Acclimatisation and adaptive capacity of sea urchins in a changing ocean: Effects of ocean warming and acidification on early development and the potential to persist

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ABSTRACT

Anthropogenic emissions of carbon dioxide are causing the oceans to simultaneously increase in temperature and acidification. As the life cycle of many marine invertebrates involves broadcast spawning, understanding the sensitivity of gametes, fertilisation and developmental stages is essential to determining species vulnerability to ocean change stressors. This thesis uses free spawning echinoids as model species to address this issue with an aim to identify effects of ocean acidification on the extracellular jelly coat of the egg with a focus on four sea urchin species; *Centrostephanus rodgersii, Heliocidaris erythrogramma, Heliocidaris tuberculata, Echinometra mathaei*. As sea urchins provide a tractable system for study of gamete and fertilisation responses to stressors, as well as investigation of genetic variation, the genetic basis of resistance to climate change stressors is also investigated in polar, tropical and temperate sea urchins: *Sterechinus neumayeri, Pseudoboletia indiana* and *Heliocidaris erythrogramma.*

With regard to the gametes and fertilisation of marine invertebrates, most studies have focused on the sperm cell and fertilisation traits. Only a handful of studies have investigated effects of ocean change stressors on the egg. The effect of ocean acidification on the extracellular jelly coat of the egg was determined for four species: *Echinometra mathaei, Heliocidaris tuberculata, Centrostephanus rodgersii* and *H. erythrogramma*. After 15 minutes, there was a significant reduction in jelly coat area for *E. mathaei* and *C. rodgersii* of ~50% at pH 7.6 with no effect of decreased pH for the other two species. The reduction in jelly coat size at lower pH suggests that sperm-egg collision rates and fertilisation success will be negatively affected by ocean acidification conditions for some species. This may contribute to the contrasting outcomes for the fertilisation trait in ocean acidification experiments. If there are differences in the vulnerability of the egg coats of marine species, ocean acidification may act as a strong selective force at the gamete stage.

Many studies report that fertilisation in marine invertebrates is robust to ocean warming and acidification scenarios predicted for 2100. For different male-female pairs across the different echinoids, the response to ocean stressors was not so straight forward, with some pairs greatly affected by stressors while others were unaffected, with some even showing enhanced performance. Male-female gamete compatibility is an important determinant in fertilisation success for sea urchins with the male-female pair influencing subsequent development as seen

for each of the urchins examined in this thesis. It was clear that not all matings were equal and this was a potentially important source of genetic variation found. The pairs that remain unaffected or perform better in ocean change scenarios would be expected to seed future populations.

As the ocean continues to change in pH and temperature, marine species will need to acclimatise or adapt to avoid extinction. If marine populations possess adequate genetic variation in tolerance to climate change stressors, species may be able to persist. Breeding designs such as the North Carolina II can be used to identify the sources of genetic and environmental variances in embryo performance. This quantitative genetic approach was used for the Antarctic sea urchin *Sterechinus neumayeri* to explore how the contribution of sire and dam influenced the performance of cleavage stage embryos and blastulae, and how these contributions differed when exposed to stress from increased temperature (+3°C) and acidification (-0.3-0.5 pH units). Both stressors decreased cleavage success and the percentage of normal blastulae, with a negative interactive effect between stressors. The response to these factors differed among the sire-dam pairs indicating the influence of parents. The significant dam by temperature interactions indicated different performance among maternal half-siblings in response to increased temperature. As adaptation depends on additive genetic variance for stress tolerance being present in populations and there were no sire by stressor interactions found, *S. neumayeri* may need to rely on phenotypic plasticity to persist through an ocean decreasing in pH and warming, at least with respect to early development.

