there was a significant increase in percentage sperm motility when compared to ambient at 750 µatm (p= 0.047), 1000 µatm (P=<0.005), and 380 µatm plus 2 °C (p=0.003). After 12 months exposure there was no significant effect of pCO_2 (p=0.826), and no significant interaction between pCO_2 and temperature (p=0.389), there was however a significant effect of temperature (p=0.049) and time (p=<0.005).

2.2.8 Fertilisation and cleavage success

After three months exposure to increased CO_2 there was a significant effect of pCO_2 (p=0.006) sperm density (p=0.002), on fertilisation success, however temperature was not a significant factor (p=0.244). There was also a significant interaction between temperature and pCO_2 (p=<0.005) (see figure 2.4). A similar pattern was observed in cleavage success after 3 months with pCO_2, and sperm density significantly impacting

cleavage success (p=0.006; p=0.00). Temperature did not have a significant effect on cleavage success (p=0.070) (see figure 2.5) but there was a significant interaction between temperature and pCO_2 (p=<0.005). After six months there was a significant impact of pCO_2 (p=<0.005), temperature (p=<0.005) and sperm density (p=<0.005), as well as a significant interaction between pCO_2 and temperature (p=0.034) (see figure 2.5). The fertilisation success at 380 µatm ambient treatment was significantly higher than at 750 µatm (p=0.005) and 1000 µatm (p=<0.005), and significantly lower than at380 µatm ambient + 2 °C (p=<0.005). Cleavage success shows an impact of pCO_2 (p=<0.005), sperm density (p=<0.005) as well as a significant interaction between pCO_2 and temperature (p=0.009) (see figure 2.5), with 380 µatm being significantly higher than 750 µatm (p=0.001), 1000 µatm (p=<0.005), and 750 µatm plus 2 °C (p=<0.005), with 380 µatm plus 2 °C showing a significant increase in cleavage success (p=0.004).

After 12 months exposure there was a significant impact of $pCO2$ (p=<0.005), temperature (p=0.029), and sperm density (p=<0.005) on fertilisation success as well as a significant interaction between pCO_2 and temperature (p=0.002) (see figure 2.4). There was a significant decrease in fertilisation success at 750 µatm (p=<0.005), and 750 µatm plus 2 °C (p=<0.005) when compared to control. There was also a significant impact of pCO_2 (p=<0.005), temperature (p=<0.005), and sperm density (p=<0.005) on cleavage success as well as a significant interaction between pCO_2 and temperature (p=0.007) (see figure 2.5). Cleavage success was significantly lower at 750 µatm (p=<0.005) and 750 µatm plus 2 °C (p=0.023) when compared to ambient. At the 12 month time point data for fertilisation and cleavage success for 1000 µatm were unable to be collected. Upon later inspection of the gonads they were found to be black and shrivelled indicating no egg mass.

2.2.9 Sperm density, and fertilisation success,

After 3 months parental exposure to increased pCO_2 there was a significant effect of sperm density on fertilisation success. At lower sperm densities (10^3 and 10^4 sperm ml^{-1}) there was a significant reduction in fertilisation success at 750 µatm (10^3 sperm ml^{-1}, p= 0.003; 10^4 sperm ml^{-1}, p= <0.005), 1000 µatm (10^3 sperm ml^{-1}, p= 0.002; 10^4 sperm ml^{-1}, p= <0.005) and 380 µatm ambient + 2 °C (10^3 sperm ml^{-1}, p= <0.005; 10^4 sperm ml^{-1}, p= <0.005), when compared to ambient pCO_2 and temperature conditions. At higher sperm densities (10^5 sperm ml^{-1}) there was a significant difference

between fertilisation success at 1000 µatm (p=0.001) and 380 µatm ambient + 2 °C (p=0.038) when compared to ambient pCO_2. In contrast to the other sperm densities, at the largest sperm density (10^6 sperm ml^{-1}) there was only a significant decrease seen between ambient and 1000 µatm (p=0.007) (see figure 2.6).

After six months exposure the effects of sperm densities on fertilisation success under differing pCO_2 conditions showed that at a sperm density of 10^3 sperm ml^{-1} there is a significantly lower fertilisation success at 1000 µatm (p=0.00) and 380 µatm ambient + 2 °C (p=0.001) when compared to ambient, however at 10^4 sperm ml^{-1} there was a significant reduction in fertilisation success at 750 µatm (p= 0.003), 1000 µatm (p= 0.002) and 750 µatm ambient + 2 °C (p=0.033). At sperm densities of 10^5 and 10^6 there was a significant reduction in fertilisation success when compared to ambient at 1000 µatm (10^5 sperm ml^{-1}, p=<0.005; 10^6 sperm ml^{-1}, p=<0.005) and 380 µatm plus 2 °C (10^5 sperm ml^{-1}, p=0.019; 10^6 sperm ml^{-1}, p=0.001) (see figure 2.6).

After 12 months exposure to increased pCO_2 sperm density showed a significant effect on fertilisation success (<0.005). There was a significant reduction in fertilisation success at 750 µatm at all sperm densities when compared to ambient (10^3 sperm ml^{-1}, p=<0.005; 10^4 sperm ml^{-1} p=<0.005; 10^5 sperm ml^{-1} p=0.001; 10^6 sperm ml^{-1} p=0.002). At the higher sperm densities (10^5 and 10^6 sperm ml^{-1}), there was also a significant reduction in fertilisation success at 750 µatm plus 2 °C (10^5 sperm ml^{-1}, p=0.007; 10^6 sperm ml^{-1}, p=0.003) (see figure2. 6).

2.2.10 Comparison of fertilisation and cleavage success

At treatment 1000 pCO_2 there was a significant difference between fertilisation success and cleavage success (6months p=0.000) both showing there is a significant increase from fertilisation success to cleavage success (3 months p=0.004; fertilisation = 63%, cleavage =69%; 6months p=0.000; fertilisation=66. %, cleavage= 73. %), (see table 2.2). These results suggest it is important to treat fertilisation and cleavage success as separate reproductive endpoints. This trend was also seen at 750 µatm high 3 months (p=0.001; fertilisation= 79%, cleavage=87%) however when looking at 750 µatm high 6 months there was a decrease in percentage success from fertilisation success to cleavage success (p=0.044; fertilisation=81%, cleavage=78%) and this result is repeated at 380 µatm 12 months (p=0.009; fertilisation=86% cleavage=79%) and 380 µatm high 12 months (p=0.034;fertilisation=84%,cleavage=83%)

2.2.11 Treatment analysis

There was no significant effect of exposure time on VCL under ambient (380 µatm) conditions (6 months, p=0.810; 12 months, p=0.082). There was a significant increase in sperm motility at 1000 µatm after 3 months exposure (p=0.022). After 6 months exposure 750 µatm, 1000 µatm and 750 µatm ambient +2°C showed a significant increase in sperm motility when compared to 380 µatm after 3 months exposure (750 µatm, p=0.001; 1000 µatm, p=0.000; 750 µatm high, p=0.026). There was no significant difference in mean VCL after 12 months when compared to 380 µatm 3 months ambient (750 µatm, p =0.074; 1000 µatm, p=0.092; 380 µatm high, p=0.116; 750 µatm high, p=0.290). After 12 months exposure all VCL values differed significantly from the values attained after 3 months except for control treatment and 750 µatm ambient +2°C (750 µatm, P=0.015;1000 µatm, P=0.000; 380 µatm ambient +2°C, P=0.000)

Exposure time did not significantly affect fertilisation success at ambient temperature and pCO_2 (380 µatm) (6 months, p= 0.649; 12 months, p=0.086). There was a significant decrease in fertilisation success after 6 months exposure at the most extreme pCO_2 (1000 µatm, p=0.000). Also after six months there was a significant increase in fertilisation success at higher temperatures under ambient pCO_2 (380 µatm high. P=0.020). After 12 months exposure there was a significant reduction in fertilisation success at 7500 µatm ambient temperature and 750 µatm increased temperature (p=0.000;p=0.000). There was a significant increase in cleavage success under ambient conditions after 6 months exposure when compared to 3 months exposure under ambient conditions but after 12 months exposure there was no significant difference (p=0.004;.p=0.379). After 6 months exposure there was a significant increase in fertilisation success at ambient pCO_2 and increased temperatures (p=0.000). After 12 months exposure there was a significant decrease in fertilisation success at increased pCO_2 when compared to 380pm ambient 3months (750 µatm ambient, p= 0.000; p=0.044). There was also a significant increase in fertilisation success at 380 µatm higher temperature after 12 months exposure (p=0.048).

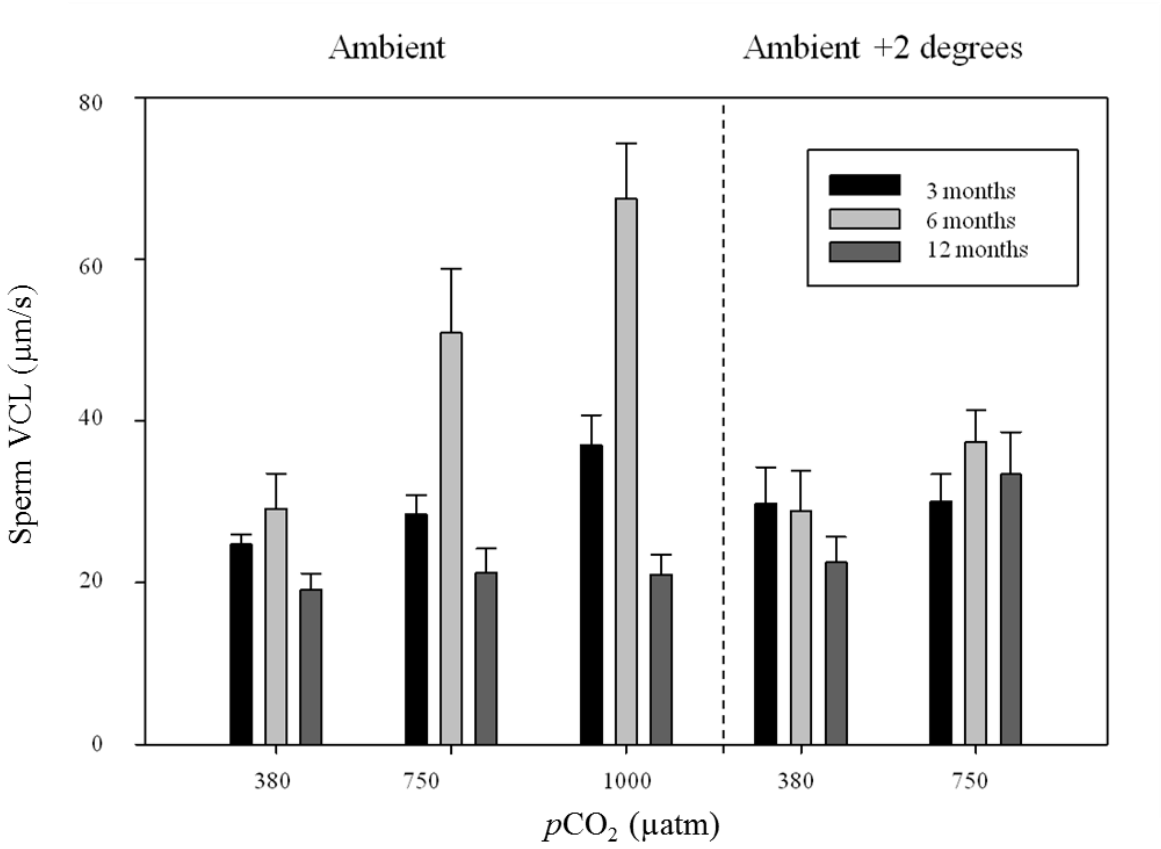

Figure 2.2: Curvilinear velocities (VCL) of sperm after 3, 6 and 12 months parental exposure to increased pCO_2 and increased temperature. After 3 and 6 months exposure to increased pCO_2, there is a significant increase in VCL (3 months p= 0.004; 6 months p=<0.005), however after 12 months there is no significant effect of pCO_2 on VCL (p= 0.132). Temperature is however a significant factor at 12 months but not 3 or 6 months (3 months p= 0.381; 6 months p=0.124; 12 months p= 0.021). Graph represents estimated marginal means + SE (standard error).

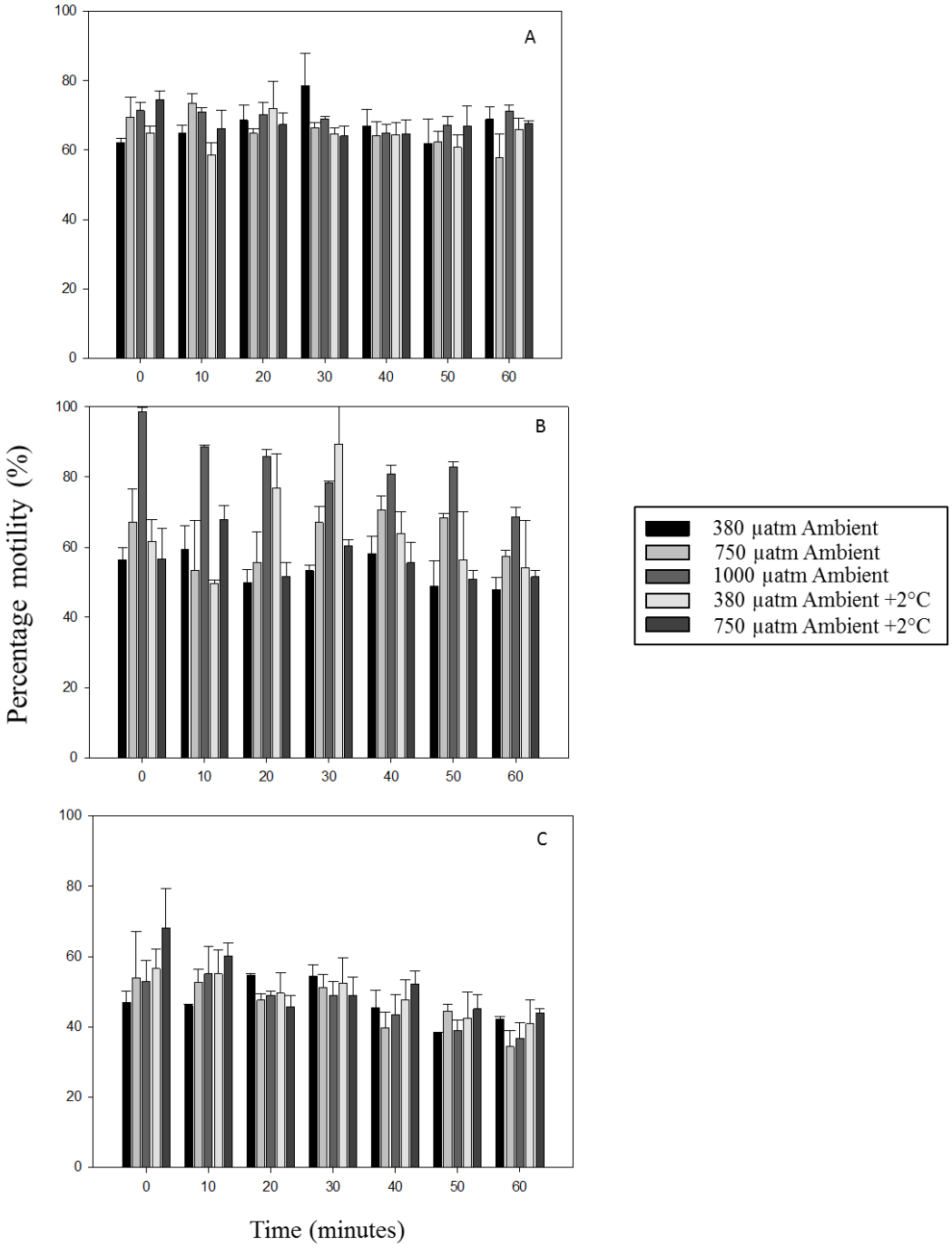

Figure 2.3: The percentage motility of sperm samples at A) 3 months, B) 6 months and C) 12 months. After 3 months exposure there was no significant effect of pCO_2, temperature or time on percentage of motile sperm, however after 6 months exposure sperm percentage motility was significantly increased under increased pCO_2 (p=<0.005), time also had a significant effect (p=0.013) and there was a significant interaction between temperature and pCO_2 (p=0.002). After 12 months exposure there was a significant effect of temperature (p=0.049) and time (p=<0.005), however pCO_2 had no significant impact on percentage motile sperm (p=0.826). Graph represents estimated marginal means + SE.

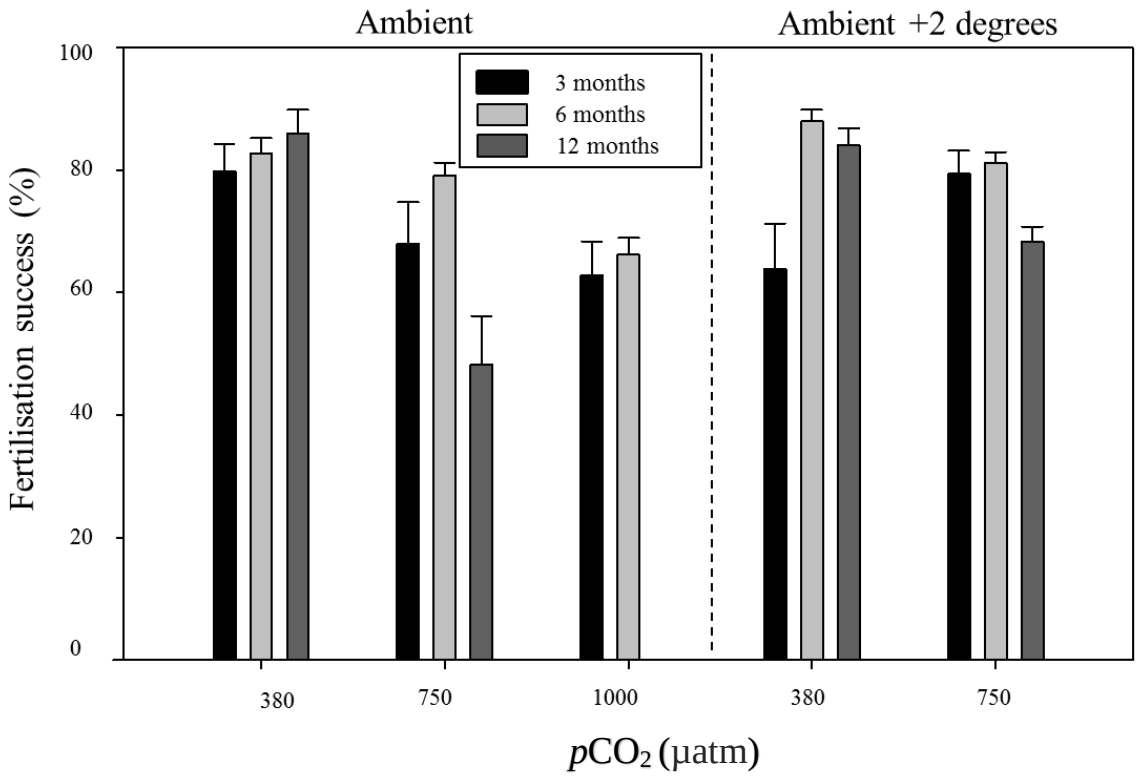

Figure 2.4: The effects of increased pCO_2 and temperature on fertilisation success after 3, 6 and 12 months exposure to increased pCO_2 and increased temperature. There is a significant effect of pCO_2 after 3 (p= <0.001), 6 (p=<0.001) and 12 months (p= <0.001). Temperature is not a significant factor after 3 months (p= 0.244), however there is a significant effect of temperature on fertilisation success after both 6 months and 12 months (6 months p=<0.005; 12 months p= 0.029), as well as sperm density being a significant covariate (3 months p= <0.005; 6 months p=<0.005; 12 months p= 0.029). There is also a significant interaction between pCO_2 and temperature at all-time points (3 months p=<0.005; 6 months p=0.034; 12 months p= 0.002). No data were obtained for 1000µatm at 12 months as no mature females were present. Graph represents estimated marginal means ± SE.

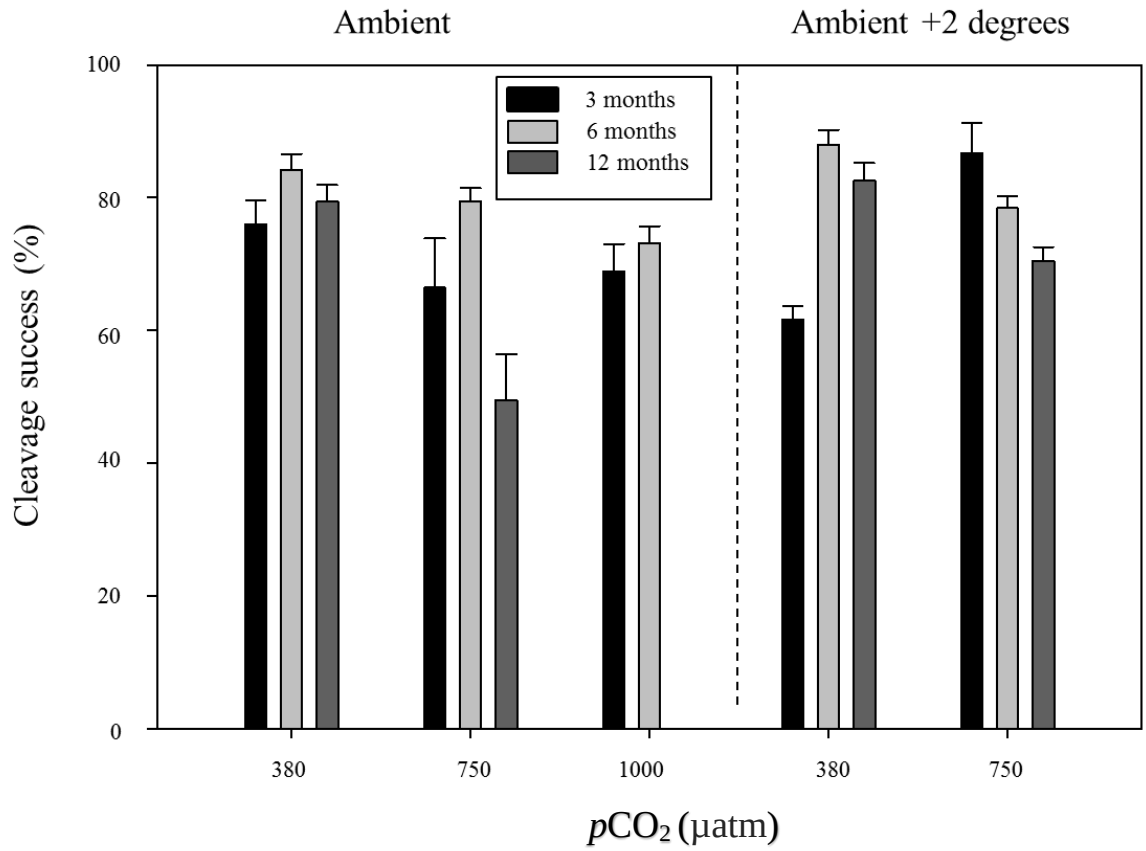

Figure 2.5: The effects of increased pCO_2 and increased temperature on the cleavage success, after 3, 6 and 12 months exposure times. There is a significant effect of pCO_2 on cleavage success after 3, 6 and 12 months (3 months p= 0.006; 6 months p=<0.005; 12 months p= <0.005). Temperature is a significant factor at 12 months but not at 3 and 6 months (3 months p= 0.070; 6 months p=0.111; 12 months p= <0.005), as well as sperm density being a significant covariate (3 months p= <0.005; 6 months p=<0.005; 12 months p= <0.005). There is also a significant interaction between CO_2 and temperature at 3, 6 and 12 months (3 months p= <0.005; 6 months p=0.009; 12 months p= 0.007). No data were obtained for 1000µatm at 12 months as no mature females were present. Graph represents estimated marginal means ± SE.

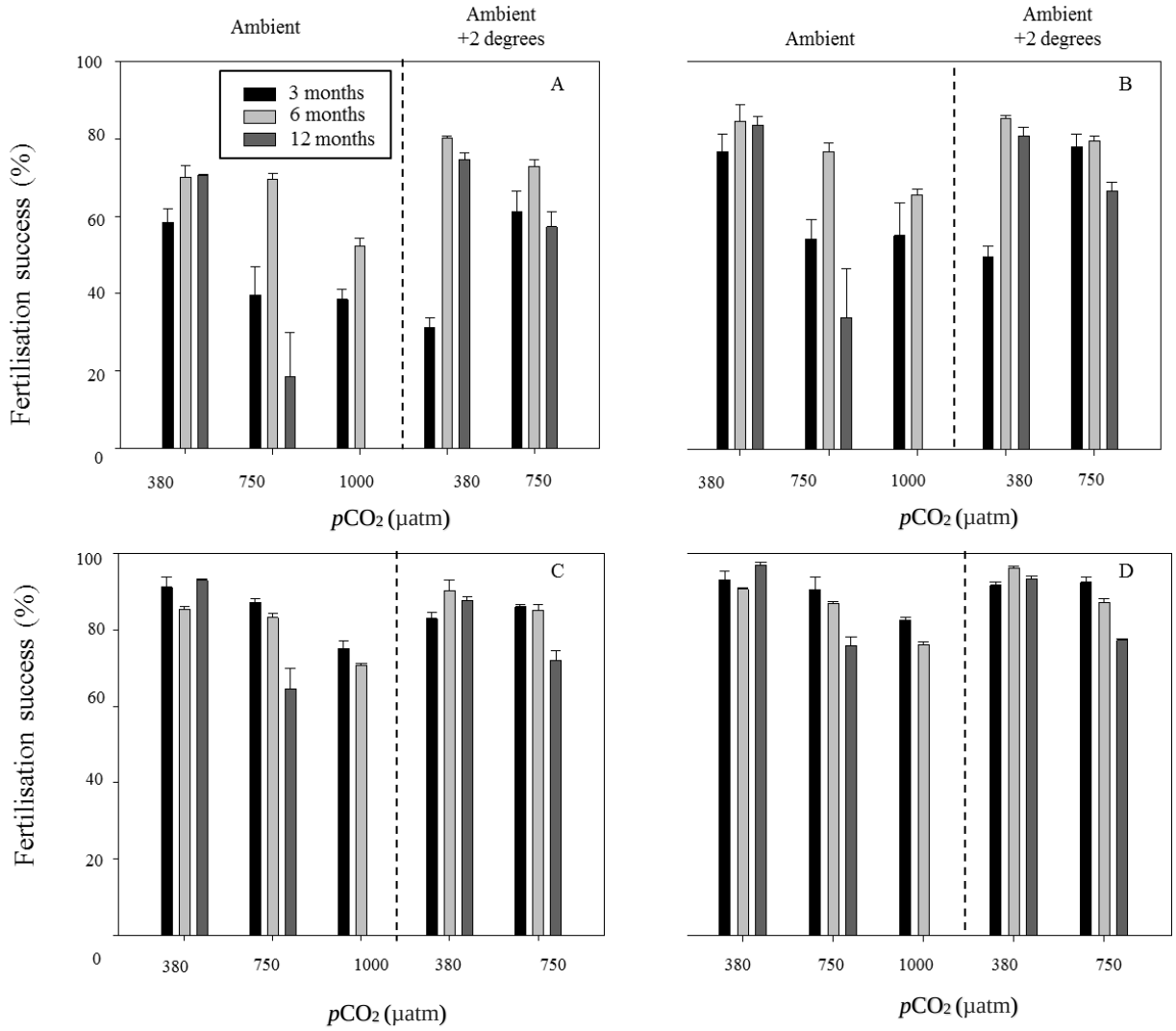

Figure 2.6: Percentage fertilisation success at differing sperm densities: A) 10^3, B) 10^4 C) 10^5 D) 10^6. Treatment and sperm density have a significant effect on fertilisation success at 3months (treatment p==<0.005; sperm density p=<0.005), 6 months (treatment p==<0.005; sperm density p=<0.005), and 12 months. At 3 months exposure there is a significant interaction between sperm density and treatment (p=0.012). Graph represents estimated marginal means ± SE.

1.1. :

Table 2.2: Total alkalinity values for all data across all time points for 5 treatments, A = Ambient

Treatment	Time Point (months)	TA (umol kg-1)	pH	Temp	Salinity	DIC (umol kg-1)	pCO2 (uatm)	Omega Cal	Omega Arg	HCO3 (umol kg-1)	CO3 (umol kg-1)
380 A	0 - 3	2131	8.02±0.09	10.49±0.71	34.33	2003	532	2.32	1.48	1883.34	97.07
750 A	0 - 3	2097	7.93±0.09	10.53±0.73	34.49	2003	663	1.89	1.20	1895.44	79.00
1000 A	0 - 3	2355	7.81±0.10	9.66±0.91	34.53	2298	1004	1.61	1.02	2186.14	67.36
380 A+2	0 - 3	2357	7.97±0.09	11.84±0.85	34.90	2233	679	2.43	1.55	2103.42	101.85
750 A+2	0 - 3	2403	7.90±0.09	12.04±0.95	34.97	2300	816	2.18	1.39	2174.98	91.53
380 A	3 - 6	2172	7.94±0.12	11.37±1.17	35.20	2060	665	2.16	1.38	1941.57	90.70
750 A	3 - 6	2091	7.86±0.10	11.60±1.21	35.21	2011	791	1.72	1.10	1905.01	72.42
1000 A	3 - 6	2352	7.75±0.09	11.03±0.36	35.33	2303	1137	1.53	0.98	2190.17	64.36
380 A+2	3 - 6	2361	7.92±0.09	12.85±1.42	35.33	2249	771	2.29	1.47	2120.75	96.41
750 A+2	3 - 6	2239	7.80±0.10	12.84±1.38	35.36	2169	991	1.71	1.09	2057.20	71.75
380 A	6 - 9	2255	8.08±0.03	15.04±0.90	34.98	2074	483	3.18	2.04	1922.43	133.40
750 A	6 - 9	2183	7.93±0.09	15.66±0.65	34.89	206	722	2.31	1.49	1939.54	96.98
1000 A	6 - 9	2251	7.79±0.05	15.63±0.37	34.91	2177	1029	1.79	1.15	2064.01	74.82
380 A+2	6 - 9	2112	8.01±0.07	17.93±0.38	35.16	1950	568	2.83	1.83	1812.23	118.49
750 A+2	6 - 9	2170	7.86±0.11	17.90±0.16	35.07	2062	872	2.17	1.40	1940.72	91.07
380 A	9 - 12	2238	8.06±0.04	13.06±1.55	34.94	2080	508	2.83	1.81	1940.61	118.65
750 A	9 - 12	2229	7.94±0.07	12.89±1.60	34.80	2117	694	2.21	1.41	1996.40	92.64
1000 A	9 - 12	2219	7.78±0.09	12.73±1.74	34.90	2157	10156	1.60	1.02	2049.70	67.11
380 A+2	9 - 12	2166	8.03±0.06	15.55±1.96	34.89	2006	537	2.81	1.81	1868.37	117.94
750 A+2	9 - 12	2165	7.93±0.08	15.63±1.75	35.07	2041	695	2.32	1.50	1918.22	97.54

Table 2.3: Results of a paired t test for differences between elevation of the fertilisation membrane and early (2-4cell) cleavage success as used as a measure of fertilisation success. Significant results are indicated by (*)

Treatment	Time point	Mean	Standard error	Degrees of freedom	Significance
380 µatm Ambient	3 months	3.59835	2.22	11	0.133
750 µatm Ambient	3 months	-1.16917	1.24	11	0.366
1000 µatm Ambient	3 months	1.99555	2.47	11	0.436
380 µatm High	3 months	-0.26870	0.76	11	0.731
750 µatm High	3 months	-3.73961	1.04	11	0.004*
380 µatm Ambient	6 months	-4.44412	0.73	11	<0.001*
750 µatm Ambient	6 months	1.48714	1.16	11	0.227
1000 µatm Ambient	6 months	-0.13911	0.74	11	0.854
380 µatm High	6 months	-5.22949	1.10	11	0.001*
750 µatm High	6 months	2.01859	0.89	11	0.044*
380 µatm Ambient	12 months	6.19348	1.72	7	0.009*
750 µatm Ambient	12 months	-1.07556	1.86	11	0.575
1000 µatm Ambient	12 months	1.28448	0.49	7	0.034*
380 µatm High	12 months	-1.34086	0.84	11	0.139

2.4 Discussion

This study addresses the issue of long-term exposure to elevated pCO_2 and temperature on reproductive fitness and success of the sea urchin *P. lividus*. The findings suggest that long-term exposure to increased pCO_2 will have significant effects on the reproductive fitness of *P.lividus*. The 3- and 12-month time points were used as a measure of reproductive capacity, as opposed to reproductive success as they fall outside the normal breeding season for *P. lividus* on the west coast of Ireland (Durrus, Bantry, Co. Cork) (Byrne, 1990).

To the author's knowledge, no previous studies have concentrated on the effect of adult exposure to increased pCO_2 on sperm motility and fertilisation success, though much previous work had been carried out on short-term exposures. In terms of sperm motility, there was a significant effect of pCO_2, temperature and exposure time on the sperm VCL and percentage sperm motility. After 6 month's exposure there was a significant increase in sperm VCL compared to at 3 and 12 months. This time point fell within the normal reproductive cycle of the sea urchin and afforded optimal temperature conditions for reproduction. The increase in both sperm motility and VCL suggests that the higher temperature from summertime conditions was not the upper thermal limit of the Irish population of *P.lividus.* After long-term exposure (12months) there was no longer a significant increase in VCL at increased pCO_2 suggesting that sperm activation was negatively affected. Previous research has shown that sperm percentage motility and swimming speed are affected by changes in pCO_2 (Havenhand et al., 2008; Havenhand and Schlegel, 2009; Frommel et al., 2010; Morita et al., 2010; Caldwell et al., 2011; Lewis et al., 2012; Schlegel et al., 2012), but only one study has shown an increase in sperm motility with decreasing pH (Caldwell et al., 2011). Sperm activation pH has been used previously to explain variations in sperm swimming speeds at different levels of ocean acidification (Caldwell et al., 2011). This process involves sperm storage at a lower pH (pH 7.2), which is below the activation threshold of sperm dynein ATPase which powers the flagellum (Johnson et al., 1983). Once the sperm is released into the water column, the pH is increased to the activation pH (pH 7.6), which causes the flagellum to activate and mitochondrial respiration to be initiated (Christen et al., 1983).

A negative impact of chronic exposure to aquatic contaminants on sperm morphology, including size, has been reported, (Sopinka et al., 2012). Similarly when sperm from *Oreochromis niloticus* were released into contaminated water (cadmium and lead), morphological deformities of the sperm flagella were noted. These deformities could decrease sperm motility with a knock-on effect on fertilisation success (Shalaby and Migeed, 2012). Similar effects have been observed for *Danio rerio* after a 2-month exposure to increased concentrations of DDT and Arochlor1254. The changes to swimming speed seen in the present study are consistent with the previous reports. Sperm morphology dictates the ability of the sperm to swim and if this is altered, sperm swimming speed will be impacted.

Increased temperature reportedly has a variable effect on sperm VCL and percentage motility. For example Caldwell et al. (2011) did not find a significant effect of elevated temperature and CO_2 on sperm motility. There was, however, a significant effect of temperature on VCL. The current study showed that after six months exposure, temperature significantly increased sperm motility, with motility increasing at 750µatm ambient +2°C. The six month time point fell in to the peak spawning season for *P. lividus* (Byrne, 1990) and therefore the optimal breeding conditions were present. This may help to explain the increase seen as individuals reproductive output. The increases in sperm VCL could have ramifications on sperm longevity. Sperm that swim faster use stored energy more rapidly and are therefore motile for less time. After 12 months exposure there was a significant effect of temperature on sperm motility but *p*CO2 had no effect. Sperm motility also decreased with time, with corresponding effects on sperm longevity. Previous studies have shown that when temperature alone is varied, sperm longevity is not significantly affected. This is because although higher temperature increases sperm metabolism, there is a corresponding decrease in water viscosity, which enables the sperm to swim more easily and enhances the likelihood of fertilisation (Naud and Havenhand, 2006). A reduction in sperm motility in the present study was coupled with a significant reduction in fertilisation success, particularly after 12 months exposure, possibly due to the altered morphology of the sperm leading to lower motility. The initial increase in sperm motility after three and six months may occur as a response to the initial stress of exposure to increased OA conditions, and this may eventually lead to detrimental effects on later life-history stages (Pineda et al., 2012).

The previous two long-term studies on echinoderms (Dupont et al., 2012; Uthicke et al 2012) did examine the effects of OA, as a single stressor, on egg

fecundity. The current study found that after 3, 6 and 12 months exposure to increased pCO_2 there was a significant decrease in the fertilisation success of *P. lividus*, and this difference was most pronounced after 12 months. There are several possible explanations for the reduction in fertilisation success at higher pCO_2. First, as found by Kurihara and Shiryiama (2004), lower pH may cause a delay in development. Their study, however, involved short-term exposure to very high levels of acidification (5000 - 10,000 µatm, pH 7.0 -6.8), which are not ecologically relevant. Effects on development may be evident at. pH 7.6 in chick embryos which found that when eggs are exposed to lowered pH values there is a delay in the rate of cell multiplication as well there being a decrease in the rate that the radioactive nucleoside, thymidine^{-3}H, was incorporated into DNA (Rubin, 1971). The significant reduction in fertilisation success is also linked to gonad growth and it has been stated that increased pCO_2 has a negative impact on gonad growth (Siikavuopio et al., 2007; Moulin et al., 2011). This reduction in gonad growth would support the results obtained in the current study as after 12 month exposure to increased OA there were no viable females at the highest pCO_2 (1000 µatm) and when the gonads were removed they appeared shrivelled and a different colour to healthier gonads. The results from the current thesis correspond with unpublished data by Kurihara et al., which showed that when the sea urchin *Hemicentrotus pulcherrimus* was reared at pH 7.8 for 10 months there was a delay in gonadal development and the developmental period of *H. pulcherrimus* was shortened to almost half (Kurihara, 2008).

It has also been suggested that a reduction in fertilisation success can be explained due to a slowing of the fast block to polyspermy, and that an increase in pHe and pHi in the unfertilised eggs interferes with the Na^+/H^+ exchange. This ion exchange prevents the fertilisation membrane from being raised (Reuter et al., 2011). More recently it has been indicated that there is a negative carry-over effect of parental exposure to increased pCO_2, exhibited by a reduction in egg fecundity (Dupont et al., 2012). It is possible that the results of the current study support the results of the previous study by way of reduction in fertilisation success, this could be due not only to developmental delay but due to the increased energy demands associated with increasing OA. The increased energy required at an early stage could have a knock on effect at later stages, as less energy is available for future developmental processes (Dupont et al., 2012). This may be further exacerbate due to changes in seaweed palatability which may occur due to increased pCO_2 (Poore et al., 2013)

The current study reports fertilisation success was significantly affected by temperature after 6 and 12 months adult exposure. Similarly there was a significant effect of temperature on cleavage success reported after 12 months parental exposure. Both fertilisation and cleavage success had interacting effects of pCO_2 and temperature at 3, 6 and 12 months. It has been suggested that echinoderm species are robust to increased temperatures and that such increases can enhance rates of fertilisation and cleavage success. These increases in fertilisation success reflect a combination of enhanced sperm motility, as a result of lower viscosity of seawater, and high thermal tolerance of eggs (Byrne, 2012). This thermo-tolerance has previously been attributed to maternal factors, which are imprinted by ovary temperature (Fujisawa, 1995; Yamada and Mihashi, 1998; Byrne et al., 2009). It is therefore possible that when parents (mother) are incubated at increased temperatures prior to spawning, eggs may have a greater thermal tolerance than those spawned from un-acclimated individuals. This concept corresponds to the results seen in this study; as after long-term incubation fertilisation success in higher temperatures was greater than at lower temperatures at corresponding pCO_2 treatments.

The relationship between fertilisation success and sperm density has previously been well documented for echinoderm species (Levitan et al., 1991). Many of the previous OA studies concerning fertilisation success have used an optimal sperm density, which can generate misleading results (Schlegel et al., 2012). However, an increasing number of studies have considered the effects that varying sperm densities will have on fertilisation success under future ocean acidification scenarios and these studies have provided contradictory results (Byrne et al., 2010a; Ericson et al., 2010; Reuter et al., 2011; Gonzalez-Bernat et al., 2013). The results of the current study show a significant reduction in fertilisation success under increased pCO_2 conditions and the effect is intensified at lower sperm densities (Figure 2.6).

In general, echinoderms may be robust to projected changes in oceanic pH (Dupont et al., 2009). However certain echinoderm species and life history stages appear to be more vulnerable than others. It is also true that inconsistent results have been obtained for the same species. For example, the current study found that there was a significant effect of pCO_2 on fertilisation success in *P.lividus*, consistent with Moulin et al. (2011). However Martin et al. (2011), utilising the same species, reported that lowered pH had no effect on fertilisation success. The inconsistencies could be attributable to changes in methodology. Martin et al. (2011) incubated eggs and sperm for 2 hours at 20°C and measured fertilisation success as elevation of the fertilisation

membrane or polar body cleavage. Moulin et al. (2011), on the other hand, allowed 15 minutes for elevation of the fertilisation envelope and 1 hour for cleavage at 11.4 - 15°C.

The results of the current study suggest that after long-term exposure to increased *p*CO2, sperm motility, sperm VCL, fertilisation success and cleavage success will be significantly negatively affected, with all of the endpoints showing significant reductions. It is also apparent that the increase in temperature shows a mediating effect in terms of fertilisation and cleavage success. The current laboratory based study strongly suggests that reproduction of *P.lividus* will be significantly affected by increasing oceanic pCO_2. These reductions seen in lab experiments are in accord with recent field based experiments which have shown a reduction in the abundance of *P.lividus* around volcanic vents (Hall-Spencer et al., 2008; Calosi et al., 2013). A reduction in sea urchin abundance due to OA could have serious ramifications on the reproductive success of the species as well as the wider ecosystem, as sea urchins are an important ecosystem engineer occupying a keystone position in many marine ecosystems. This study highlights the need for further long term studies especially in to the reproduction and development of marine invertebrates. It is also important for future research to determine whether population level effects are wholly or in part attributable to impacts on reproduction.

Chapter 3: Developmental stability of the sea urchin *Paracentrous lividus* to increased pCO2 and temperature

3.1 Introduction

There has been a significant body of work carried out in to the effects of climate change on marine invertebrate. Many previous short term studies have shown that increases in environmental stresses such as, *p*CO2 and temperature, will have a negative impact on species reproduction and development. (Fabry et al., 2008; Parker et al., 2009; Talmage and Gobler 2009; Dupont et al., 2010a; Dupont et al., 2010b; Chan et al., 2011; Lischka et al., 2011; Martin et al., 2011; Moulin et al., 2011; Byrne et al., 2012; Catarino et al., 2012; Doo et al., 2012; Ericson et al., 2012; Nguyen et al., 2012). Larval development, in particular developmental fitness of invertebrate larvae is crucial as it plays a large role in survival and settlement.

A tool used to determine the developmental fitness (the chance of an organisms surviving development to have the chance of passing genes to the next generation) of invertebrate larvae is fluctuating asymmetry (FA). This term is applied to subtle and random variations from perfect bilateral symmetry (Palmer and Strobeck, 1992; Palmer, 1996; Palmer and Strobeck, 1997; Vollestad and Hindar, 2001; Lewis et al., 2004; Caldwell et al., 2005; Graham et al., 2010). It is used as a measure of developmental noise (Lewis et al., 2004; Graham et al., 2010) and it can also be a measure of the developmental stability of an organism (Palmer and Strobeck, 1992; Palmer, 1996; Vollestad and Hindar, 2001; Lewis et al., 2004; Graham et al., 2010). Developmental stability reflects an organism's ability to achieve a genetically predetermined optimum phenotype under any environmental conditions (Vollestad and Hindar, 2001; Lewis et al., 2004). Disruptions in developmental stability and therefore deviations from perfect bilateral symmetry can reflect an individual's ability to cope with environmental stressors (Vollestad and Hindar, 2001; Lewis et al., 2004; Graham et al., 2010). Due to this, developmental stability, and especially fluctuating asymmetry, can be used as a measure of phenotypic and genetic quality (Thornhill and Moller, 1997).

Changing oceanic conditions, such as OA, represents an environmental stressor. When exposed to these conditions it is often necessary to redirect energy from processes that are not necessary to the organism's immediate health, such as maintaining perfect bilateral symmetry, to more crucial processes. This change in energy allocation can lead to a reduction in developmental stability of invertebrate larvae (Palmer, 1996). Changes in the symmetry of an individual suggest an inability to buffer from environmental stressors (Lewis et al., 2004; Graham et al., 2010).

Previous investigations have suggested that increasing levels of ocean acidification will cause changes in morphology including symmetry of marine invertebrates (Dupont et al., 2008; Byrne et al., 2009; Byrne et al., 2011a; 2011b; Crim et al., 2011; Martin et al., 2011; Catarino et al., 2012; Dorey et al., 2013). So far the effects of increasing OA on developmental stability have not been examined through use of fluctuating asymmetry. The study of developmental stability is important as it is essential for the continued growth and development of marine invertebrate larvae. This is especially relevant when looking at early developmental stages of organisms, as small fluctuations in symmetry may have greater effects on organisms at this stage, and can cause changes in development at later stages.

The aim of the current study is to assess the effects of increasing temperature and CO_2 on developmental stability, through use of fluctuating asymmetry index, on the ecologically and economically important sea urchin *Paracentrous lividus* (Martin et al 2011; Moulin et al., 2011). *P. lividus* has a broad distribution in the Mediterranean as well as along the NE Atlantic coast from Morocco to Ireland; it is also a keystone species and the dominant grazer in many shallow benthic marine environments along its distribution. It also is an important prey species for many larger marine animals (Boudouresque and Verlaque, 2001; Martin et al 2011). The life history of *P.lividus* is also well documented and allows for comparison between studies.