A quantitative genetic investigation of the effects of near-future ocean conditions on the early development success of the tropical sea urchin *Pseudoboletia indiana* showed that ocean acidification conditions (-0.3-0.5 pH units) decreased fertilisation across all dam-sire combinations with effects of pH differing among the pairings. Decreased pH reduced the percentage of normal gastrulae with negative effects alleviated by increased temperature (+3°C). A low genetic correlation indicated that genotypes that performed well at gastrulation in low pH did not necessarily perform well at higher temperatures thus different gene sets influence performance for the two stressors. Significant sire by environment interactions indicated the presence of heritable variation in tolerance of stressors at gastrulation and thus the potential for selection of resistant genotypes, which will enhance population persistence *P. indiana*. This species has recently colonised temperate latitudes and southern range edge populations of

P.indiana may benefit from future warming with potential for extension of their distribution in south east Australia.

The quantitative genetic approach was also used to investigate the effects of near-future acidification and warming across the life cycle of the temperate sea urchin *Heliocidaris erythrogramma*. This fast developing species, with access to the juvenile in 3–5 days, was used as a model system to assess the response of different genotypes. Across fertilisation to metamorphosis, maternal legacy was important, with dam identity significantly interacting with stressors. Mothers enhanced offspring performance likely through the influence of maternal environmental history and developmental plasticity. Across the genotypes tested, fertilisation was negatively affected by increased temperature, but not pH. Larval development was compromised in low pH, but not temperature. By the settled juvenile stage no impact of warming or acidification was evident and this was likely due to selective mortality of sensitive individuals. Across all environments tested, the juveniles exhibited a similar ability to calcify. The impact of warming and acidification on development after fertilisation was influenced by parents, with the offspring of some dam-sire pairs more sensitive than others. That the progeny of some sire-dam pairs showed high stress tolerance indicates the potential for selection of resistant genotypes, adaptive variation to facilitate persistence of *H. erythrogramma* populations.

Data from the quantitative genetic experiments across the three species show inherent differences in the response of gametes to ocean stressors, as well as differences in gamete compatibility which can drive differing responses to ocean change. Across polar, tropical and temperate sea urchins, the mechanisms that may facilitate persistence in a changing ocean differ, revealing the potential winners and losers. For *S. neumayeri*, as no genetic variation was present in response to ocean change stressors likely due to its stenothermal characteristics, maternal effects and sire-dam effects will be essential in buffering development. For *P. indiana*, the species which covers the largest latitudinal distribution and shows the greatest amount of heritable genetic variation in responses to stressors, increased temperature will facilitate persistence and expansion of populations in NSW. For *H. erythrogramma*, inherent resilience likely due to preadaptation to a habitat which highly fluctuates in temperature and pH will facilitate survival. Furthermore maternal effects were significant in this species indicating that dams will buffer offspring through phenotypic plasticity. *Heliocidaris erythrogramma* have a significant maternal investment in production of large eggs, preloaded with maternal protective

factors and this is likely a source of phenotypic plasticity. Maternal effects are likely to be an important mechanism in persisting through a changing ocean for this species.

This thesis provides a novel contribution to our understanding of the potential for climate adaptation in the face of ocean acidification and warming in using the multistressor approach and incorporating gametes and dam-sire compatibility traits at fertilisation in quantitative genetic selection tests. The results indicate that the impacts of ocean change stressors are different across species and so different mechanisms will likely be used to acclimatise and adapt. The species investigated will likely have different outcomes in a changing ocean. However, further research across complete life cycles as well as multigenerational studies are needed for more accurate predictions on how species' distributions may change as the ocean continues to increase in temperature and acidification.

CHAPTER ONE: GENERAL INTRODUCTION[1]

1.1 Climate Change

Climate change refers to the long-term change in weather patterns and distributions, where the statistical properties of the world's climate system are considered (Hoegh-Guldberg and Bruno, 2010; Howes et al., 2015). The Earth's climate fluctuates naturally due to drivers such as the El Niño-Southern Oscillation, one of the most powerful sources of yearly climate variability (Tudhope et al., 2001). Additionally, anthropogenic influences have caused a significant increase in climate fluctuation. Since the industrial revolution, the burning of fossil fuels, agricultural practices and deforestation have led to an increase in the emission of carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O) and halocarbons, with CO_2 being the major greenhouse gas (IPCC, 2013). Global CO_2 atmospheric concentration has already risen from 280 to 400 parts per million (ppm) since pre-industrial times (IPCC, 2013; Howes et al., 2015).