3.2 Methods

For animal husbandry and carbonate chemistry please see Chapter 2. Sections 2.2.1 and 2.2.2, and Figure 2.2.

3.2.1 Spawning induction

Sea urchins, *P. lividus*, were acclimated for six months prior to experimentation. Individuals were collected at random from experimental tanks (four per treatment and five experimental treatments) and spawning was induced through intra-coelomic injection of 1.0ml of 0.5 M KCl. Gametes were collected from the first three individuals of each sex to spawn. Sperm was collected dry (i.e. undiluted) and stored on ice for no more than two hours. Females were left to express eggs for 1 hour into control and experimental treatments comprising 200ml of FSW (0.22μm filtered) adjusted to a range of CO_2 (380, 740, and 1000 μatm and at ambient and ambient +2°C) and then removed.

3.2.2 Fertilisation

Sperm density was determined by haemocytometer counts and mean egg density was calculated from 3 x 50 μL aliquots of eggs suspended in 200ml of FSW. An egg density of 5 eggs per ml was added to 250ml of 0.22μm FSW and a sperm density of 2.5 x $10^5 ml^{-1}$ was added; these amounts had been predetermined prior to experimentation. Fertilisation success had been checked previously to ensure the eggs and sperm were viable. After 1 h the water level was increased to a total volume of 1 L with the appropriate CO_2 adjusted seawater and covered with parafilm. Continuous aeration of pCO_2-adjusted air was added to the beakers (three per treatment). Beakers were then incubated at ambient and ambient +2°C. Higher experimental temperatures were maintained through use of water baths, with the temperature monitored daily. Water changes (25%) were performed daily using seawater that had been adjusted to the relevant pCO_2 to avoid salinity levels becoming too high. Larvae were raised until the echinopleutus stage was reached, (96 hours at ambient temperature; 72 hours at ambient +2°C).

3.2.3 Fluctuating asymmetry

Fluctuating asymmetry analysis was conducted on echinopleutus larvae using the procedures outlined by Lewis et al. (2004) and Caldwell et al. (2004). In brief, fluctuating asymmetry was determined after 72 (ambient temperature +2°C), and 96 hours (ambient temperature). Larvae were collected and fixed in 70% ethanol. Between 5 and 10 individuals were pipetted, using a Pasteur pipette, onto concavity slides and a coverslip added. Excess water was removed with absorbent blue roll. Measurements were taken on 30 individuals and then averaged. Individuals that showed gross abnormalities, which did not allow for all measurements to be taken, were discounted. Four measurements were used: full length (FL); body rod length (BR); post-oral arm length (POR); and anterolateral rod length (AR) (Figure 3.1). Measurements were taken on both the left and the right side and then the difference between the two compared. Asymmetry analysis was conducted using Palmer's online fluctuating asymmetry calculations worksheet (version 11) (http://www.biology.ualberta.ca/palmer/asym/FA/FA-refs.htm#tools). Briefly tests for anti-symmetry and directional asymmetry were performed using right minus left data (R-L). Skew and kurtosis values with standard error were determined and compared to the expected value (zero for a normal distribution) using an independent t-test. A one-way ANOVA was performed to test for directional asymmetry; this compared mean left to right measurements for all traits. A sequential Bonferroni correction was performed to protect against false positives and type 1 error.

Further measurements of body length (BL), body width (BW), cavity length (CL) and cavity width (CW) were made to assess variation in these morphological traits under increasing pCO_2 and temperature conditions. These measurements were not, however, suitable FA analysis. A test for normality (Kolmogorov-Smirnov test) determined that the data were normally distributed so a two-way ANOVA was applied with pH and temperature as fixed factors. Significant differences within and between pCO_2 treatments and temperature were made using pairwise comparisons generated by estimated marginal means. Length measurements (L) were taken and the ratio of POR (r+l/2): L was calculated to determine total larval skeleton and length of feeding structure relative to larval size. This measurement can be used as an index for biomineralisation (O'Donnell et al., 2010).

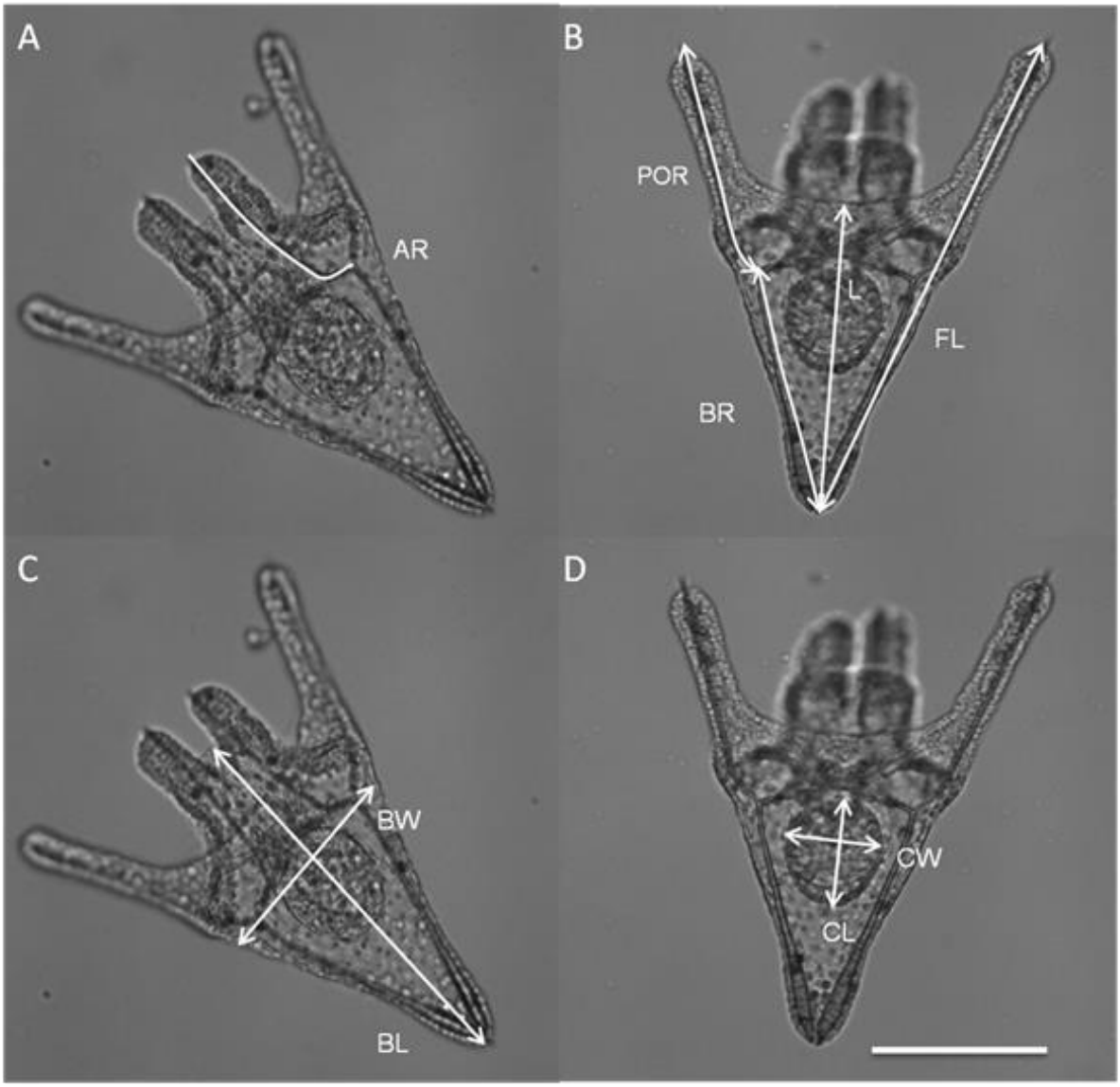

Figure 3.1: The range of morphometric measurements taken from each echinopluteus larvae: AR= anterolateral rod; POR=post oral rod; BR=body rod; L= length; FL= full length; BL= body length; BW= body width; CL= cavity length; CW cavity width. Scale bar = 100 μm

3.3 Results

Analysis of morphological measurements for *P. lividus* plutei revealed a significant effect of elevated pCO_2 on trait size compared with the ambient treatment (Table 3.2). Both pCO_2 and temperature had significant impacts on: body length (pCO_2 P=<0.001; temperature P=<0.001); body width (pCO_2 P=<0.001; temperature P=0.003); cavity length (pCO_2 P=<0.001; temperature P=<0.001); cavity width (pCO_2 P=<0.001; temperature P=<0.001); full length (pCO_2 P=<0.001; temperature P=<0.001); body rod (pCO_2 P=0.001; temperature P=0.002); post-oral rod (pCO_2 P=<0.001; temperature P=<0.001); and anterolateral rod length (pCO_2 P=<0.001; temperature P=0.026) (Table 3.1). Moreover, for all traits except body width there was a significant interaction of pCO_2 and temperature (BL p=0.000; BW p=0.139; CL=0.000; CW p=0.000; FL p=0.000; BR p=0.001; POR p=0.009; AR p=0.000). There was a significant increase in body length when compared to ambient at 1000µatm and 380µatm ambient +2°C and a decrease at 750µatm ambient and 750µatm ambient +2°C with the same pattern seen for body width. Both cavity length and width showed a significant decrease in size at higher pCO_2 and at higher temperatures.

Echinopleutus full length and body rod length showed an increase at 750µatm and 750µatm +2°C, (750µatm FL – p= 0.250; BR – p= 0.069; 750µatm +2°C, FL – p= 0.112; BR – p= 0.102), which was not significant. A significant increase did, however, occur at 1000µatm and 380µatm +2°C, = (FL - p<0.001, p<0.001; BR - p<0.001, p<0.001). There was no significant increase in post-oral rod length at 750µatm or 750µatm +2°C (p=0.608; p=0.517) but there was a significant increase at 1000µatm and 380µatm +2°C (p= <0.001; p= <0.001). Anterolateral rods were significantly longer at 1000µatm and 380µatm +2°C when compared to ambient (p= <0.001; p= <0.001) and there was a significant decrease in size at 750µatm ambient and 750µatm +2°C (p= 0.000, p=0.000).

There was no significant directional asymmetry for any of the treatments assayed (data not shown). FA indexes (Table 3.3) were greater under increased pCO_2 and temperature conditions (750µatm ambinet+2°C), except for anterolateral rod length where the greatest FA index value was in 380µatm ambient +2°C. Larvae in the latter treatment also had the lowest FA index value, followed by 380µatm ambient. FA indexes for 750µatm and 1000µatm remained high for all treatments. Percentage gross abnormalities (Table 3.3) were highest in the 750µatm +2°C treatment, followed by

750µatm at ambient and 1000µatm ambient; this pattern is similar to that reflected in the FA indexes (Table 3.1).

Univariate analysis of BHL(r+l/2): L showed a significant effect of CO_2 ($p=<0.001$), but no significant effect of temperature ($p=0.539$) and no significant interaction between temperature and CO_2 ($p=0.231$). The larval skeletons were larger than under ambient conditions (figure 3.5).

Table 3.1: ANOVA table for all traits measured in combination with pCO2 and temperature

	Df	Sum squared	Mean squared	F value	P (>f)
Body length					
pCO_2	2	130655.470	65327.735	88.119	<0.001
Temperature	1	28596.047	28596.047	35.573	<0.001
pCO_2*temperature	1	17099.014	17099.014	23.064	<0.001
Residuals	745	552311.708	741.358		
Body width					
pCO_2	2	126392.664	63196.332	278.283	<0.001
Temperature	1	2002.476	2002.476	8.818	0.003
pCO_2*temperature	1	497.948	497.948	2.193	0.139
residuals	745	169184.754	227.094		
Cavity Length					
pCO_2	2	27266.375	13633.188	123.907	<0.001
Temperature	1	4354.404	4354.404	39.576	<0.001
pCO_2*temperature	1	2230.053	2230.053	20.268	<0.001
residuals	745	81970.372	110.027		
Cavity width					
pCO_2	2	16308.819	8154.410	77.844	<0.001
Temperature	1	2046.979	2046.979	19.541	<0.001
pCO_2*temperature	1	3097.020	3097.020	29.565	<0.001
residuals	745	78040.815	104.759		
Full length					
pCO_2	2	294676.037	147338.018	96.078	<0.001
Temperature	1	27342.968	27342.968	17.830	<0.001
pCO_2*temperature	1	19870.346	19870.346	12.957	<0.001
residuals	745	1142476.672	1533.526		
Body rod					
pCO_2	2	6196.560	3098.280	7.008	0.001
Temperature	1	4276.886	4276.886	9.674	0.002
pCO_2*temperature	1	5016.203	5016.203	11.346	0.001
residuals	745	329370.141	442.108		
Post-oral rod					
pCO_2	2	201884.778	100922.389	168.315	<0.001
Temperature	1	10935.214	10935.214	18.237	<0.001
pCO_2*temperature	1	4144.671	4144.671	6.912	0.009
residuals	745	446706.141	599.606		
Anterolateral rod					
pCO_2	2	295192.795	1457596.397	264.795	<0.001
Temperature	1	276.538	2766.538	4.963	0.026
pCO_2*temperature	1	24412.688	24412.688	43.798	<0.001
residuals	745	415262.054	557.399		

Table 3.2: ANOVA P values for trait size compared with ambient at three pCO2 treatments and two temperature treatments. FL = full length, BR = body rod, POR = post-oral rod, AR = anterolateral rod, BL = body length, BW = body width, CL = cavity length, and CW = cavity width.

pCO_2 (µatm)	**Temp (°C)**	**FL**	**BR**	**POR**	**AR**	**BL**	**BW**	**CL**	**CW**
750	A	0.250	0.069	0.608	<0.001	0.035	<0.001	<0.001	<0.001
1000	A	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
380	A+2°C	<0.001	0.000	0.006	<0.001	<0.001	0.293	<0.001	<0.001
750	A+2°C	0.112	0.102	0.517	<0.001	0.266	<0.001	<0.001	<0.001

Table 3.3: Mean FA index value ±SD for *P. lividus* echinopleutus larvae under varying pH and temperature treatments. See table 3.2 for abbreviations.

pCO_2 (µatm)	Temp. (°C)	FL	P	BR	P	POR	P	AR	P
380	A	79.94 ± 11.5	<0.001	2.2± 4.4	<0.001	85.26± 13.0	<0.001	59.3± 10.4	<0.001
750	A	211.18± 17.9	<0.001	24.91± 7.0	<0.001	120.95± 15.4	<0.001	48.84± 8.1	<0.001
1000	A	95.42± 15.3	<0.001	42.34± 7.1	<0.001	120.83± 13.1	<0.001	53.54±15.3	<0.001
380	A+2	6.09± 10.2	<0.001	1.59± 8.3	0.030	29.53± 4.6	<0.001	180.81±10.1	<0.001
750	A+2	237.86± 18.1	<0.001	219.72±3.6	<0.001	106.35± 10.4	<0.001	61.41± 9.6	<0.001

Table 3. 4; Average larval trait measurements (µm) with ± standard error at varied pCO_2 and temperature treatment, n=30

Treatment	FL	SE	BR	SE	POR	SE	AR	SE	BL	SE	BW	SE	CL	SE	CW	SE
380A	313.72	6.38	188.55	3.88	128.75	3.21	136.51	5.00	276.46	5.75	123.95	2.10	102.98	2.34	81.74	2.54
750A	318.93	6.05	192.97	3.01	127.30	3.84	114.96	3.44	269.83	4.37	104.73	2.33	88.96	1.27	67.86	1.39
1000A	370.06	7.55	198.45	4.11	172.19	4.91	160.19	4.10	302.43	4.78	138.83	3.25	85.12	1.95	69.09	1.82
380A+2	338.73	7.87	199.67	3.23	142.54	5.71	153.56	5.64	300.94	5.30	122.12	3.76	93.74	2.28	73.50	1.95
750A+2	318.23	9.37	191.01	5.22	129.27	4.96	104.84	3.35	264.62	9.29	99.25	1.80	74.47	2.12	68.13	1.30

Table 3 5: The percentage of individuals that exhibited gross abnormality (phenodeviants) in each treatment.

pCO_2 (ppm)	**Temp (°C)**	**Percentage gross deformity (%)**	**Other (%)**
380	15.5±0.5	12	88
750	15.5±0.5	18	82
1000	15.5±0.5	18	82
380	18±1.0	14	86
750	18±1.0	21	79

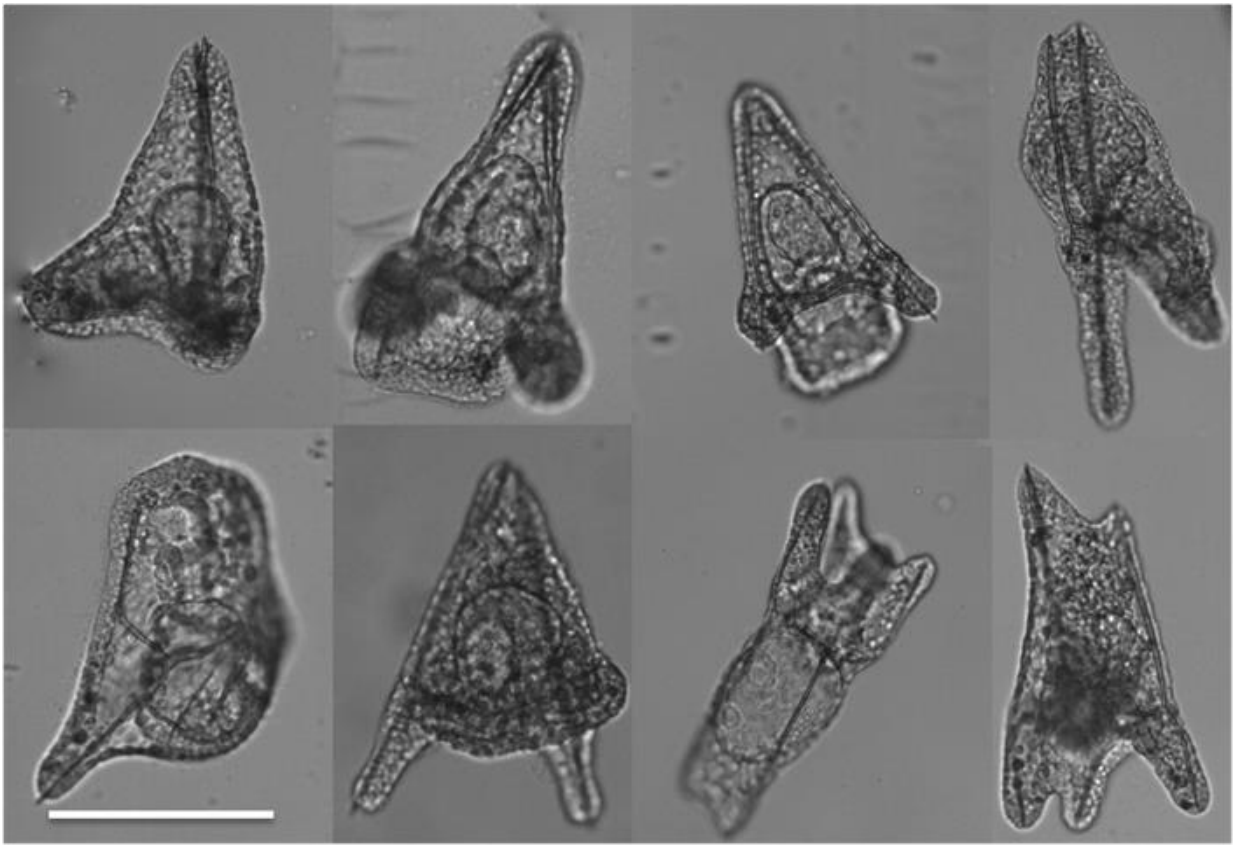

Figure 3.2: Examples of phenodeviant larvae exhibiting gross morphological abnormalities at varying CO_2. Phenodeviants show morphological deformities that were too extensive to permit the application of fluctuating asymmetry analysis. Such individuals are unlikely to survive to the settlement stage and the abnormalities may negatively impact feeding and swimming behaviour. Scale bar = 100µm

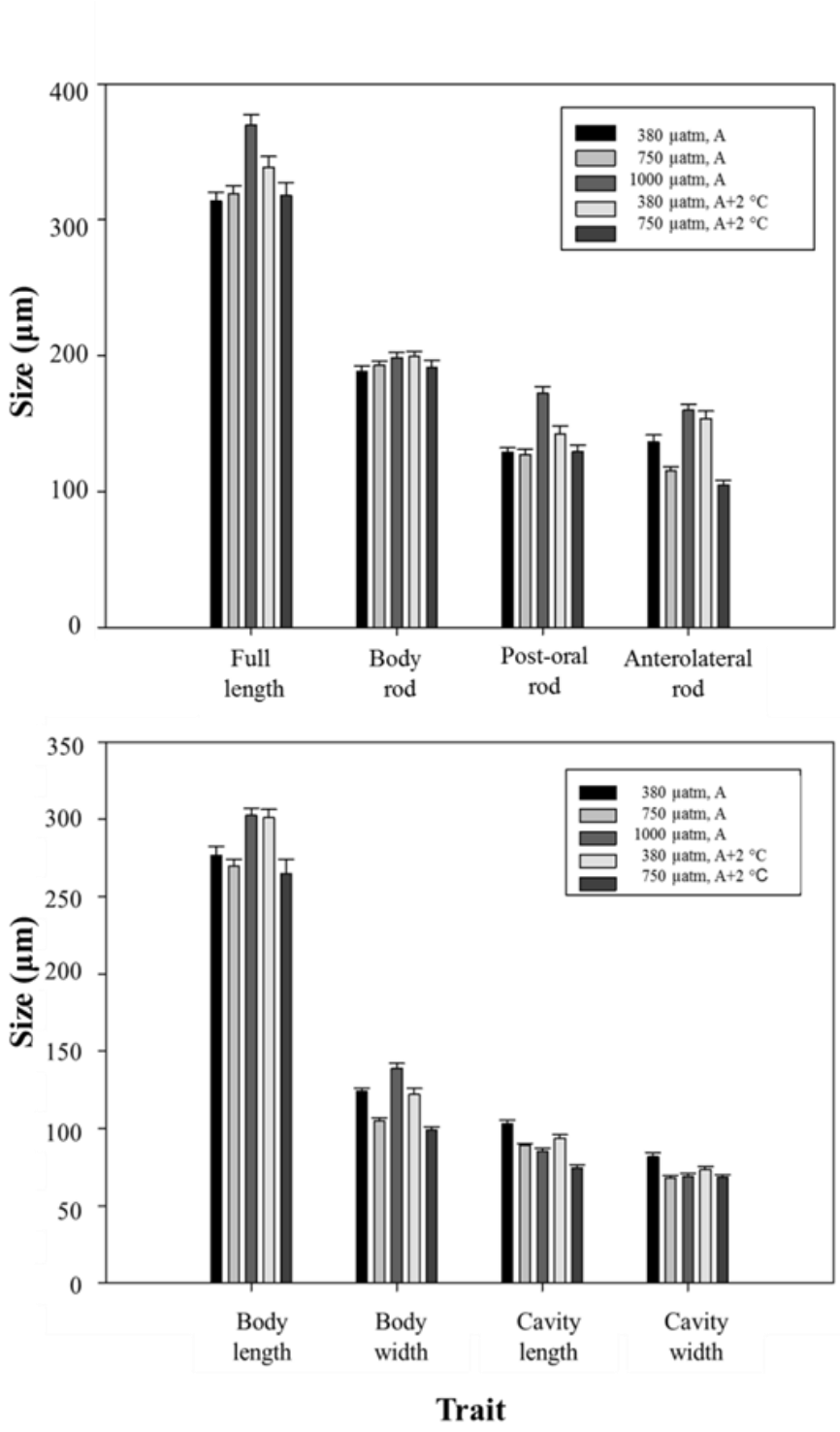

Figure 3.3: The Mean trait lengths ± standard errors of *P. lividus* pluteus larvae cultured under differing CO_2 and temperature treatments.

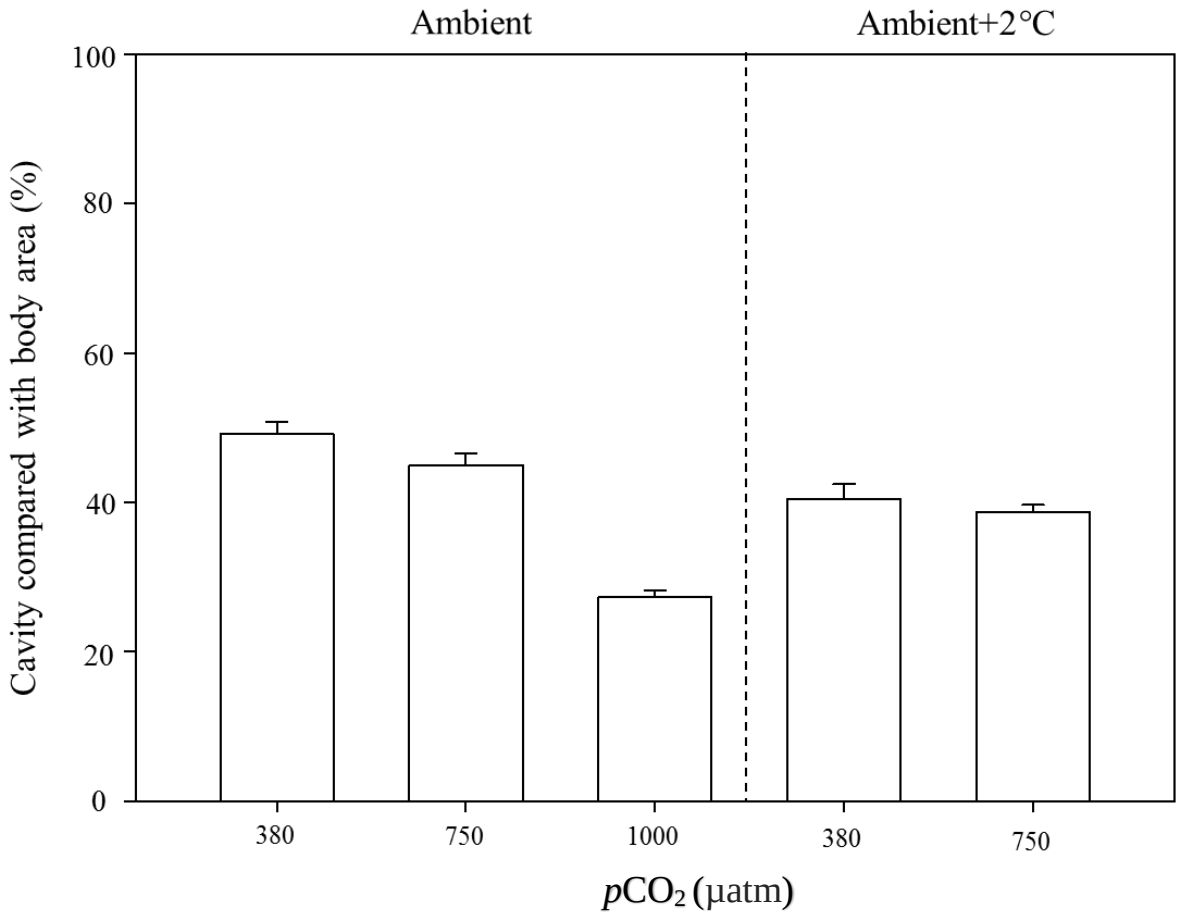

Figure 3.4: Mean percentage ± standard error of the total body area (two dimensional) occupied by cavity (stomach) at each of the five experimental treatments

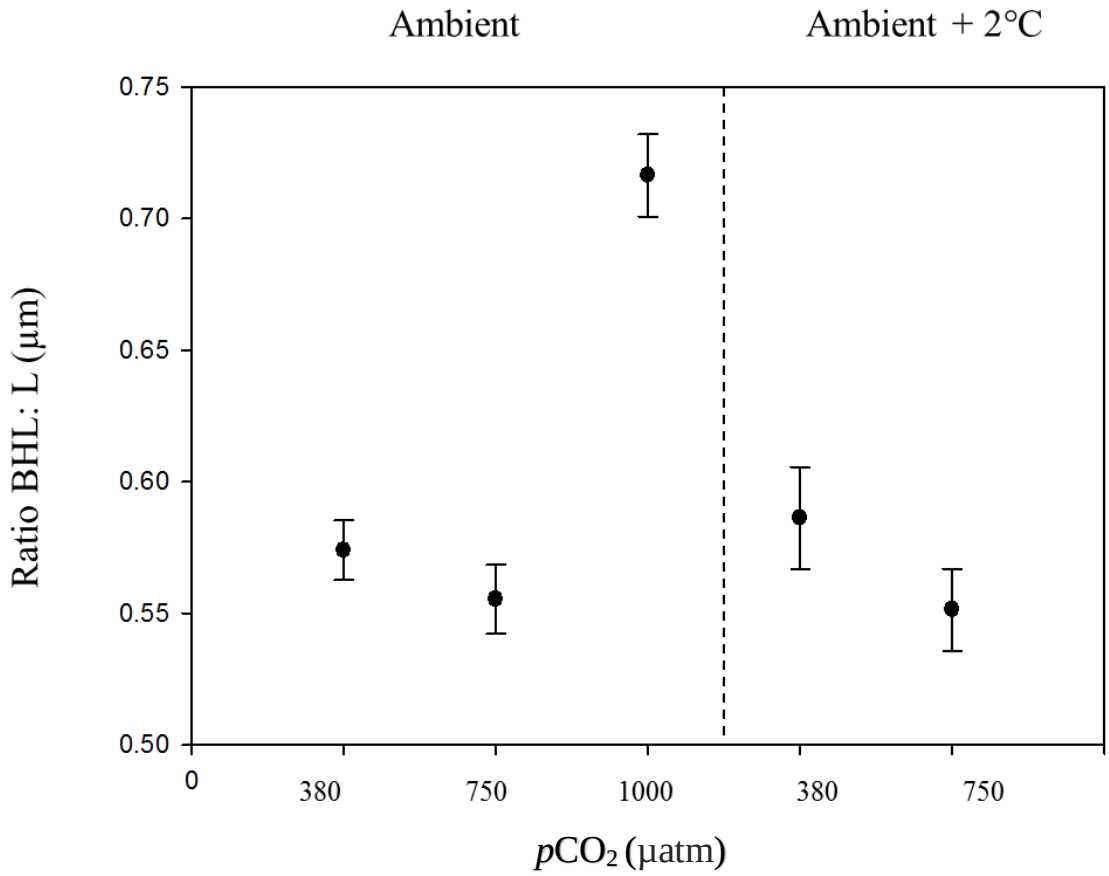

Figure 3.5: Biomineralisation index calculated as the measure of total larval skeleton and length of feeding structure relative to larval size. (Ratio of POR (r + l/2): L) Data are means ± standard error.

3.4 Discussion

The current study addresses the impacts of increases in pCO_2 and temperature on larval growth and developmental stability after 6 months parental acclimation. Variations in the developmental stability of an organism can have serious ramifications for later development, and this can have further implications for survival. The results of the present study suggest that there will be a significant negative impact on larval stability with increasing pCO_2 and temperature. It is also suggested that larval size will increase in increasing OA, but stomach size will decrease.

Overwhelmingly, previous studies concerned with larval development and growth have observed increasing pCO_2 to have a negative impact (Dupont et al., 2008; Kurihara 2008; Kurihara and Shirayama, 2004; Byrne et al., 2009; Clark et al., 2009; Parker et al., 2009; Talmage and Gobler, 2009; O'Donnell et al 2010; Sheppard-Brennand et al., 2010; Byrne et al., 2011a; Byrne et al., 2011b; Chan et al., 2011; Gaylord et al., 2011; Gazeau et al., 2011; Lischka et al., 2011; Moulin et al., 2011; Stumpp et al., 2011a; Stumpp et al., 2011b; Yu et al., 2011; Catarino et al., 2012; Chan et al., 2012; Dickinson et al., 2012), with few finding no significant effect on growth rate (McDonald et al., 2009; Munday et al., 2011). Only three previous studies have indicated an increase in growth rate under acidified conditions (Munday et al., 2009; Dupont et al., 2010a; Parker et al., 2012). There have been several suggestions as to why this increase in growth occurs. For example Dupont et al. (2010) suggested these increases in growth seen in the sea star *Crossaster papposus*, may be temporary and there may be negative effects that manifest themselves later in development. It is important to note that *C. papposus* is a direct developer so may have different tolerances to increasing OA.

These results correspond to those found presently which suggest where increases in growth rate were seen, it also highlights the need for longer observations as this initial increase in growth rate could have implications on later development. Parker et al. (2012) also reported an increase in larval growth rate in the Sydney rock oyster *Saccostrea glomerata* when reared under acidified conditions. Selectively bred individuals and juveniles from parents acclimated to acidified conditions showed a higher resilience to future OA scenarios. Both of the groups showing increased growth rate in the present study were acclimated as adults to experimental CO_2 prior to experimentation. Parker et al. (2012) suggested that the acclimation of adults to

environmental stressors prior to spawning allows for increased egg size (Moran and McAlister, 2009), and in broadcast spawners increased maternal investment (Podolsky and Moran, 2006; Moran and McAlister, 2009; Parker et al., 2012). Studies by Munday et al., (2009b) utilising the clownfish, *Amphiprion percula*, also saw a favourable effect of OA on larval length. A possible reason for this increase in length seen was that marine fish have a greater capacity for acid-base regulation that marine invertebrates.

With increases in pCO_2 and temperature it was found that there were increases in gross abnormalities, also termed "phenodeviants" (Moller and Swaddle, 1997; Lewis et al., 2004). These are a gross indicator of developmental instability (Moller and Swaddle, 1997). Increased levels of these phenodeviants occurred alongside increased FA index similar to results found in previous work (Lewis et al., 2004). The phenodeviants were removed from the analysis as they were not viable.

The current study is unique in that adults were kept under acidified conditions for 6 months prior to experimentation, which allowed for gametogenesis to occur under experimental conditions. Higher fluctuating asymmetry (FA) indexes are likely translated to reduced feeding (Chan et al., 2011; Doo et al., 2012), swimming (Doo et al., 2012) and settlement ability, as well as greater susceptibility to predation (Doo et al., 2012). The reduction in cavity size (stomach) of the echinopleutus larvae under acidified conditions supports the suggestion that increasing FA values may reduce feeding ability. Significant reduction in the stomach size of sea urchin echinopleutus larvae has been seen previously, when subjected to increased pCO_2 (Chan et al., 2011; Dupont and Thorndyke, 2009) and it was suggested that this may lead to a reduction in feeding ability and disrupt growth and potentially survival.

It has long been realised that there is a negative correlation between asymmetry and fitness (Swaddle et al., 1994). In accord with prior work, the present study which utilised increasing levels of OA and temperature as a stressor showed an increase in larval abnormality /asymmetry (Dupont et al., 2008: Byrne et al., 2009; Byrne et al., 2011a; Byrne et al., 2011b; Crim et al., 2011; Martin et al., 2011; Catarino et al., 2012). As far as the author is aware, this is the only study to be concerned with the effects of ocean acidification on FA index which, as mentioned previously, is concerned with small fluctuations from normality, and is a quantitative measure.

Studies on the effects of exposing marine invertebrates to increased CO_2 have had variable results. Some studies showed that even a small reduction in pH (0.2)

yielded 100% mortality in the brittle star *Ophiothrix fragilis* due to larval malformation (Dupont et al., 2008), whereas other studies (Yu et al., 2011) found no significant difference between treatments in absolute asymmetry, which has a characteristic "half normal" distribution (R-L) (Moller and Swaddle, 1997) of *Strongylocentrotus purpuratus*. Variations in the results found suggest that different species have different tolerances to increasing CO_2. In contrast to the current study Martin et al. (2011) showed only slight effects of increasing OA on symmetry index below pH 7.25 in *P. lividus*, however there was no acclimation period for organisms prior to experimentation. This may account for there being no significant effect on symmetry index. Parental acclimation is a key factor in OA experimental design and has been shown previously to have significant effects on experimental outcome. Parker et al., (2012) exposed adults to acidified conditions for two weeks prior to spawning and found that this exposure promoted faster development and greater growth in larvae when compared to adults conditioned at ambient CO_2. It has been suggested that adult acclimation to environmental stressors prior to spawning may lead to increased maternal investment, for example via egg size (Podolsky and Moran, 2006; Parker et al., 2012). It is possible that this increase in egg size may increase larval stability and improve survival.

Fluctuating asymmetry has been used to determine the developmental stability of organisms to a range of environmental challenges including environmental stressors and Eco toxicants. It has previously been reported that short-term exposures of *Nereis virens* larvae to a bioactive diatom-derived aldehyde (2-trans, 4-trans Decadienal) resulted in a significant increase in FA values for eye spot, peristomial cirri length and anal cirri length (Lewis et al., 2004). Similarly studies using *Psammechinus miliaris* larvae showed that asymmetrical development and mortality increased with increasing 2, 4-decadienal concentration (Caldwell et al., 2005). Long-term studies by Saucede et al. (2006) showed that in the adult sea urchin *Echinocardium flavescens* there was an increase in size relevant FA values in areas of high anthropogenic stress. However shape-related FA values were higher in areas of low anthropogenic stress. .

It is clear that the effects of increasing CO_2 on larval development are likely to be species specific with different species showing different tolerances to increased CO_2 (Dupont et al 2010b; Ericson et al., 2010; Moulin et al., 2011; Byrne, 2012; Catarino et al., 2012; Dickinson et al., 2012; Dupont et al., 2012). The present study along with previous studies (Martin e al., 2011; Moulin et al., 2011) utilising *P. lividus* suggest that

this species is particularly susceptible to the effects of OA, with the current study showing increased abnormality in juveniles spawned form adults acclimated to increased CO_2 and temperature for 6 month. Moreover, the current study indicates that increasing temperature intensified the effect of elevated CO_2 on FA, by increasing the FA index of individuals exposed to increased CO_2 and temperature. If current predictions for future CO_2 will give rise to a marked increase in larval asymmetry, especially when combined with increased temperature, then this increase in asymmetry may significantly affect larval fitness. It is possible that with decreases in larval fitness there may be further consequences on larval swimming and feeding ability which may further affect larval settlement and survival. What the present study highlights is the need for further long-term studies on the effect of ocean acidification and ocean warming on the growth and development of marine invertebrates? To gain a better perspective on this growth increase further experiments would need to be carried out in to juvenile metabolism, acid base balance and egg size. It would also be beneficial to utilise field experimentation, by utilising natural CO_2 vents, to test not only the effects of increased CO_2 levels larval morphology but also the wider ecosystem effect that this may have.

Chapter 4: Sperm motility and fertilisation success in an acidified and hypoxic environment

4.1 Introduction

For introduction to OA see chapter 1

One factor which affects population level distribution of an organism is its reproductive capacity. Reproductive processes and early ontogenetic stages of marine animals are particularly vulnerable to changing seawater properties (Pörtner and Farrell, 2008; Byrne et al., 2010a; Cooper et al., 2012). Broadcast spawning, a reproductive strategy common to many marine animals, exposes gametes directly to the seawater environment (Crimaldi, 2012), Previously, reductions in seawater pH have been shown to impact sperm swimming ability by causing changes in internal pH (pHi) of sperm and affecting motility of the flagellum (Havenhand et al., 2008; Fitzpatrick et al., 2009; Morita et al., 2010; Caldwell et al., 2011). These changes in sperm pHi have been shown to affect fertilisation by slowing the fast block to polyspermy by interfering with the Na^+/H^+ exchange and preventing the fertilisation membrane being raised (Reuter et al., 2011; Gonzalez-Bernat, et al., 2013). Spawned gametes have been used extensively in efforts to describe the potential impacts of OA on reproductive processes (Havenhand and Schlegel, 2009; Byrne et al., 2010; Ericson et al., 2010; Frommel et al., 2010;Morita et al., 2010: Cooper et al., 2012).

Numerous previous studies have focused on the negative effects that increasing pCO_2 will have on reproduction as a single stressor. As well as effects of pCO_2 there is, however, the combination of rising pCO_2 and increasing sea surface temperatures which will exact an additional toll on marine systems by reducing oxygen solubility (Hoffmann and Schellnhuber, 2009). Consequently, increased frequencies of ocean hypoxic events are predicted (Pörtner and Langenbuch, 2005; Oschlies et al., 2008; Pörtner , 2008). Over the past decade, the dissolved oxygen content of coastal waters has changed dramatically and this has led to widespread occurrences of hypoxia, especially in coastal areas which have shown an exponential increase of hypoxic events of 5.54% $year^{-1}$ (Diaz and Rosenburg, 1995; Diaz, 2001; Vaquer-Sunyer and Duarte, 2008). Normal dissolved oxygen levels range between 5.0 and 8.0 mg O_2 l^{-1} in coastal waters. Hypoxia is defined as occuring when levels of dissolved oxygen fall below 2.8

mg O_2l^{-1} (30% oxygen saturation or less) (Diaz and Rosenburg, 1995). Hypoxia has been shown to negatively affect reproduction and development of marine invertebrates (Riveros et al., 1996; Siikavuopio et al., 2007a; Spicer and El-Gamal, 1990; Marcus et al., 2004; Sedlaceck and Marcus, 2005; McAllen and Brennan, 2009; Cheung et al., 2005).

In addition to direct effects of water chemistry on reproduction, indirect effects may also result from energy partitioning, with less energy being available for reproductive processes (Kroeker et al., 2010; Kroeker et al., 2011; Long et al., 2013). With marine hypoxic events set to increase there will be increased stress on marine invertebrates. Increases in hypoxic events and temperatures (thermal extremes) expose animals to environmental extremes. Animals respond to these extremes by metabolic depression, which in turn decreases internal oxygen stores and respiratory CO_2 accumulates. Similarly, when exposed to increased CO_2 (hypercapnia), metabolism is also supressed by shifting the steady state of acid-base equilibria, along with a reduction in trans-membrane ion exchange This shift in acid-base equilibria limits the rate of protein synthesis, which can have a long-term negative affect on growth and reproduction of an organism (Pörtner et al., 2005).

As elevated pCO_2 and hypoxia are reported to have similar negative effects on reproduction in isolation, they may be expected to have synergistic or additive effects in combination. Consequently, the effects of long-term exposure (6 months) to elevated pCO_2 prior to gamete exposure to hypoxia before and during fertilisation were examined. This was designed to represent the effect of a hypoxic event in a high pCO_2 ocean, and the effect(s) that this may have on the sperm motility and fertilisation success of the sea urchin *Paracentrotus lividus*, which is an ecologically and economically important marine grazing species. With the occurrence of hypoxic events set to rise, it is important to understand the potential impacts this may have on animal reproduction in an already acidifying ocean and perhaps consequently on the future abundance and distribution of ecologically and economically important marine species.

4.2 Methods

For animal husbandry and carbonate chemistry please see Chapter 2 Sections 2.2.1 and 2.2.2, an and Figure 2.2

4.2.1 Experimental incubations

Adult *Paracentrotus lividus* were kept for six months prior to spawning in varying pCO_2 (Figure 2.2). Oxygen content was manipulated through input of nitrogen into sealed chambers; pH was monitored continually through use of a micro pH probe. Oxygen content was determined through use of an OxySense® system (OxySense® 5250i, Dallas, USA) for both normoxic and hypoxic conditions. Normoxic conditions were set at 80% Dissolved oxygen and maintained by bubbling air or CO_2 enriched air in to the experimental chambers. Temperature was monitored throughout the experiments and maintained through use of a temperature controlled room.

4.2.2 Spawning induction

Twenty randomly selected individuals (seven from the 380 µatm treatment and 13 from the 750 µatm treatment) were induced to spawn by intra-coelomic injection of 0.5-1.0ml of 0.5 M KCl. Sperm was collected dry (i.e. undiluted) and stored on ice for no more than one hour. Sperm was not pooled and males were treated as individuals. Females were allowed to express their eggs for 1 h. Egg densities were determined by counting 3 x 50 µL aliquots of egg suspension. Sperm densities were determined using a Neubauer haemocytometer.

4.2.3 Sperm motility

Sperm samples were taken from three males, from both *p*CO2 treatments (380µatm and 750µatm). Sperm densities were then adjusted to 10^7 sperm ml^{-1} using either hypoxic or normoxic adjusted FSW from sealed chambers and sperm were held at 18°C in a pH and oxygen controlled environment. Subsamples were taken at 10-min intervals (from 0 to 60 min) and immediately transferred to a glass slide. Sperm motility, determined as percentage motility and swimming speed (curvilinear velocity, VCL), was measured by computer-assisted sperm analysis (CASA) at 18^{o}C according to Caldwell et al. (2011). In brief, the CASA sperm tracking was performed by a Microptic sperm class analyser®

(Microm UK), which incorporated a Nikon Eclipse 50*i* negative phase contrast microscope (200x magnification) fitted with a Peltier cooled stage. Image capture was by a Basler A602F camera at a rate of 100 frames s-1. Multiple fields were captured, each for 0.5 seconds, until a minimum of 200 sperm were scored per treatment.

4.2.4 Fertilisation success

Fertilisation assays were conducted at varying CO_2 and oxygen levels (380 µatm and 750 µatm; 30% and 100% O_2 saturation) in 6-well multi-plates with gametes collected from three males and three females at densities of 2.5 x 10^5 ml^{-1} for sperm and 500 eggs per well, containing 10ml FSW. Fertilisation success was determined after 1 h using evidence of first mitotic cleavage as an indicator.

4.2.5 Data analysis

Motility data from sperm with a head area < 5 µm² and >35 µm² were discounted to eliminate false negatives attributable to sperm clumping. A test for normality (Kolmogorov-Smirnov) was carried out and data transformed using a natural log when not normally distributed. A 2-way ANOVA was conducted on the log VCL data to determine significant factors and interactions. Percentage sperm motility data were arcsine transformed prior to statistical analysis and a test for normality (Kolmogorov-Smirnov) was carried out. A two-way ANOVA was conducted using time as a cofactor.

4.3 Results

The results obtained from the current study show that if a hypoxic event was to occur after long-term exposure to increased pCO_2 there would be a significantly detrimental effect on the fertilisation success of *P.lividus*. Conversely, however, it would appear that sperm motility and sperm VCL would be robust to such an event.

4.3.1 Sperm motility

Neither time ($p = 0.141$) nor pCO_2 ($p = 0.37$) as single variables significantly affected percentage motility (Table 4.1, Fig. 1a). Swimming speed increased at 750 µatm under normoxic conditions relative to 380 µatm treatments (table 4.1, figure 4.11b). Percentage motility decreased ($p = 0.002$) under hypoxia (table 4.1; 380 µatm $p = 0.032$; 750 µatm $p = 0.005$) with 750 µatm with hypoxia having the lowest percentage motility, however this did not differ significantly from the 380µatm hypoxic level, and there was no significant interaction (figure 4.1a). Swimming speed increased at 750 µatm under hypoxic conditions relative to 380 µatm treatments (table 4.1, figure 4.11b). Swimming speed was significantly reduced under 380 µatm hypoxic conditions ($p<0.05$). pCO_2 and hypoxia separately showed significant effects on swimming speed (both $p <0.01$), however there was no significant interaction (table 4.1b). There was a significant decrease in percentage motility and sperm swimming speed with time ($p <0.01$).

4.3.2 Fertilisation success

Fertilisation success reduced at 750 µatm under both normoxic and hypoxic conditions (Figure 4.2; Table 4.1; $p=<0.05$; $p<0.01$). There was a significant decrease in fertilisation success under hypoxic conditions ($p=<0.01$). Fertilisation success at 380 µatm was almost fourfold lower under hypoxic versus normoxic conditions. Fertilisation was further compromised at 750 µatm with an approximate 27-fold reduction under hypoxic versus normoxic conditions. The detrimental effect of elevated pCO_2 on fertilisation success shows a significant interaction when combined with hypoxia (pCO_2*oxygen $p= <0.005$).

Table 4.1: ANOVA table for (*a*) percentage sperm motility; (*b*) sperm curvilinear velocity; and (*c*) fertilisation success at elevated pCO_2 (750 versus 380 µatm) in combination with hypoxic and normoxic conditions. Sperm motility data are corrected for time.

	Df	Sum squared	Mean squared	F-Value	P(>f)
(*a*)					
pCO_2	2	49.961	49.961	0.813	0.370
Oxygen	2	643.293	643.293	10.470	0.002
Time	7	135.788	135.788	2.210	0.141
pCO_2*Oxygen	4	1.023	1.023	0.017	0.898
Residuals	79	4853.940	61.442		
(*b*)					
pCO_2	2	3.253	3.253	9.105	0.003
Oxygen	2	3.445	3.445	9.642	0.003
Time	7	4.013	4.013	11.233	0.001
pCO_2*Oxygen	4	0.069	0.069	0.194	0.661
residuals	79	28.223	0.357		
(*c*)					
pCO_2	1	1621.303	1621.303	62.735	<0.005
Oxygen	1	20801.082	20801.082	804.876	<0.005
pCO_2*Oxygen	1	521.013	521.013	20.160	<0.005
residuals	32	827.002	25.844		

Table 4.2: Measured pH and oxygen of experimental wells throughout the fertilisation success experiment.

***p*CO$_2$ treatment (µatm)**	**Oxygen content (%)**	**Time**	**Measured pH**
380	100	0	8.08 ± 0.01
		10	8.08 ± 0.01
		20	8.08 ± 0.02
		30	8.07 ± 0.01
		40	8.07 ± 0.02
		50	8.07 ± 0.01
		60	8.08 ± 0.01
750	100	0	7.95 ± 0.01
		10	7.95 ± 0.01
		20	7.93 ± 0.01
		30	7.94 ± 0.01
		40	7.94 ± 0.02
		50	7.94 ± 0.02
		60	7.94 ± 0.01
380	30	0	8.08 ± 0.01
		10	8.07 ± 0.01
		20	8.07 ± 0.02
		30	8.06 ± 0.01
		40	8.07 ± 0.02
		50	8.07 ± 0.02
		60	8.07 ± 0.01
750	30	0	7.95 ± 0.01
		10	7.94 ± 0.01
		20	7.94 ± 0.02
		30	7.93 ± 0.02
		40	7.94 ± 0.02
		50	7.94 ± 0.02
		60	7.94 ± 0.02

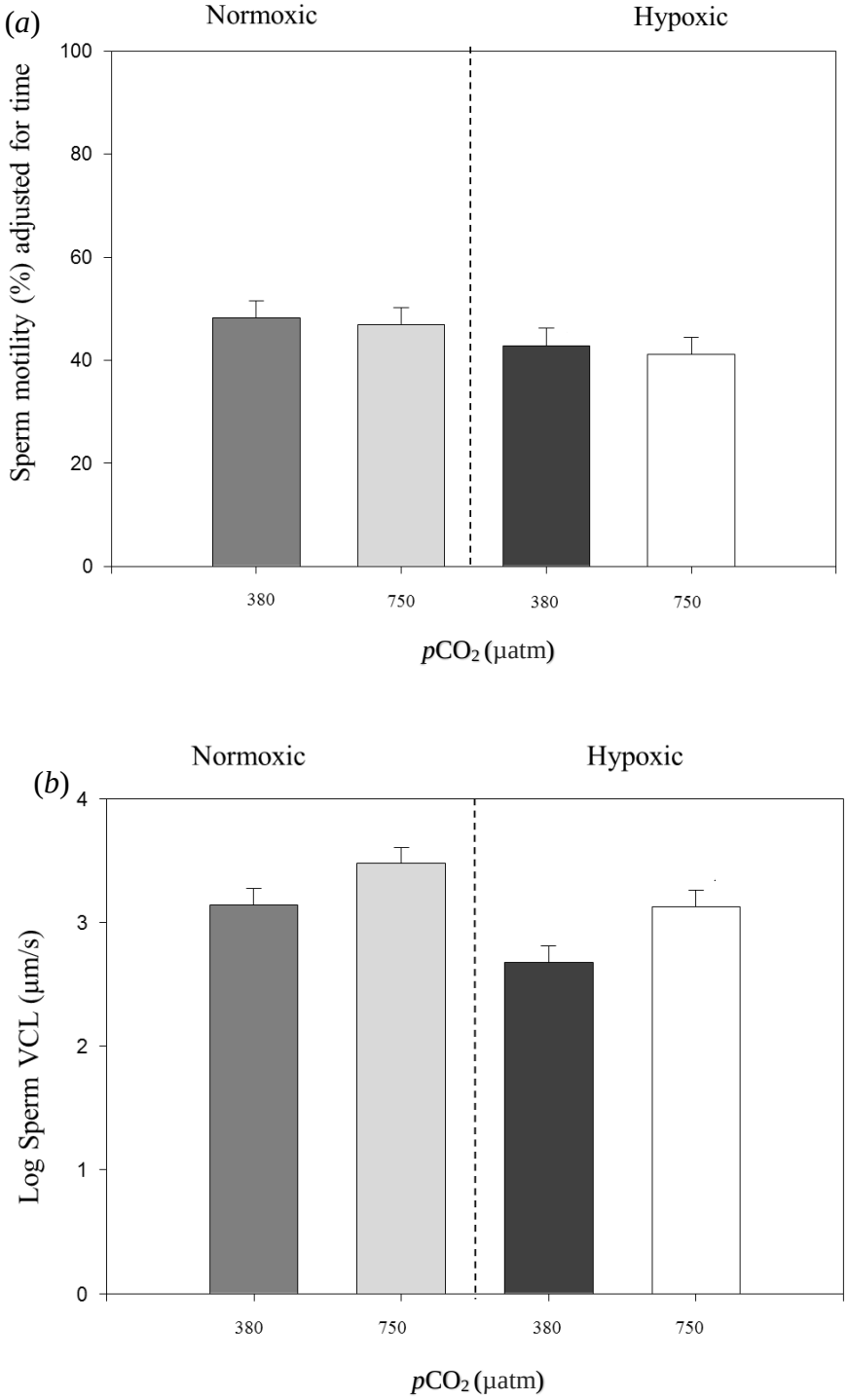

Figure 4.1: Effects of CO_2-induced acidification in combination with hypoxia on *Paracentrotus lividus* sperm (*a*) percentage sperm motility adjusted for time and (*b*) log VCL. Means±95% confidence intervals. Graphs show estimated marginal means, graph (*b*) adjusted for time at 30 minutes.

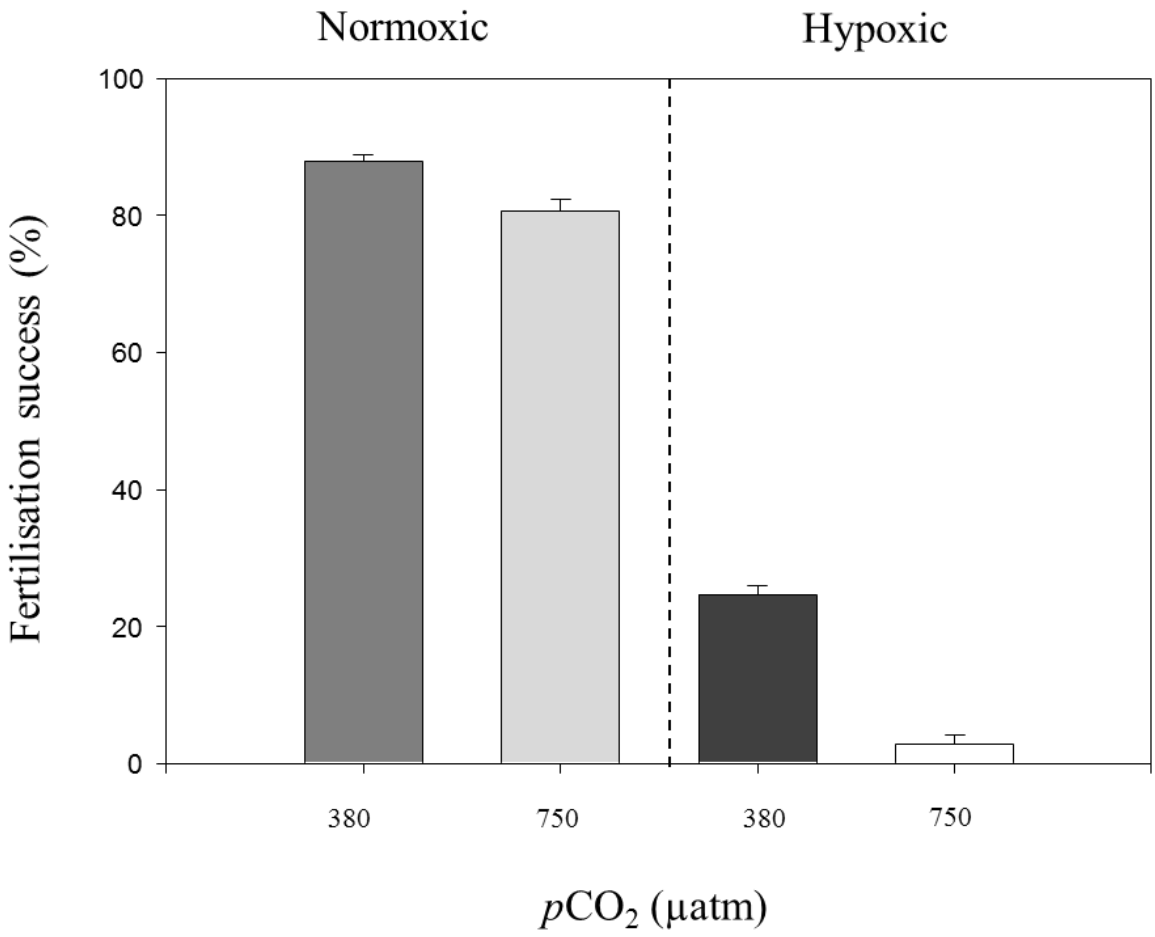

Figure 4.2: Effects of CO_2-induced acidification in combination with hypoxia on *Paracentrotus lividus* fertilisation success. Data are means ±95% confidence intervals.

1.1.

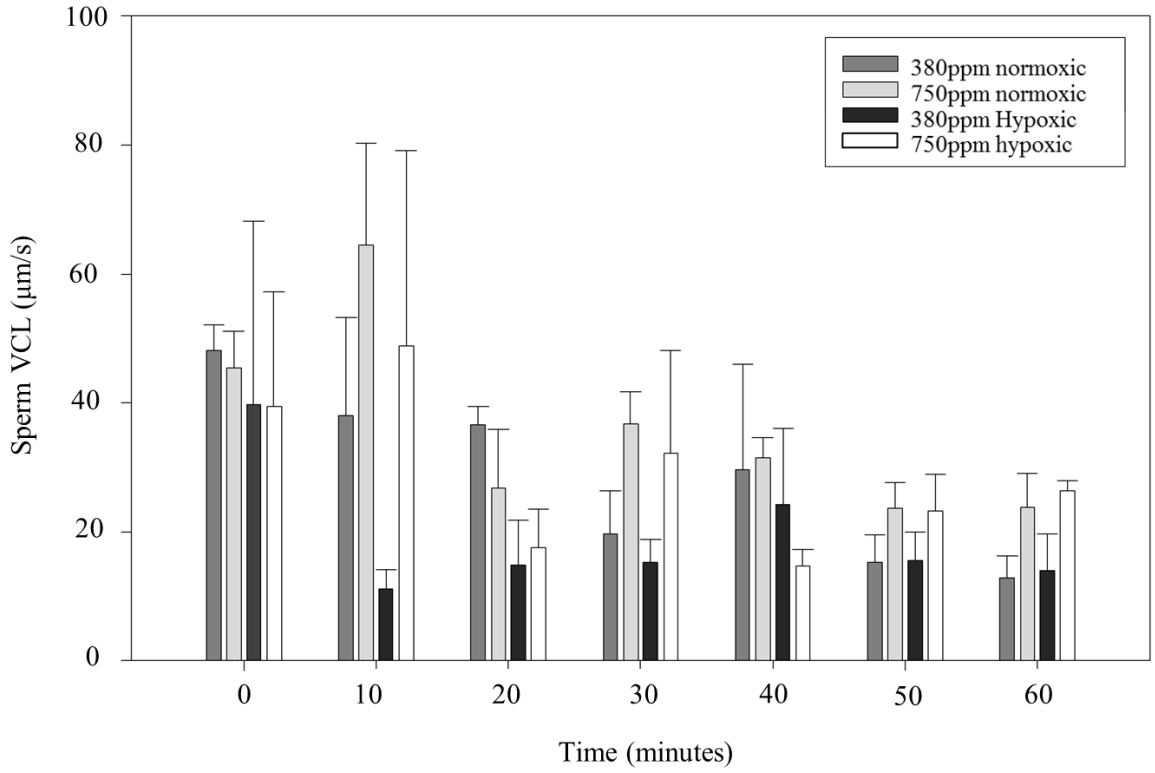

Figure 4.3: Sperm swimming speed (VCL) over time. Data are means ±95% confidence intervals

4.4 Discussion

To date no work has been published on simulated combined effects of ocean acidification and hypoxia on the reproduction and development of invertebrates. The results of the current study suggest that if a hypoxic event were to occur under future ocean acidification scenarios, there would be a significant decrease in fertilisation success, although sperm motility would not be affected significantly. The results also highlight the need for further studies into the synergistic effects of abiotic factors, as ocean acidification is unlikely to occur in isolation.

Although sperm swimming speed itself will be reduced during a hypoxic event, this reduction will be counteracted by the increase in pCO_2 predicted for the next 100 years. This would infer that sperm swimming speed will not be negatively affected by hypoxia under future predicted atmospheric pCO_2. The majority of previous studies (Havenhand and Schlegel, 2008; Frommel et al., 2010; Morita et al., 2010) concerned with sperm motility found that there was a reduction in sperm swimming speed under acidified conditions contrary to the current study. A rationale for an increase in sperm swimming speed is offered in previous work (Caldwell et al., 2011) by means of sperm activation pH. This is the mechanism whereby sperm are stored in an immotile state at pH 7.2, below the activation threshold of sperm dynein ATPase that powers the flagellum (Johnson et al., 1983). When the sperm is released into the water column the pH of the sperm is increased to 7.6 and the flagellum is activated as well as the initiation of mitochondrial respiration (Christen et al., 1983b). This indicates that there will be an increase in sperm VCL, perhaps modulated by sperm-activating peptides (SAP's), which are released by the egg jelly coat. These SAP's evolved 70 million years ago when atmospheric CO_2 was at far higher levels than the present day and ocean were more acidic (pH 7.4-7.6) (Neill et al., 2004; Darszon et al., 2008; Caldwell et al., 2011).

Hypoxia is also an important factor in relation to sperm motility. Previous research into the effects of hypoxia on sperm swimming speed has found contrasting results, with the majority of studies seeing a reduction in sperm swimming speed when exposed to hypoxic conditions (Bencic et al., 1999a; Bencic et al., 1999b; Wu et al., 2003). Sperm motility is an energetically demanding process requiring ATP, which is generated in the mid piece of the sperm. However, in the absence of oxygen ATP cannot be synthesised from ADP via oxidative phosphorylation, limiting energy availability for flagellum activity. Therefore under hypoxic conditions there is little

available oxygen and sperm are unable to become active (Billard and Cosson, 1990; Fitzpatrick et al., 2009). When this reduction in sperm motility through hypoxia is considered with the increase in sperm motility due to increasing pCO_2, it could be suggested that there is a mediating effect of hypoxia on OA. This means that a hypoxic event may have less effect on sperm swimming speed in a future elevated CO_2 ocean than previous studies would suggest.

Although sperm swimming speed remained high, results indicate that the motility of the sperm was negatively affected in hypoxic conditions The results also indicated, similarly to previous studies (Havenhand and Schlegel, 2008), that pCO_2 do not have a significant effect on the motility of sperm. Nevertheless, under hypoxic conditions there was a marked reduction. This suggests that if a hypoxic event occurred in a high CO_2 world there may be fewer motile sperm. The ability of a sperm to become motile is crucial for fertilisation success. If sperm are unable to become motile, fertilisation success will be severely impacted.

The current study differs from much of the previous literature as sperm motility and swimming speed were tracked over a one hour period. This longer track time was used as fertilisation of broadcast spawners may not happen immediately, as gametes need to disperse, so by tracking for one hour this study is better able to access what may happen naturally. Consistent with this reasoning, sperm swimming speed was on average at its highest 10 minutes after activation (Figure 4.3); a point which may have been missed previously due to shorter tracking times. This suggests that previous sperm motility experiments which only track for seconds straight after activation (e.g. Schlegel et al., 2012) may miss important trends which occur in time.

In contrast to sperm motility, fertilisation success shows a reduction under both hypoxic conditions and increased pCO_2 conditions. The effects of increased pCO_2 conditions on fertilisation success have been widely studied and are believed to be attributable to developmental delay (Kurihara and Shiryiama 2004) or to the slowing of the fast block to polyspermy (Reuter et al., 2011) (see Chapter 2 for further explanation). Previous short-term studies on the effects of OA on fertilisation success have shown varying results with the majority of studies showing no significant effect of increased pCO_2 on fertilisation success in echinoderms (e.g. Byrne et al., 2009; Byrne et al., 2010a; Byrne et al., 2010b; Martin et al., 2011). A previous study (Moulin et al., 2011) concerning *P.lividus* also showed a reduction in fertilisation success which corresponds to the results found in the current study (see chapter 2). Havenhand et al.

(2008) also reported a significant reduction in fertilisation success at pH 7.7 in the sea urchin *Heliocidaris erythrogamma*. These intra- and inter-specific differences in the results have previously been attributed to variations in experimental design. For instance, as mentioned previously, Havenhand et al (2008) found a reduction in fertilisation success at pH 7.7. On the other hand, Byrne et al. (2010a), using the same species, did not find a significant effect of pH on fertilisation success. Variations between the two methodologies used in these studies include temperature, gamete acclimation and gamete density, all of which dramatically affect fertilisation success.

Presently a significant reduction in fertilisation success was found when adults were exposed to increased pCO_2 for six months and a hypoxic event was simulated. There was also a significant effect of hypoxia alone. In contrast to previous studies concerning reproduction under increased pCO_2, studies on effects of hypoxia on reproductive capacity show a significant negative effect on reproductive endpoints including fertilisation success. This significant reduction suggests that early embryonic development is reliant on aerobic respiration. Studies concerned with respiration rate at fertilisation found that fertilised sea urchin eggs show a marked increase in the respiratory rate after fertilisation (Yasumasu et al., 1996), which would account for the reduction seen here under hypoxic conditions. After fertilisation, oxygen is required primarily for the oxygenation of glycogen, which is stored in the eggs and is an essential energy reserve for development. The oxygen used is attained through diffusion across the membrane. As this diffusion is determined by the difference in oxygen partial pressure between the egg and the external environment, for broadcast spawners the relevant conditions are those of the external marine environment (Herreid, 1980; Wang and Zhan, 1995). Hypoxic conditions may cause a decrease in this gradient and the eggs are therefore less capable of acquiring adequate oxygen, which in turn may lead to the inhibition of embryonic development. Riveros et al. (1996) showed a significant reduction in fertilisation success (below 40%) when the sea urchin *Arbacia spatuligera* was exposed to oxygen levels of 30% and below. Similarly in the sea urchin *Strongylocentrotus droebachiensis* there was a significant negative effect of hypoxia on gonad growth (Siikavuopio et al., 2007a). Reductions in reproductive ability and output have also been seen in brine shrimp (Spicer and El-Gamal, 1990), copepods (Marcus et al., 2004; Sedlaceck and Marcus, 2005; McAllen and Brennan, 2009), and gastropods (Cheung et al., 2005). The results from previous studies also indicate a reduction in energy allocation for reproduction (Cheung et al. 2008) as well as a reduction in

developmental rate indicating developmental delay (see Chapter 2) (McAllen and Brennan, 2009).

The results suggest a synergistic effect between increased pCO_2 and hypoxia; this can be seen by the significant reduction in fertilisation success under hypoxic conditions and by the significant difference which occurs between the 380 µatm and 750 µatm treatments. The results suggest that increased pCO_2 conditions and a hypoxic event will have severe negative impacts on fertilisation success. The diffusion of pCO_2 created during respiration is reliant on a diffusion gradient similar to that for oxygen and under increased pCO_2 the CO_2 molecules do not move as readily across the membrane, leading to reductions in fertilisation success. This synergistic effect may lead to severe negative effects on species recruitment and distribution.

It is the aim of the current study to assess the impact that a hypoxic event would have on the reproductive parameters of sperm motility and fertilisation success in a high CO_2 world, and to assess the importance of these parameters in terms of reproduction and development. Sperm swimming speed is not necessarily the most important factor in fertilisation success as can be seen from the results of this study. It is in fact possible that increased sperm swimming speed reduce fertilisation success, because sperm that swim faster use up the available energy faster and therefore die quicker. Broadcast spawning is affected by many factors, including water currents and chemistry, and as fertilisation may not happen immediately sperm need to be motile for longer (Levitan, 2000). Here evidence is provided that under ambient conditions sperm swimming speed is lower when compared to those subject to CO_2-induced ocean acidification, which supports the idea that for optimal fertilisation success in the sea urchin *P. lividus* there needs to be a trade-off with sperm speed and longevity. It appears that a hypoxic event will negatively affect fertilisation success regardless of oceanic pCO_2, but this effect will be intensified at near future pCO_2 conditions. This idea is supported by the results of this study, which suggest that during a hypoxic event sperm VCL may remain high, although this is not the most important factor in fertilisation success. This significant reduction in fertilisation success will have a consequential effect at later life stages as if fewer individuals are available, recruitment and thus distribution will be affected. There are likely to be serious ramifications on the food chain as *P.lividus* is not only an important grazing species but also an important source of prey for larger organisms.

Chapter 5: Multi-generational effects of ocean acidification on the tube-building polychaete *Ficopomatus enigmaticus*

5.1 Introduction

The effects of ocean acidification on reproductive and developmental endpoints including fertilisation success, sperm motility and sperm swimming speed have previously been studied in a range of species. The majority of studies, however, have concentrated on short-term effects of decreasing oceanic pH on reproduction, leaving a gap in knowledge relating to possible long-term and multi-generational impacts of increasing OA on reproduction and development. Short-term studies have shown a variety of responses to decreased pH conditions. Dupont et al. (2012) reported that within the Echinodermata 61% of all studied species showed a negative response to decreased pH. There was no significant effect of decreasing pH in 34 % and a positive effect in 5%. The majority of studies have focused on calcifying species, which are predicted to be most susceptible under increasing OA (see Chapter 1 Table 1.1). Fewer studies have concentrated on non-calcifying organisms (Chapter 1 Table 1.2)

The early ontogenetic stages of marine invertebrates have been previously shown to be particularly sensitive to changing oceanic chemistry (Pörtner and Farrell, 2008; Byrne et al., 2010a; Cooper et al., 2012). The current study utilises the reproductive endpoints of sperm motility, sperm swimming speed (measured as curvilinear velocity (VCL)) and fertilisation success. Previously, the majority of studies concerned with sperm motility and sperm swimming speed have shown a negative effect of decreasing oceanic pH (Havenhand et al., 2008; Morita et al., 2011; Lewis et al., 2012). Similarly, the majority of studies concerned with fertilisation success saw a reduction in success when exposed to future ocean pH scenarios (Kurihara et al., 2004a; Kurihara and Shirayama, 2004; Havenhand et al., 2008; Parker et al., 2009; Parker et al., 2010; Foo et al., 2012; Lewis et al., 2012; Barros et al., 2013; Gonzalez-Bernat et al., 2013). Effects of decreasing pH seen at early reproductive stages could be problematic for later stages of development.

Only one study has addressed the effects of OA on reproduction and development in polychaetes. Lewis et al. (2012) examined the effects of decreased pH on sperm motility, fertilisation success, survival and larval morphology in the polychaete *Pomatoceros lamarckii*. They found that sperm motility and swimming speed were not significantly affected until pHs of 7.4 and 7.2 and fertilisation success showed a significant reduction at pH 7.6 and 7.4. This study only concentrated on short-term exposure to decreased pH with no allowance for parental conditioning to reduced pH conditions prior to experimentation.

It has been suggested that increased CO_2 levels will have significant effects on marine biota (Fabry et al., 2008; Hoffmann et al., 2010, Kroeker et al., 210; Byrne et al., 2011). As yet, however, there are relatively few robust indicators of the long-term biological consequences of ocean acidification. Multi-generational studies act as an analytical tool for predicting the long-term biological impacts of ocean acidification as well as gauging a species' ability for survival, recruitment and adaptation. To date there have been two multi-generational studies concerning the effects of ocean acidification on reproduction and development of copepods, with contrasting results (Kurihara and Ishimatsu, 2008; Fitzer et al., 2012). The difference between previous studies and the current study is the generation time of the species. Early studies by Kurihara et al., (2008) used the calanoid copepod *Acartia tsuensis*, which has a generation time of nine days between egg and maturity (225 degree days at 25ºC). More recent studies by Fitzer et al. (2012) used the harpacticoid copepod *Tisbe battagliai*, which has a generation time of 14 days. The current study utilises the tube worm *Ficopomatus enigmaticus*, which has a generation time of 90 days. *F. enigmaticus* is a broadcast spawning serpulid polychaete with a non-calcifying feeding larval stage, and it is a novel study species for use in Ocean acidification studies. It is able to be reproductively viable within a 3 month time period which makes it a good test species for multigenerational studies. The onset of sexual maturity can be seen as the lower thorax changes colour, in the male this is green and in the female a pinkish. Reproduction occurs externally as gametes are expelled from the body and the calcareous tube (Gabilondo et al., 2013). After fertilisation early embryonic development occurs and after approximately 18 Hours a motile trochophore stage is reached. (Gabilondoo et al., 2013). The larvae. Approximately 4 days post fertilisation larvae were motile metatrochophores with 2 eye spots and 3 abdominal segments. Between 5 and 9 days post fertilisation larvae became competent to metamorphose and settle. Prior to settling larvae with the neurotroch band

of cilia and the chaetae. During the early stages of settlement the larvae secrete a primary tube which is un-calcified, this was moulded by the larvae through body movement. After this the secondary calcareous tube was secreted by the collar gland (Gabilondoo et al., 2013).

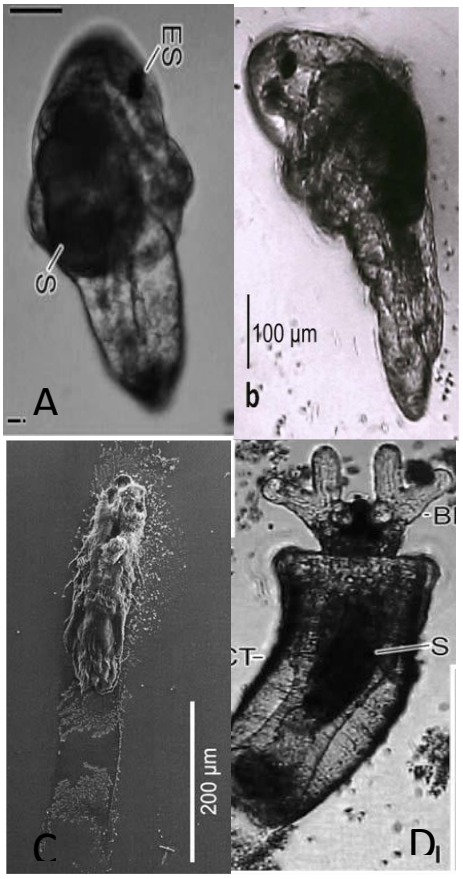

Figure 5. 1, adapted from Gabilondo et al, (2013) depicts the varying life stages of the polychaete *Ficopomatus enigmaticus*, a) Metatrochophore larvae with eye pot and stomach visible, b) competent larvae prior to settlement, c) ESEM of larvae 5 days post fertilisation and newly settled, D) metamorphosed larvae with Calcareous tube.

F. enigmaticus has a broad geographic distribution through temperate and sub-tropical waters in both the northern and southern hemispheres (Obenat rand Pezzani, 1994, Obenat et al., 2006). It is also an ecosystem engineer and has a well-documented reproductive cycle. The current study aims to determine the impact of decreased pH on sperm motility, sperm VCL and fertilisation success over multiple generations to determine the long-term multi-generational effects that decreased oceanic pH will have on reproduction and development of this species.

5.2 Methods

5.2.1 Experimental conditions

F. enigmaticus were obtained from Swansea marina (51°29'21"N, 2°45'37"W) and cultured in a flow-through aquarium system at 20°C with a 16:8 photoperiod prior to submersion in experimental treatments. Natural seawater was collected from the Blue Reef Aquarium®, Tynemouth, Tyne and Wear, UK from an inshore sub-sea pipeline. Worms were fed ad libitum on a mixed microalgae diet (*Isochrysis galbana* and *Tetraselmis suecica at cell concentrations of* 4.4×10^5 cells.ml^{-1} and 2.0×10^5 cells.ml^{-1} respectively). *F.enigmaticus* were allowed to acclimate for 1 month prior to spawning in experimental treatments. This period allowed for gametogenesis to occur. Experimental tanks were maintained at 20°C (±0.5) at treatment pHs (which were nominally 8.12, 7.95, 7.82, and 7.67) with a 16:8 photoperiod in aerated 0.22 μm filtered seawater, with water changes every 14 days. Twelve experimental chambers (3 per treatment) were utilised, with stocking densities of approximately 30 worms in their tubes per experimental chamber.

5.2.2 Carbonate chemistry

pH was regulated via injection of CO_2 utilising a solenoid valve, automatically controlled by and Aqua-medic™ (Loveland, Colorado) pH computer and probes. A Dr DAQ™ (Cambridgeshire, UK) data logging system continually monitored pH, and temperature and daily salinity measurements were recorded with total alkalinity (TA) titrations taking place prior to water changes (see Table 5.1). Carbonate system parameters, which were not directly measured, were calculated from pH, salinity TA and temperature using CO2SYS (Pierrot et al., 2006), employing constants from Mehrbach et al. (1973) refitted to by Dickson and Milero (1987) and the KSO dissociation constant from Dickson (1990).

5.2.3 Destructive spawning

After acclimation the zero generation *F. enigmaticus* were spawned following published methodology (Gabilondo et al., 2013). Briefly, adult *F enigmaticus* were removed from their tubes using watchmaker's tweezers and placed into watch glasses. Sex was determined with the use of a light microscope. Females were placed into 10ml of pH-

adjusted 0.22 μm filtered seawater (FSW). In the case of males, 2 μl of seawater adjusted to experimental pHs was added to induce the release of the sperm and then the sperm were collected and stored on ice until use.

5.2.4 Sperm motility

For methodology see chapter 2 section 2.2.4

5.2.5 Egg Diameter

Egg samples were taken for all generations at each pH treatment, giving a total of 15 samples. They were fixed in 70% ethanol for microscopic analysis. Egg diameter measurements were made using Image J software from images captured with a digital camera (Sanyo) attached to an inverted dissection microscope (Olympus CH × 41 at × 10 objectives).

5.2.6 Controlled Fertilisation

Eggs from nine females were fertilised with sperm from nine males. For Generations 1 and 2, samples were mixed between experimental chambers to prevent inbreeding. Egg densities were determined from the average of three counts of 50 μL aliquots. Between 450 and 500 eggs were added to individual wells with 10ml of FSW. Fertilisation assays were conducted under pH-adjusted conditions in 6-well multi-plates with sperm at densities of 2. 5 x 10^3, 10^4, 10^5 ml^{-1} and 500 eggs per well. Fertilisation success was determined after 90 minutes as the presence of the 2-4 cell cleavage.

5.2.7 Fertilisation for multigenerational experiments

For multi-generational studies, individuals were removed from their tubes and gamete density determined as described in Section 2.2. At least nine males and nine females were used for controlled fertilisations. These were carried out in crystallising dishes (500ml) containing 250ml of pH-adjusted FSW, 5 eggs per ml and 2. 5 x 10^5 sperm per ml. The FSW volume was increased to 500ml and the crystallising dishes were gently aerated with a solenoid-controlled mix of O_2 and CO_2 (see section 2.1).

5.2.8 Continued culture of F. enigmaticus

Once the worms had settled, in that they had begun to secrete the primary tube (Gabilondo et al., 2013) (7 d for Generation 0, 9 d for Generations 1 and 2) the FSW volume was increased to 2 L and continuous aeration applied. There were three tanks per treatment spread across two incubators. Worms were allowed to develop for three months post fertilisation and fed *ad libitum* with a diet of mixed microalgae (*Isochrysis galbana* and *Tetraselmis suecica* at densities of 4.4×10^5 cells.ml^{-1} and 2.0×10^5 cells.ml^{-1} respectively. Water changes were performed every two weeks to maintain constant salinity.

5.2.9 Statistical analysis

Sperm motility, curvilinear velocity (VCL) and fertilisation success data were not normally distributed (Kolmogorov-Smirnov), therefore a general linear mixed model was used for analysis, with restricted maximum likelihood (REML). Covariates of incubator and tank were used and these were found to be non-significant and therefore removed from the model. As time (the time sperm were tracked for) was found to have a significant effect on sperm motility and VCL, these variables were adjusted to 30 minutes, generating estimated marginal means from the GLMM for each combination of pH and generation. Similarly as sperm density was found to have a significant effect on fertilisation success, estimated marginal means were generated for fertilisation success adjusted to a sperm density of $2.5x10^4$ cells.ml^{-1}. Significant differences in sperm motility, VCL and fertilisation success between pH treatments within and between each generation were detected using pairwise comparisons generated from the estimated marginal means. For egg diameter, a test of normality was carried out (Kolmogorov-Smirnov test) and when data were normally distributed a two-way ANOVA was carried out using pH and generation as fixed factors. Significant differences within and between pH treatments and generation were tested for using pairwise comparisons generated by estimated marginal means. All statistical analyses were carried out using SPSS (Version 19, New York, United States) and all data are presented as estimated marginal means ± standard error.

5.3 Results

5.3.1 Carbonate chemistry

Table 5.1 summarises the sea water chemistry for the duration of the experiment. As expected there was a significant difference in pCO_2, pH, and aragonite saturation (ΩAr) (ANOVA: pCO_2, $F_{3,8}$=56.643, p=0.000; pH, $F_{3,8}$= 11652.0, p=0.000; ΩAr , $F_{3,8}$= 265.718, p=0.000). There was no significant difference in salinity, total alkalinity (TA) and temperature among treatments (salinity, $F_{3,8}$=4.00, p=0.052; TA, $F_{3,8}$=40.852, p=0.503; and temperature, $F_{3,8}$=3.017, p=0.094).

5.3.2 Sperm motility

There was no significant effect of incubator or tank on percentage sperm motility (incubator, $F_{1,736}$=0.616, *P*= 0.433; tank, $F_{1,736}$=2.673, *P*= 0.102), so they were removed as a covariate from further analysis. Time was a significant factor and was, therefore, used as a covariate in the analysis ($F_{1,738}$=25.200, *P*= 0.000). Both pH and generation were adjusted for time to take in to account the time-frame used. There was no significant effect of pH on the percentage motility of sperm ($F_{3,738}$=1.821, *P*= 0.142). Generation had a significant effect on percentage sperm motility ($F_{2,738}$=19.467, *P*= 0.000). The results showed that Generation 0 did not differ significantly from Generation 1 (p=0.076) but it did from Generation 2 (*P* =0.001). There was no significant difference between Generation 1 and Generation 2 (*P*=0.116). There was a significant interaction between pH and generation ($F_{6,738}$=2.778, *P*= 0.011).

5.3.3 Sperm VCL

There was no significant effect of incubator or tanks (incubator, $F_{1,736}$=0.435, *P*= 0.510; tank, $F_{1,736}$=0.522, *P*= 0.470) so they were removed as covariates for further analysis. Time was a significant factor and used as a covariate in the analysis ($F_{1,738}$=21.218, *P*= 0.000). Both pH and generation were adjusted for time to take in to account the time frame used. Similarly to percentage motility, there was a significant effect of generation on sperm swimming speed ($F_{3,\ 738}$=18.947, *P*= 0.000). Generation 0 differed significantly from Generation 1 and 2 (G1, *P*= 0.007; G2 *P*= 0.025) but there no significant difference between Generation 1 and 2 (*P*= 0.631). Sperm VCL was highest at Generation 0 and lowest at Generation 1. The results indicate pH had no

significant effect on sperm VCL ($F_{2,738}$=0.989, *P*= 0.397). There was no significant interaction between pH and Generation ($F_{6,738}$=1.483, *P*= 0.181).

5.3.4 Egg diameter

There was a significant effect of pH ($F_{3,}$= 10.038, p=<0.001) generation ($F_{2,}$=21.103, p=<0.001) and a significant interaction between the two factors (pH*Generation $F_{6,}$=13.758, p= p=<0.001). At pH 7.67, pH7.82, and pH 8.10 there was a significant increase, between Generation 0 and Generation 1 (pH p=<0.001; 7.82 p=<0.001; 8.1, p=0.015). pH 7.95 showed a significant increase between Generation 0 and Generation 1 (p=0.000). By Generation 2 there was a significant increase in egg size at pH 7.95, which differed significantly from all other pHs (8.1 p=<0.001; 7.82, p=<0.001; 7.67, p=<0.001).

5.3.5 Fertilisation success

There was no significant effect of incubator or tank on fertilisation success (Table 5.2; incubator, $F_{1,948=}$0.007, *P*= 0.935; Tank, $F_{1,948}$=0.006, *P*= 0.937) and this was therefore removed as a covariate from further analysis. There was no significant effect of individual on fertilisation success and were therefore removed as a covariate (Table 5.2: male, $F_{1,948}$=0.018, *P*= 0.893; female $F_{1,948}$=0.608, *P*= 0.436). Sperm density was a significant factor (Table 5.2; $F_{1,948}$=1316.008, *P*= 0.000) and was used as a covariate within the analysis with both pH and generation being adjusted for sperm density. Both pH and generation had a significant effect on fertilisation success (Table 5.2: pH, $F_{3,948}$=3.888, *P*= 0.009; Generation $F_{2,948}$=8.460, *P*= 0.000). There was also a significant interaction between the two factors (Table 5.2: $F_{6,948}$=5.768, *P*= 0.000). Fertilisation success in all pH treatments differed significantly from each other (8.1, $F_{2,948}$=82.011, *P*= 0.000; 7.95, $F_{2,948}$=160.570, *P*= 0.000; 7.82, $F_{2,948}$=187.156, *P*= 0.000; 7.67, $F_{2,948}$=126.878, *P*= 0.000) and all generations differed significantly from each other (G0, $F_{3,948}$=119.184, *P*= 0.000; G1, $F_{3,948}$=124.737, *P*= 0.000; G2, $F_{3,948}$=177.135, *P*= 0.000). There was an increase in F value with each generation suggesting that more of the variance can be described by pH. Percentage fertilisation success was consistently higher in control pH treatments (8.1) across all generations, with the lowest pH treatment (7.67) showing consistently lower percentage fertilisation success (See figure

5.4) Generation 1 yielded the highest fertilisation success when compared to Generation 0 and 2.

Table 5. 1: Seawater chemistry data within experimental system. Temperature, salinity pH and total alkalinity (TA) measured to calculate seawater parameters using CO2Sys software.

Generation	pH	Mean pH	Salinity	Mean Temperature (°C) in	Mean TA in (µmol/ kg)	TCO_2 in (mmol/kg SW)	pCO_2 in (µatm)	HCO_3^- in (mmol/kg SW)	CO_3^{2-} in (mmol/kg SW)	Ω Ca in	Ω Ar in
1	**8.1**	8.09 ± 0.04	36 ± 1	19.66 ± 1.1	2560.30	2321	547	2124	178.82	3.95	2.72
2	**8.1**	8.09 ± 0.04	36 ± 0.5	19.66 ± 1	2531.15	2294	541	2100	176.73	3.90	2.69
3	**8.1**	8.09 ± 0.04	36± 1	19.66 ± 1.5	2547.70	2309	544	2114	177.91	3.93	2.71
1	**7.95**	7.95 ± 0.05	36 ± 0.5	19.83 ± 1	2439.57	2279	775	2126	127.40	2.81	1.94
2	**7.95**	7.95 ± 0.05	36 ± 1	19.83 ± 1.8	2427.07	2267	771	2115	126.71	2.80	1.93
3	**7.95**	7.95 ± 0.05	36 ± 0.5	19.83 ±1.8	2364.54	2207	751	2059	123.39	2.72	1.88
1	**7.82**	7.82 ± 0.03	36 ± 1	19.82 ± 1.2	2377.12	2271	1053	2141	95.26	2.10	1.45
2	**7.82**	7.82± 0.03	37 ± 1	20.22 ± 1.5	2235.57	2129	989	2006	91.57	2.03	1.40
3	**7.82**	7.81± 0.03	37 ± 0.5	20.22 ± 1.9	2406.17	2295	1066	2162	98.70	2.19	1.51
1	**7.67**	7.67± 0.03	36 ±1	19.55 ± 0.7	2701.84	2649	1743	2514	77.77	1.71	1.18
2	**7.67**	7.67± 0.03	36 ± 0.5	19.55 ± 1.6	2539.46	2488	1637	2361	73.06	1.61	1.11
3	**7.67**	7.67± 0.03	36 ± 0.5	19.55 ± 1.3	2107.01	2059	1355	1954	60.48	1.33	0.92

Table 5.2: General linear mixed model table for *(a)* Sperm curvilinear velocity, *(b)* percentage sperm motility and, *(c)* fertilisation success. Non-significant results were removed from further analysis. Significant differences indicated by (*)

	Numerator df	Denominator df	F-Value	P(>f)
a)				
Generation	2	736	18.909	<0.001*
pH	3	736	0.987	0.398
Time	1	736	21.176	<0.001*
Incubator	1	736	0.435	0.510
Tank	1	736	0.522	0.470
Generation*Time	2	736	20.889	<0.001*
pH*Time	3	736	0.620	0.602
Generation*pH*Time	6	736	1.480	0.182
b)				
Generation	2	736	19.527	<0.001*
pH	3	736	1.827	0.141
Time	1	736	25.278	<0.001*
Incubator	1	736	0.616	0.433
Tank	1	736	2.673	0.102
Generation * Time	2	736	14.667	<0.001*
pH * Time	3	736	0.619	0.603
Generation*pH*Time	6	736	2.786	0.011*
c)				
pH	3	948	235.464	<0.001*
Generation	2	948	47.884	<0.001*
Male	1	948	0.018	0.893
Female	1	948	0.608	0.436
Sperm Density	1	948	1292.504	<0.001*
Incubator	1	948	0.007	0.935
Tanks	1	948	0.006	0.937
pH * Sperm Density	3	948	3.819	0.010*
pH*Generation*Sperm Density	8	948	8.825	<0.001*

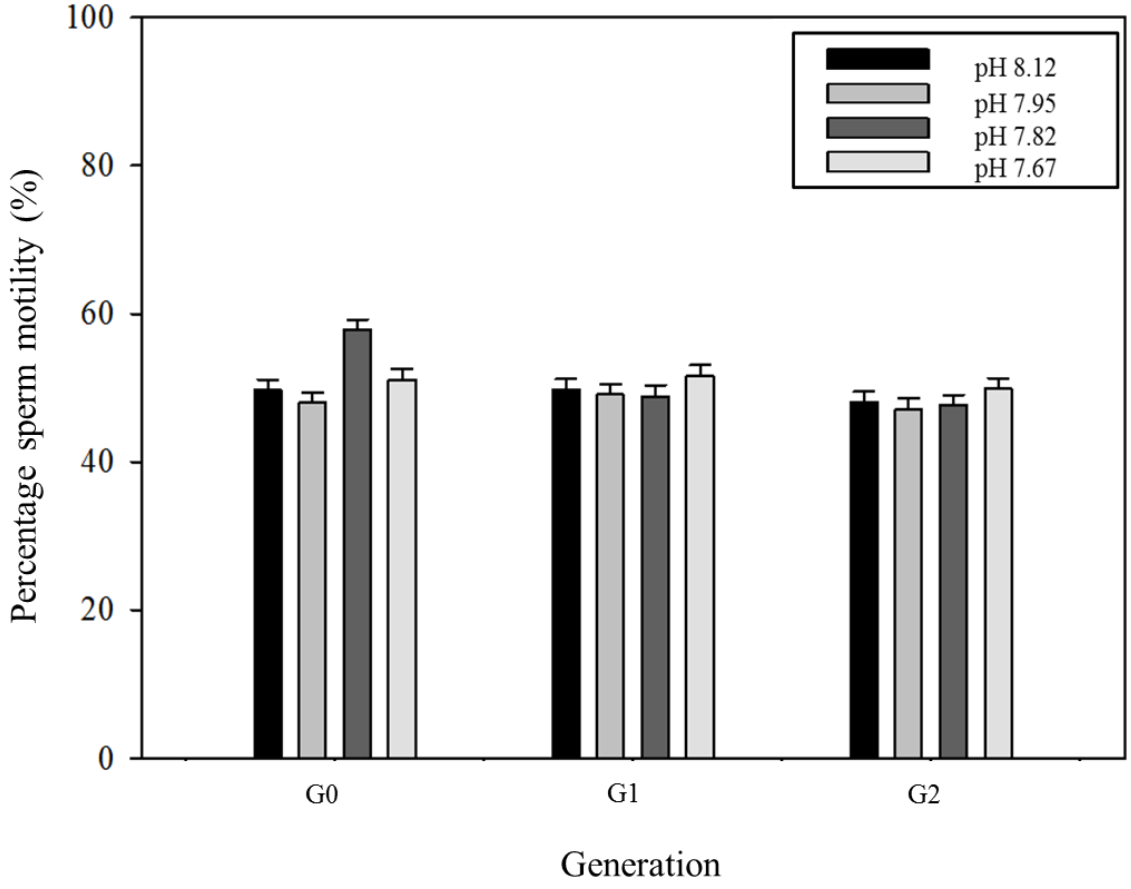

Figure 5.2: Percentage sperm motility adjusted for time at 30 minutes; data shown are estimated marginal means and SE (standard error).

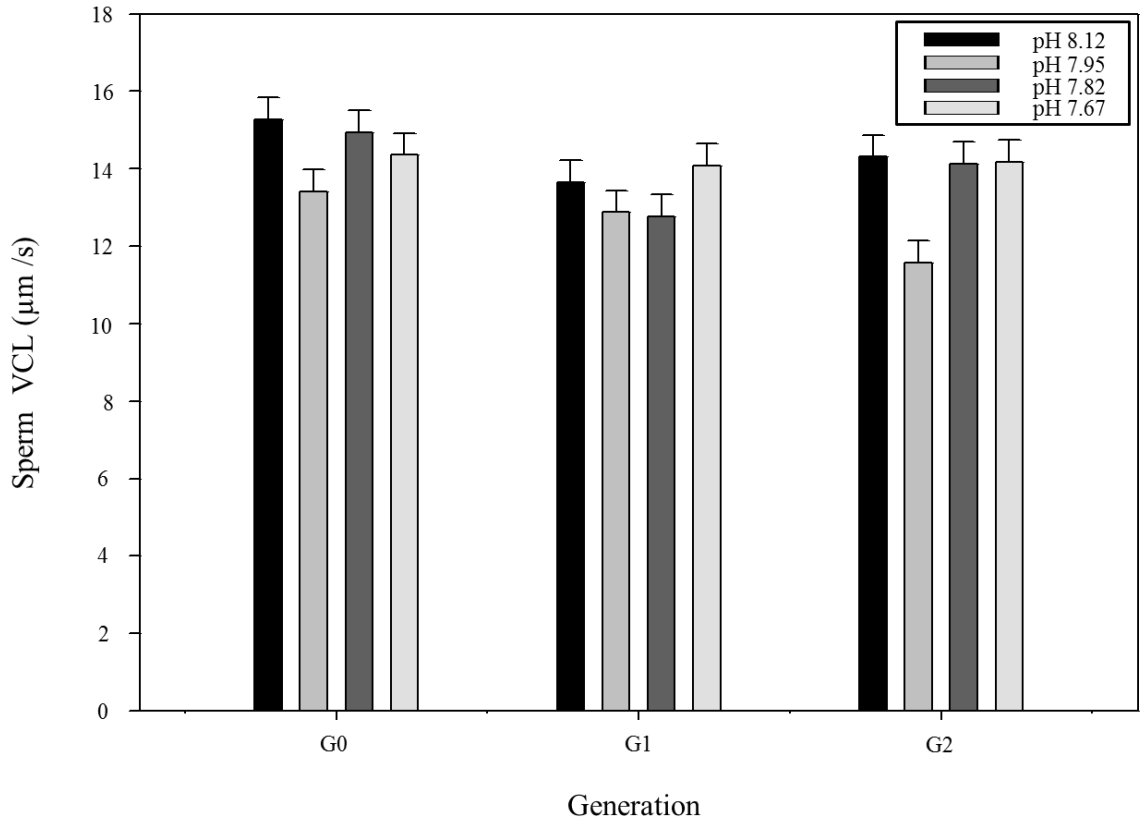

Figure 5.3: Sperm VCL adjusted to 30 minutes, data shown are estimated marginal means and SE.

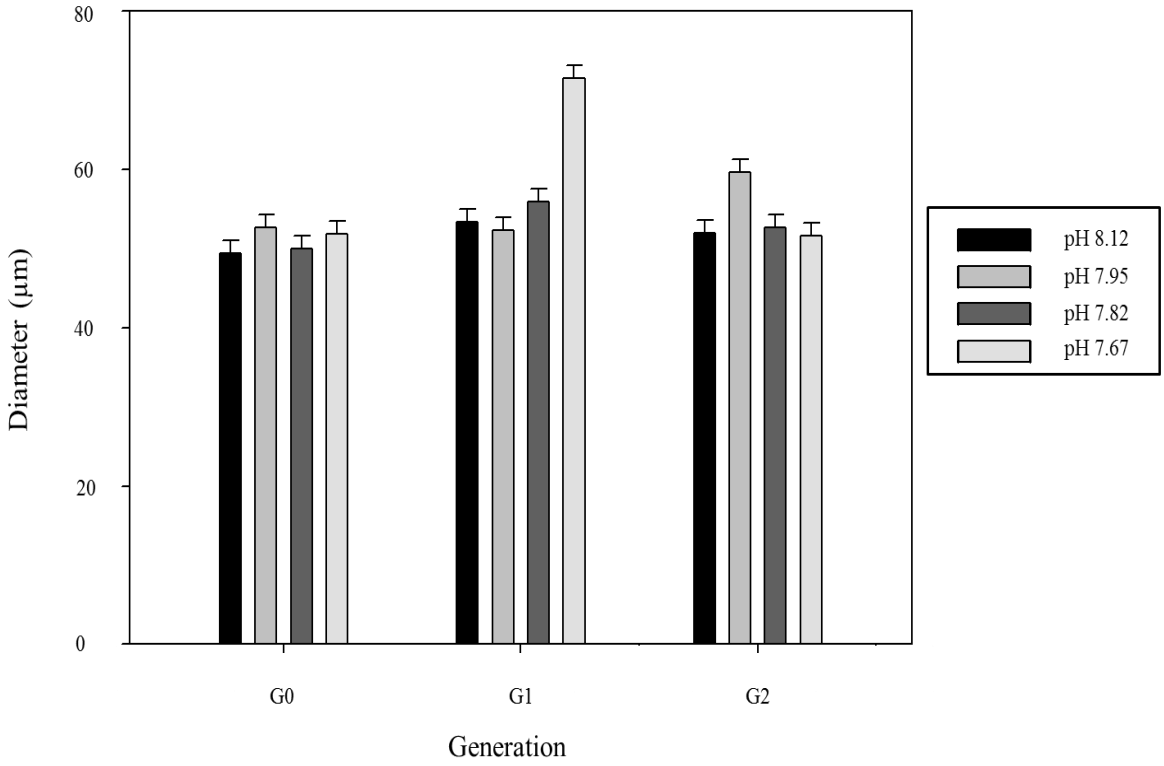

Figure 5.4: Mean egg diameter at all pH treatments for all Generations. Data shown are estimated marginal means and SE.

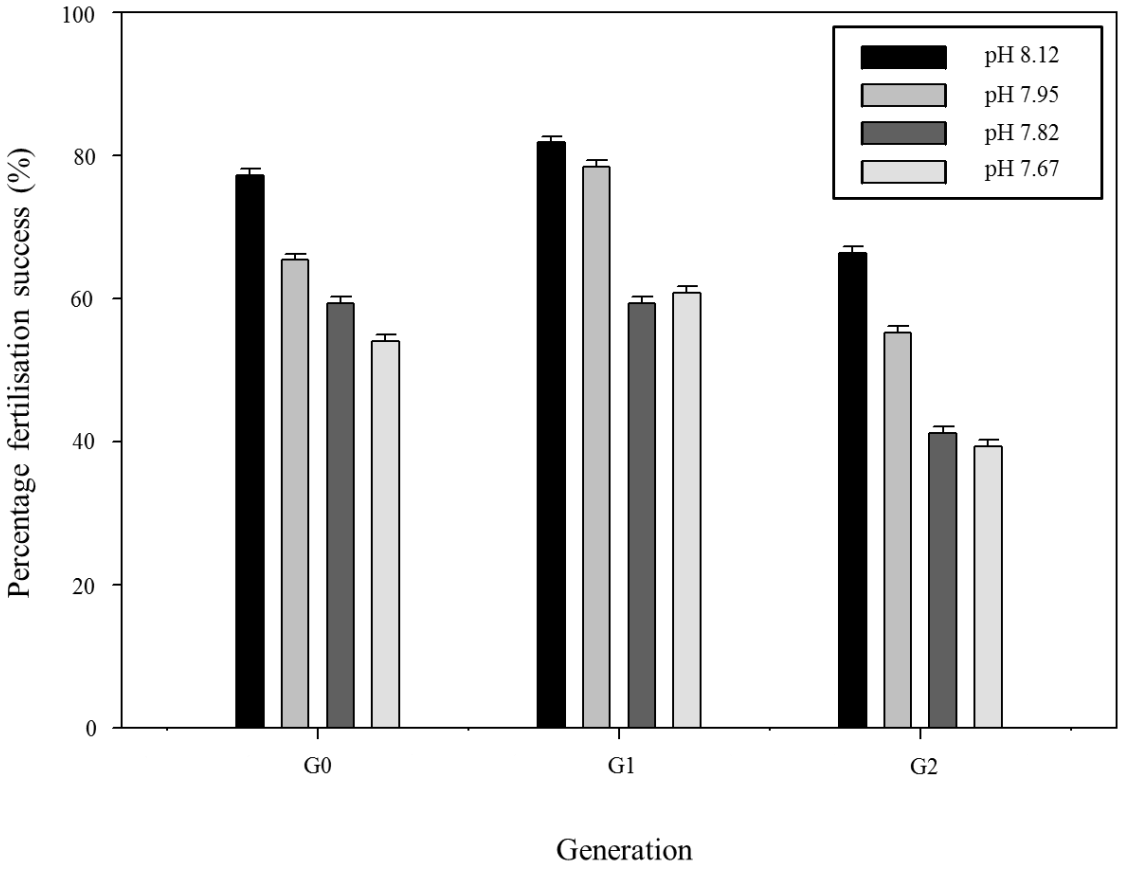

Figure 5.5; Percentage fertilisation success adjusted to a sperm density of $2.5x10^4$sperm ml^{-1}, data shown are estimated marginal means and SE.

5.4 Discussion

This study addresses multi-generational exposure to decreased pH on the reproductive fitness of the polychaete *Ficopomatus enigmaticus*. It is important to determine the effects that decreasing oceanic pH will have on the reproductive processes of organisms as it may compromise further development, as well as population growth and recruitment. The results of the current study show that when *F.enigmaticus* is exposed to lower pH conditions over multiple generations there is a significant effect on its reproductive success. Percentage sperm motility and sperm VCL, however, showed no significant impact of pH, although there was a significant impact of generation, with both sperm motility and sperm swimming speed significantly decreasing with subsequent generations.

Negative effects of increasing OA on reproductive endpoints have previously been reported in a range of marine organisms (see Chapter 1 Table 1.2). The current study is to the author's knowledge the first to examine the multi-generational effects of increasing OA on sperm motility and fertilisation success. This study reports a significant decrease in fertilisation success when exposed to decreased pH conditions and this trend is increased when exposed for subsequent generations. There was also a significant effect of generation on fertilisation success with all generations showing significantly different results. Moreover, increases in pH caused a significant reduction in fertilisation success, while decreases in pH resulted in lower fertilisation at all sperm densities.

Many studies have addressed the effect of OA on fertilisation success with contrasting results (see Chapter1, Table 1.2). Several theories have been proposed for a reductions in fertilisation success, including a slowing in the fast block to polyspermy (Reuter et al., 2011) reduction in egg fecundity (Dupont et al., 2012) and developmental delay (Kurihara and Shiryiama, 2004), all of which are viable explanations for the decrease seen here. A unique aspect of the current study, however, is the mutigenerational approach to sperm motility, fertilisation success and egg size. Two previous studies have examined multi-generational effects of OA on reproduction and development utilising copepods as study species (Kurihara and Ishimatsu, 2008; Fitzer et al., 2012), however neither looked at the same reproductive endpoints as the current study. Fitzer et al., (2012) found a significant reduction in number of nauplii of *Tisbe*

battagliai when exposed to lowered pH across three generations. Conversely, Kurihara and Ishimatsu (2008) reported no significant effect of decreased pH on survival, developmental rate, growth, fecundity or hatching success in the copepod *Acartia tsuensis*.

Studies on multi-generational effects of pollution on the killifish, *Fundus heteroclitus*, showed that when individuals were raised under high pollutant levels, they were more resistant to acute toxicity. This suggested genetic adaptation to the contaminants present in their environment. Adaptation came at a cost, however, as they were more susceptible to other environmental stressors such as hypoxia and photo-enhanced toxicity (Meyer and Di Giulio, 2003). More recent studies have considered the effects of parental exposure to decreased pH prior to spawning on subsequent generations. Parker et al. (2012) reported that by conditioning adults oysters (*Saccostrea glomerata*) to future oceanic pCO_2 conditions during reproductive conditioning, there was a positive carry-over effect on the resulting larvae, with those spawned to previously conditioned adults showing increased growth and increased developmental rate. These results are comparable to those of the current study as there was a significant increase in fertilisation success at Generation 1 when compared to generation 0, followed, however, by a significant decrease at Generation 2. The increase in fertilisation success seen here could be attributed to an adaptive maternal effect (Untersee and Pechenik, 2007; Marshall et al., 2010; Sanford and Kelly, 2011; Parker et al., 2012), which posits that in response to environmental stress females are able to increase maternal energy investment per individual by increasing egg size, and consequently also offspring size. This response has been shown previously to be beneficial for offspring as large offspring generally exhibit faster development, spending less time as planktonic larvae (Podolsky and Moran, 2006; Allen et al., 2008: Moran and McAllister, 2009; Matson et al., 2012; Parker et al., 2012).

An increase in maternal investment is often seen as an increase in egg size (Parker et al., 2012). The current study shows that under control conditions egg size stays relatively similar across all generations. The same pattern is repeated at pH 7.95 which shows a similar egg size at Generation 0 and 1. However, there is a significant increase in egg size at Generation 2, which is similar to the pattern seen at pH 7.82 and 7.67 where there is a significant increase in egg size at Generation 1. In contrast this increase in egg diameter is not seen at Generation 2 at pH 7.82 and 7.67 where eggs size

decreases. The results agree with the theory of adaptive maternal effect though the increase in energy input to producing larger eggs did not occur at lower pH treatments over subsequent generations. Rather egg size decreased significantly between Generation 1 and 2 in pH 7.82, and 7.67. This result suggests that the extra energy needed for egg provisioning is not available owing to possible energy reallocation. Larval development in non-feeding larvae is fuelled exclusively by the energy sequestered in the eggs. It has been suggested previously, however, that under environmentally stressful conditions, for example decreased pH, energy is reallocated from growth and reproduction to maintain whole organism homeostasis (Matson et al., 2012). It is possible that individuals at pH 7.95 were able to cope with decreased pH conditions for the initial generation without increasing maternal investment in the eggs, but this was not able to be maintained over the longer term as in subsequent generations (G2) egg size increased. These changes in maternal investment and egg size correlate with fertilisation success because at pH 7.82 and 7.67 fertilisation success was significantly higher than generation 0 at Generation 1, when eggs are at their largest, but decreased significantly at Generation 2 where there is a significant reduction in fertilisation success as well as egg size. In the short-term, therefore, organisms that are able to cope with decreased pH conditions may be unable to maintain high output through subsequent generations and longer time scales.

Previous studies concerned with the effects of eco-toxicants on invertebrate egg size have shown varying results. Studies concerned with pCO_2 on egg size in the brittle star *Amphuria filiformis* found no effect when exposed to increased CO_2 conditions (Wood et al., 2008). This study, however, collected gametes during the latent period of egg growth, after completion of vitellogenesis, which means eggs had finished growth prior to experiments. Studies utilising the sea urchin *Echinus mathaei* also showed there was no significant effect of pCO_2 treatment on egg size (Uthicke et al., 2012), although studies concerned with other environmental stressors, such as intraspecific competition and decreased temperatures, have shown an increase in egg size (Allen et al., 2008; Moran and McAllister 2009; Parker et al., 2012). It has been suggested previously that increased maternal investment in eggs can have effect on population dynamics (Benton et al., 2005) and phenotypic plasticity (Holbrook and Schal, 2004)

Percentage sperm motility and swimming speed are important to fertilisation success. The results of the current study indicate that the sperm of *F. enigmaticus* are

generally robust to decreasing pH with no significant effect of pH on sperm motility or swimming speed (VCL) though there were significant intergeneration differences. The few studies that have considered the effects of increasing pH on sperm motility (Havenhand et al., 2008; Havenhand and Schlegel 2009; Morita et al., 2009; Frommel et al., 2010; Caldwell et al., 2011; Lewis et al., 2012) obtained contrasting results, with the majority reporting a negative effect or no significant effect on percentage sperm motility and VCL (see chapter 1 table 1.2). Variations in sperm response to decreasing pH have previously been attributed to species specific-responses and differing experimental methodologies (Lewis et al., 2012). When compared to previous reports on sperm swimming speed, the sperm of *F.enigmaticus* appear to have a slower VCL than other species. For example, Havenhand et al. (2008) working with sea urchin *H. erythrogramma*, obtained VCLs of 26.3µm s^{-1} at pH 8.1 (control) and 23.5 and 26.3µm s^{-1} under decreased pH conditions. Similarly studies by Havenhand and Schlegel (2009) found sperm VCLs of 92.1 µm s^{-1} at pH8.1 and 94.3 µm s^{-1} at pH 7.8) in the oyster *C. gigas*; significantly higher values than obtained in the current study. These variations in sperm swimming speed could account for some of the interspecies differences in fertilisation success in the OA literature. It is possible that species with slower sperm swimming speeds may require a longer time for fertilisation and because fertilisation success times often remain the same between species, those with slower VCL may not have chance for fertilisation to occur.

In contrast to the current study, Havenhand et al. (2008) showed a significant negative effect of decreasing pH on sperm motility in the sea urchin *Heliocardaris erythrogramma*. A pH decrease of 0.4 units caused a significant reduction in percentage motile sperm and swimming speed. Similarly, Morita et al. (2009) showed significant effects of increased pH on sperm flagellar motility in the sea cucumber *Holothuria* sp. and the coral *Acropora digitifera*. As in the current study, previous research on the polychaete *Pomatoceros lamarckii* did not find a significant effect of decreasing pH on percentage motile sperm or VCL until pHs 7.4 and 7.2. In contrast to the current study, however, there was no significant effect of time, although sperm were tracked for only 30 minutes in total (Lewis et al., 2012). There was no significant impact of decreased pH on sperm motility or swimming speed of the oyster *Crassostrea gigas* although there was a significant difference between the male's sperm performance (Havenhand and Schlegel, 2009). Caldwell et al. (2011) obtained a positive effect of reduced pH on sperm motility measured as an increase in percentage motility and VCL.

In marine broadcast spawners, sperm released to the external environment are activated by the change in pH (Schlegel et al., 2012). For *F. enigmaticus*, however, a significant negative effect of reduced pH was seen on sperm swimming speed. It is possible that the reduced pH had a negative effect on the mitochondrial activity of the sperm. It is apparent from the results presented here that the effect of ocean acidification on sperm motility will be species specific with some species showing a greater robustness. It is possible that sperm motility is unaffected by decreasing pH is due to the robust nature of *F. enigmaticus*, which has shown resilience to other abiotic factors including salinity and temperature (Straughan, 1972; Fornós et al., 1997).

To the author's knowledge this is the first study to examine multi-generational effects of decreasing pH on sperm motility and VCL in a marine broadcast spawning invertebrate. Recent studies have concentrated on the effects of parental exposure to increased pCO_2 and the effects this has on their progeny. Uthicke et al. (2012) showed that decreasing pCO_2 had a significant effect on the ability of males to spawn. Although the results were presented as the averages of a scale of 1-3, it is difficult to use this as a reproductive endpoint as there will be high variability between individual males.

This is also thought to be the first study that addresses the issue of marine eco-toxicants on sperm motility and VCL. There have, however, been a few studies that have examined the effects of multi-generational exposure to chemical toxicants on sperm quality and gonad development. Nash et al. (2004), for example, found that when the zebra fish *Danio rerio* were subjected to environmental concentrations of ethynylestradiol (the bioactive oestrogen in the female contraceptive pill) over multiple generations, there was a significant effect on gonad growth and sperm quality. At the concentration of 5ng L^{-1} EE there was no expressible sperm present at generation one and none of the males sampled had normal testes.

In summary, this study suggests that exposure to reduced pH over multiple generations will have a significant negative effect on reproductive success but sperm motility will remain relatively unchanged. There is a need for further multi-generational studies to be conducted, as little is known about what effects multigenerational exposure to OA will have on organism's reproduction and development. It is possible that the reductions seen in fertilisation success will lead to a reduction in recruitment and survival. This is due to the developmental domino effect where exposure to stress during early development can result in latent deleterious effects, and this is because later

development is dependent on early stages of development (Byrne, 2011). As an ecosystem engineer if there is a significant reduction in fertilisation success of *F. enigmaticus* then repercussions will not be confined to a species effect but will encompass the ecosystem also.

Chapter 6: Final perspective

Oceanic conditions are changing due to increasing chemical inputs, such as CO_2 from the atmosphere, causing processes such as OA. Combined with these increases in OA it is predicted that there will be increases in temperature due to increases in the global heat budget as well as increases in occurrences of hypoxic events (Pörtner and Langenbuch, 2005; Guinotte and Fabry, 2008; Oschlies et al., 2008; Pörtner , 2008; Reid et al., 2009; Wohlers et al., 2009). These changes in oceanic conditions are posing new challenges to organisms. It has been suggested that the reproductive processes and early life stages of an organism are more sensitive to changing oceanic conditions than later life history stages (Pörtner and Farrell, 2008; Byrne et al., 2010a; Cooper et al., 2012). This could have serious consequences for species survival because early life stages and fertilisation success are known to act as a population bottleneck. Variations in reproductive capacity will cause problems for future generations cumulating in the possibility of reduced population size and abundance. It is therefore imperative to determine the effects that increasing OA and temperature will have on reproductive processes of an organism.

Previous research has been concerned with the effects that OA will have on the reproductive and developmental processes of marine invertebrates. However these studies have left gaps in our knowledge, with few of concentrating on the long term or multigenerational effects that OA and combined stressors will have on reproductive and developmental processes. The data presented here aim to fill the knowledge gaps left by previous studies as well as providing a novel approach to determining developmental fitness of organisms within the field of OA.

This thesis has increased our understanding of the reproduction and development of two important marine invertebrate species within a rapidly changing ocean. In this final chapter the data obtained will be discussed in terms of the effect of long-term and multigenerational exposure to predicted climate change scenarios on reproduction and development and the effects this may have on species abundance and survival. For the first species, the sea urchin *Paracentrotus lividus,* the effects of long-term parental exposure to ocean acidification (OA) on sperm motility, fertilisation

success and developmental stability were examined in isolation and in combination with temperature (Chapters 2 and 3) and hypoxic stress (Chapters 4) . The multigenerational effects of OA on the polychaete *Ficopomatus enigmaticus* were also studied in relation to sperm motility, fertilisation success, and egg size, to increase our current understanding of future effects of OA.

To date there have been several studies addressing the short term effects of increasing OA and temperature on reproductive processes (Lacoue-Labarthe et al., 2009; Parker et al., 2009; Byrne et al., 2010b; Findlay et al., 2010a ; Findlay et al., 2010b ; Parker et al., 2010; Parker et al., 2012;Thiyagarajan and Ko, 2012). These studies have looked in general at limited gamete and parental exposure to OA. However, there have been no studies that concentrate on long-term parental exposure and the effect that this will have on fertilisation success and only one which determine long term effects on early development (Dupont et al., 2012). The current thesis aimed to rectify this knowledge gap by providing results for long term exposure to OA and temperature and their possible effects. It is important to determine these effects as marine invertebrates form integral components of marine ecosystems and food chains. For example, the sea urchin *P. lividus* acts as an ecosystem engineer in many of the habitats it resides in, which means it has a large influence on the habitat and environment relative to its abundance. Changes in the ocean may alter the abundance of this species leading to changes in community structure, which in turn may have a detrimental effect on other organisms within an ecosystem, and on the ecosystem itself. Previous work has shown that energy allocation is another important consideration. Stumpp et al. (2011) found that when *Strongylocentrotus purpuratus* were raised under high CO_2 conditions, energy spent on somatic growth dropped to 39-45% from 78-80% under control conditions (pH 8.1 at 14°C). These variations in energy investment may have consequences for partitioning of the energy budget, as well as development in terms of developmental delay (Stumpp et al., 2011).

With the predicted increase in OA and temperature it is important to determine the long term effects this may have on invertebrate reproduction and development. One of the main hypothesises of the present study was that long term exposure to increased OA and temperature would negatively impact sperm motility, and fertilisation success. It was found that there was a significant negative effect of OA on fertilisation success and cleavage success of *P. lividus* at three, six and twelve months of exposure (chapter

two). The extent of the negative effects increased with increasing exposure and at 12 months there were no viable females found in the highest pCO_2 treatment. It is therefore possible that the lack of significant reductions in fertilisation or cleavage success reported in the majority of previous studies (Byrne et al., 2009; 2010a; 2010b; Ericson et al., 2010; Bechmann et al., 2011; Martin et al., 2011; Nguyen et al., 2012; Byrne et al., 2013) may be a result of short-term exposures in which gametogenesis did not occur under acidified conditions. In general the results of previous short exposure studies do not portray an accurate insight in to the effects of future OA as they do not take in to consideration vital process such as vitollogenesis occurring under acidified conditions. As all processes occur under ambient conditions it is likely that the gametes produced are far more resilient to decreased pH conditions as the stress during production was low, allowing for a greater amount of parental investment.

The reductions in fertilisation success could be attributed to energy being reallocated from gonad growth and maturation towards increased costs of maintaining cellular homeostasis (Wood et al., 2008). This reduction in parental investment could also have a knock-on effect on duration of development. Delaying development is likely to occur more frequently within a changing ocean leading to a mismatch between spawning and available food for larvae. For example, Søreide et al. (2010) reported that the breeding season of the Arctic grazer *Calanus glacialis* coincides with two major ice algae phytoplankton blooms. One of the ice phytoplankton blooms are followed by the break-up of the sea ice two months later. However reductions in sea ice thickness and earlier melting can advance the peak of primary production, so that less food is available for later spawning invertebrates. It is possible this phenomenon will be seen in more habitats with negative effects on species and community structure.

In contrast to the results seen for fertilisation success there were significant increases in sperm swimming speed at increasing pCO_2 at three and six months exposure; however after 12 months exposure this pattern was no longer seen (chapter 2). Variations in sperm motility have previously been attributed to the activation pH of sperm of many broadcast spawning marine invertebrates being activated at pH 7.6 (Christen et al., 1983) which is a significantly lower pH than that of the oceans currently. This means that under increased pCO_2 sperm may swim quicker as the pH is closer to that required for activation. In contrast to the current thesis previous studies concerning sperm swimming speed have reported a significant decrease under increased

pCO_2 (Havenhand et al., 2008; Morita et al., 2010; Lewis et al., 2012; Schlegel et al., 2012). It is possible that these variations are due to the different exposure times the study organisms are subjected to. By only testing sperm motility parameters at a single time point it is possible that the sperm may not have fully activated, thusly giving a false result of sperm swimming speed and percentage motility. It is also important to use sperm from acclimated individuals as it allows for an insight in to future effects of OA with gametogenesis occurring under acidified conditions. It has previously been reported that sperm spawned from adults raised under acidified conditions exhibit abnormal features with smaller head size and shorter tale length being exhibited (Dey et al., 2009). It is possible that these morphological variations could lead to changes in the swimming ability of sperm and thusly affect sperm motility and sperm swimming speed.

The variations and reductions seen in fertilisation and cleavage success and sperm motility will have an effect not only at the organism level but also at a population level, as reductions in larval fertilisation success could lead to fewer viable larvae and therefore reduced recruitment. Changes in population levels of sea urchins have previously been shown to affect the biodiversity and function of the environment. For example the over hunting of sea otters in the kelp forests of Alaska led to a population explosion of sea urchins which caused overgrazing of the kelp forests causing their collapse (Steneck et al., 2001). Similarly studies carried out in the North West Mediterranean found large variations in the population structure of *P. livdus*. These differences were attributed to variation in predator abundance. In areas with lower predation there were more urchins and they were larger. This increase in urchin population can have a cascade effect on the community by leading to negative effects on sea weed and sea grass populations (Sala and Zabala, 1996)

The results presented in Chapter 2 do not fully map the impacts of increasing OA and temperature throughout an organism's development. This is important as different stages of ontogeny may react differently to future changes in CO_2 and temperature, and so it may be dangerous to assume future abundances based only on fertilisation success. The majority of previous research has shown negative effects of increasing OA and temperature on larval development (Dupont et al., 2008; Kurihara 2008; Kurihara and Shirayama, 2004; Byrne et al., 2009; Clark et al., 2009; Parker et al., 2009; Talmage and Gobler, 2009; O'Donnell et al 2010; Sheppard-Brennand et al.,

2010; Byrne et al., 2011a; Byrne et al., 2011b; Chan et al., 2011; Gaylord et al., 2011; Gazeau et al., 2011; Lischka et al., 2011; Moulin et al., 2011; Stumpp et al., 2011a; Stumpp et al., 2011b; Yu et al., 2011; Catarino et al., 2012; Chan et al., 2012; Dickinson et al., 2012), with only three showing increases in growth (Munday et al., 2009; Dupont et al., 2010a; Parker et al., 2012).

Similar to previous research the current study hypothesised that increasing OA would negatively impact the developmental stability of *P.lividus* (chapter three). Presently it was found that there was a significant negative impact of OA and temperature on larval development, which was manifested as an increase in fluctuating asymmetry at increased pCO_2 and temperature treatments. It is possible that these increases in fluctuating asymmetry may have ramifications on later development as they are unlikely to fully halt an individual's development but they can cause subtle variations from perfect bilateral symmetry and decrease the fitness of an organism. Variations in larval fitness can have serious effects on several aspects of life history, such as echinopleutus swimming and feeding performance. Similar to the data presented presently, studies by Chan et al, (2011) found a reduction in larval stomach size under acidified conditions and they suggested this could significantly impact larval feeding. The developmental stability of organisms is important because if developmental stability is low this can compromise the precision by which adaptions can be reached (Pélabon et al., 2004), and if an organisms ability to adapt is compromised it could have serious effects on species survival. Along with reductions in developmental stability there were also increasing incidents of phenodeviants occurring. Phenodeviants are individuals which exhibit gross abnormalities, often these will not allow for larvae to develop further; therefore increases in their occurrence could have serious effects on survival. With the greatest occurrence of phenodeviants seen in the increased pCO_2 and temperature treatments it could have ramifications on future ecosystems, especially when combined with reductions in fertilisation and cleavage (chapter two) and developmental stability mentioned previously (chapter three).

As OA is not likely to happen in isolation it is also important to consider the effects that combined multiple stressors are likely to have on reproduction and development. There has so far been a narrow range of combined stressors studied, with the most common being temperature (Lacoue-Labarthe et al., 2009; Parker et al., 2009; Byrne et al., 2010b; Findlay et al., 2010a ; Findlay et al., 2010b ; Parker et al., 2010;

Parker et al., 2012;Thiyagarajan and Ko, 2012) and fewer concentrating on salinity and UV radiation and copper (Egilsdorrit et al., 2009; Parker et al., 2009; Parker et al., 2010; Dickinson et al., 2012; Foo et al., 2012; Thiyagarajan and Ko, 2012; Davis et al., 2013, Fitzer et al., 2013; Lewis et al., 2013). From the current study it appears that increasing temperature shows varying effects at different life stages with a synergistic effect seen of increased temperature and pCO_2 for fertilisation success and cleavage success (Chapter Two) but a negative impact seen for developmental stability and the occurrence of phenodeviants (Chapter three). These differences highlight the importance of considering a wider array of life history end points and environmental stressors which are likely to occur with increasing global climate change.

With the predicted increases in oceanic pCO_2 and temperature it is hypothesised that there will be an increase in hypoxic events (Diaz and Rosenburg, 1995; Diaz, 2001; Vaquer-Sunyer and Duarte, 2008). To date no studies have looked at the effects of increasing oceanic pCO_2 and increased occurrence of hypoxic events on reproduction and development of marine invertebrates. The current study aimed to try and fill some of the knowledge gap left previously by determining the effects of six months parental exposure to OA combined with a one off hypoxic event (Chapter four). The study found that there was a significant negative impact on fertilisation success when gametes and embryos were exposed to increased pCO_2 conditions and a hypoxic event. The lack of oxygen availability during the crucial process of fertilisation limits the organism's ability to produce viable larvae, with early embryonic development being reliant on aerobic respiration. With the increasing occurrence of hypoxic events especially in costal zones (Diaz and Rosenburg, 1995; Diaz, 2001; Pörtner and Langenbuch, 2005; Oschlies et al., 2008; Pörtner , 2008; Vaquer-Sunyer and Duarte, 2008) there could be serious negative effects on populations of *P.lividus*.

In contrast to fertilisation success, sperm motility was not significantly affected by increasing pCO_2 and hypoxia. It has however been widely suggested that sperm swimming speed is not the most influential factor in terms of fertilisation success and that an increase in sperm swimming speed could actually have increased negative impact on fertilisation success, as sperm which swim faster use up energy more quickly and may die quicker. It is important to consider previous literature regarding changes in species abundance and the effects it may have on a community and population level as this may give insight in to the effect of continuing global climate change. It is important

to bridge the gap between previous literature and current research to try and predict what effects may occur. It is also important to increase the knowledge of ecosystem level effects of OA.

The data presented here highlight the differing effects seen on varying life history stages; however, all endpoints examined here suggest that long-term exposure will have significant negative effects on reproduction and early larval development. However, one of the major limiting factors with the studies concerning *P. lividus* is that it is conducted using a single generation, and this limits the studies' ability to assess multigenerational effects and the possibility for acclimation across generations. The reason that multigenerational experiments were not attempted on *P. lividus* is due to the long generation time of the species. It was therefore necessary to identify another model species which could be used for multigenerational experiments. The polychaete *Ficopomatus enigmaticus* fulfils this need. It is a calcifying marine invertebrate which has a well-documented life cycle (Gabilondo et al., 2013), and is an ecologically and economically important species. *F. enigmaticus* is an invasive biofouling species which is becoming increasingly problematic. To date, there have been few studies concerning the effects of OA on biofouling species, and polychaetes as a phylum are particularly understudied (Lewis et al., 2012).

So far literature concerning the multigenerational impacts of OA on reproduction and development has concentrated primarily on copepods and has found varying results. There was no significant effect of increased pCO_2 on *Acartia tsuensis* which showed no significant effect on survival, egg production or hatching rate (Kurihara and Ishimatsu, 2008). In contrast *Tisbe battagliai* experienced a significant reduction in growth under increased OA (Fitzer et al., 2012). However, there are no previous studies concerning the multigenerational effects of increased OA on sperm motility and fertilisation success. These processes are, as mentioned previously, vital to future development and recruitment and need further attention.

The current thesis presents data regarding the effects of OA on sperm motility and fertilisation success across multiple generations in *F. enigmaticus* (Chapter five). It was hypothesised that multi-generational exposure to future OA conditions would inhibit sperm motility and fertilisation success. Presently it was found that there was a significant negative impact of both pH and generation on fertilisation success and egg diameter. The significant differences seen in egg size across generations could be

attributed to differences in maternal investment with increased maternal investment occurring at generation one for decreased pH conditions especially at pH 7.67 followed by a decrease at generation two. This pattern would suggest that individuals are able to increase the energy invested in the eggs when exposed to stressful conditions; however, this cannot be maintained long-term as seen by the reductions at generation two. When this reduction in maternal investment is coupled with a significant reduction in fertilisation success, possibly caused by energy reallocation and developmental delay, the consequences of increasing OA could be catastrophic. It has previously been found that when there are large aggregations of *F. enigmaticus* the water quality is significantly increased due to their filtration of the water (Keene, 1980; Davies et al., 1989). As the species generally inhabit marinas and polluted bodies of water it is possible that a reduction in the population could lead to a decrease in water quality which in turn can have an effect on the organisms living there.

Sperm motility and sperm swimming speed were not negatively affected by pH but there was a significant effect of generation. In contrast to the increases in sperm motility seen in chapter two and four there was no initial increase of sperm motility with increasing pCO_2 condition for *F.enigmaticus*. This further highlights the hypothesis that all species will react differently to increased OA conditions. Further studies are required to test the effect of increasing OA on sperm longevity as this would help to identify if variations in sperm swimming speed are linked to variations in sperm longevity.

Multigenerational effects of OA on life histories have begun to be investigated. OA is likely to happen over long time periods and by using multigenerational studies we are better able to determine the adaptive capacity of marine invertebrates. A shortfall of current research is the lack of literature pertaining to future populations as the majority of studies concentrate on modern populations and their ability to adapt to future conditions and not the future populations. By conducting multigenerational studies it is possible to see the effects on future generations. This is a factor highlighted in a recent study by Kelly et al (2013) which looked at the adaptive capacity of the purple sea urchin *Strongylocentrotus purpuratus* under future OA scenarios. It was found that there was a degree of genetic variation for body size under elevated pCO_2 which indicates that this trait can evolve. The ability to evolve was incorporated in to a model and it was predicted that the OA driven decrease in population growth rate would be 50% smaller than predicted in non-adaptation models.

An important avenue for future research would be to use multigenerational experiments combined with models to try and assess the future implications. This method has previously been used by Fitzer et al (2012) using the copepod *Tisbe battagliai*. However, all previous multigenerational work, concerned with reproduction and development, has concentrated on a maximum of three generations and it would be beneficial to increase the number of generations as this will allow for more comprehensive models to be created. It is important to compare with studies concerning different life history endpoints. Studies on the coccolithophore *Emiliania huxleyi* found that after exposing individuals to increased CO_2 for 500 generations, there were increased growth rates under acidified conditions *(*Lohbeck et al., 2012). Similarly the diatom *Phaeodactylum tricornutum* also exhibited increased growth after 20 generations of exposure to increased CO_2 (2012). These results show the importance for further multigenerational work to be carried out as the results differ from much previous short term research.

An issue which has become clear is that the response to OA and climate change is likely to be species specific (Dupont et al., 2013). To better understand this it is imperative that a wider array of organisms from a variety of habitats which encompass a range of trophic levels are used, This would allow for not only species specific variations to be identified but also an ecosystem perspective of future OA scenarios to be created. Currently studies are primarily concerned with single species effects of OA. However, OA will not just affect a single organism it will affect whole ecosystems and if certain species show vulnerability there could be serious consequences for ecosystems as a whole by causing shifts in community structure and composition.

The ability of marine invertebrates to survive a changing ocean is likely to differ with varying life history strategies. With changing oceanic conditions including increases in oceanic pCO_2 levels, temperature increases, increases in salinity and UV radiation, and increases in the occurrence of hypoxic events, there is likely to be a significant effect on reproductive processes of marine invertebrates. A large proportion of an organism's energy is allocated for gonad development and growth; however in times of stress energy is reallocated from these processes to more essential functions. If stressful conditions were to continue it is possible for certain species (e.g. Sea urchin) to reabsorb their gonads to access stored energy

It is clear from the data presented here that species utilising a broadcast spawning will experience a significant reduction in reproductive success and capacity as well as problems pertaining to later development. As this is a life history trait employed by numerous benthic marine invertebrate species, there are likely to be significant changes in populations and abundance in the future oceans. It is possible that species which utilise different reproductive strategies such as internal brooding of eggs may not experience the same severe reduction in fertilisation success and so may become more prevalent within the benthic community. Where fertilisation and larval development takes place within the animal, pH can be controlled by maintaining internal acid-base homeostasis via ion regulatory mechanisms and bicarbonate buffering. However it is also important to consider the increased energetic investment per juvenile associated with this strategy. Variations in reproductive strategy can be attributed to r- and k-selection theory, with broadcast spawners (such as *P. lividus* and *F. enigmatcus*) falling into the category of r selection as their reproduction is a low cost per individual offspring and there are large numbers produced. It is known that r-selected species deal better with a variable environment such as the high intertidal where pH and temperature may change on seasonal and tidal cycles and that along latitudinal gradients may out compete more k-selected species as temperatures rise (Rastrick and Whiteley, 2011; Whiteley et al., 2011; Rastrick and Whiteley, 2013). However, despite slower generation times and therefore lower evolutionary capacity, in a more acidic ocean it is possible that k-selected invertebrates by not releasing gametes or larvae into a suboptimum environment, as well as, providing more energy to cope with the elevated ATP demand of maintaining cellular homeostasis may offer the juveniles an advantage.

Although the current thesis has begun to fill the gaps left by previous research there are still areas in which the current research could be expanded. It would be possible to build on the results seen currently by taking further measurements and using further analysis. For example developmental stability has been studied presently through use of FA index. It is possible by taking further morphometric measurements analysis of echinopleutus swimming and feeding performance could have been analysed. This analysis may give insight in to developmental fitness at later stages than currently studied. It may also have been advantageous to measure gonad index throughout long term and multigenerational study which would have allowed data to be modelled with fertilisation success to better gauge an organism's response to OA. Another area which could have been explored would be to use sperm motility and

fertilisation success data to be modelled using a fertilisation kinetics model. This type of model will also allow for the input of physical sea water parameters as well as biological aspects. This may allow for the modelling of fertilisation kinetics in future oceans. This would allow for data from OA studies to be used to model the possible effects of abiotic factors on sperm swimming speed which in turn would allow for predictions to be made on the amount of fertilisation which is likely to occur.

It is apparent that the majority of OA research is conducted in a laboratory setting with few in-situ experiments occurring (Hall-Spencer et al., 2008; Calosi et al., 2013). A novel approach to determine the effects of OA on a natural ecosystem is through the use of naturally occurring volcanic vents which create a future OA environment. Using these natural laboratories would allow for insight into a future OA world and show an organisms ability to survive under increased OA conditions as well as helping to bridge the gap between laboratory and field studies. It also allows for the ecosystem effects of OA to be studied however it can be difficult to study combined multiple stressors using this methodology as manipulating temperature or other stressors in the field and on a large scale can be difficult. However through use of further mesocosom experiments it may be possible to examine the future effects of OA and combined stressors such as temperature and hypoxia on organisms. Previously mesocosom experiments have primarily concentrated on single species effects of OA. However it would be possible to utilise mesocosm experiments to examine ecosystem level effects of OA in a controlled environment. Unlike natural laboratories mesocosm offer the opportunity to control external factors such as pH level and temperature and to better control experimental parameters.

It is also important to consider a wide variety of species during OA research. The species used here fulfilled the need for the research as a whole. However two species were required to fulfil all of the research undertaken and it would have been beneficial to find an organism in which all of the reproductive endpoints could have been measured such as the polychaete *Plateneris dumerilii* as it has a short generation times (3-6)month as well as a well-documented life history. The current study was initially attempted with this species however problems occurred with synchronous spawning, as males and females were not reproductively viable at the same time and, therefore the time to reach sexual maturity was too long (minimum 6 months) for the current study it was not possible to use the species.

Early life history stages of organisms including fertilisation success, growth and settlement operate as a population bottleneck and control general patterns of abundance and distribution. Changes to these early life history stages such as fertilisation, and larval development could have serious impacts on these processes, by having a knock-on effect to species recruitment and survival. It is important to determine the impacts that OA will have on these vital life history stages as the ecological success of species is likely to be tied to the changing ocean. Changes in an organism's ability to reproduce and develop successfully may have serious ramifications on community structure causing animals which have a higher tolerance to OA to inhabit niches left by others. These changes in community structure could have negative effects on ecosystem functioning as well reductions in global biodiversity.

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