Anthropogenic-driven climate change is causing increases in atmospheric temperature, solar radiation, storminess and precipitation. Furthermore, climate change is also affecting the oceans through changes in upwelling, currents, sea levels, sea surface temperature (SST), ocean stratification and ocean pH (IPCC, 2013; Howes et al., 2015). These changes will influence the abundance, distribution, phenology and physiology of many marine species, in particular marine ectotherms that have limited physiological regulative capacity (Poloczanska et al., 2013; Przeslawski et al., 2015). Most importantly, as these stressors occur concurrently, their potential interactions may have a negative influence on the world's ecosystems (Hoegh-Guldberg, 1999; Howes et al., 2015).

1.2 The Impact of Climate Change on the Oceans

The emission of CO_2 and other greenhouse gases have enhanced the greenhouse effect, whereby atmospheric gases trap radiation from the sun and surface of the Earth. This is causing an increase in both atmospheric temperature and SST (IPCC, 2013). Since 1960, 90% of the excess

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[1] The introduction has been submitted for publication as two reviews:
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heat in the atmosphere has been absorbed by the ocean and over the past century, SST has risen 0.4-0.8°C, with warming observed at depths of 6000 feet (Sabine et al., 2004; Roemmich et al., 2015). Coincidentally, thermal expansion and melting of glaciers due to warming has contributed to a rising sea level (IPCC, 2013). The Intergovernmental Panel on Climate Change (IPCC) predicts that the surface ocean temperatures will increase by 1.2 to 3.2°C by 2100 (IPCC, 2013; Howes et al., 2015; Gattuso et al., 2015).

The oceans are a sink for atmospheric CO_2 and have absorbed around 40% of global emissions (Zeebe et al., 2008; IPCC 2013). In seawater, dissolved CO_2 forms carbonic acid and causes a decrease in carbonate ion concentration and an increase in bicarbonate ion concentration. This results in a release of hydrogen ions to maintain equilibrium thus lowering pH, a phenomenon known as 'ocean acidification'. Since the industrial revolution, the mean pH of ocean surface water has decreased from pH_{NIST} 8.13 to 8.05, corresponding to an increase of 26% in hydrogen ion concentration. Continued uptake of CO_2 by the oceans will continue to reduce ocean pH (Caldeira and Wickett 2003). By 2100, ocean pH is expected to drop by 0.14 to 0.4 units (Caldeira and Wickett, 2003; IPCC, 2013; Gattuso et al., 2015; Howes et al., 2015).

For marine ectotherms, with cephalopods being a potential exception (Melzner et al., 2009), two factors co-vary with CO_2-driven decrease in ocean pH: (1) hypercapnia, the increase in organism partial pressure of CO_2 (pCO_2) and (2) the decrease in saturation of calcium carbonate ($CaCO_3$). Hypercapnia is coupled to acidosis of cells which can hinder metabolism, leading to impaired growth and reproduction (Raven et al., 2005; Fabry et al., 2008; Melzner et al., 2009). Carbonate ions combine with the hydrogen ions to form bicarbonate thus decreasing the concentration of $CaCO_3$ in seawater. With increasing ocean acidification $CaCO_3$ seawater saturation decreases and this will reduce the amount available for marine calcifiers, such as those who build shells or skeletons (Guinotte and Fabry, 2008; Kerr, 2010; Howes et al., 2015, Figure 1.1).

1.2.1 Ocean Change in Eastern Australia

In eastern Australia, rates of ocean warming are approximately 3-4 times faster than many regions. Australian marine invertebrates are facing considerable warming, especially intertidal and shallow subtidal species (Ridgeway, 2007; Hobday and Lough, 2011; Poloczanska et al.,
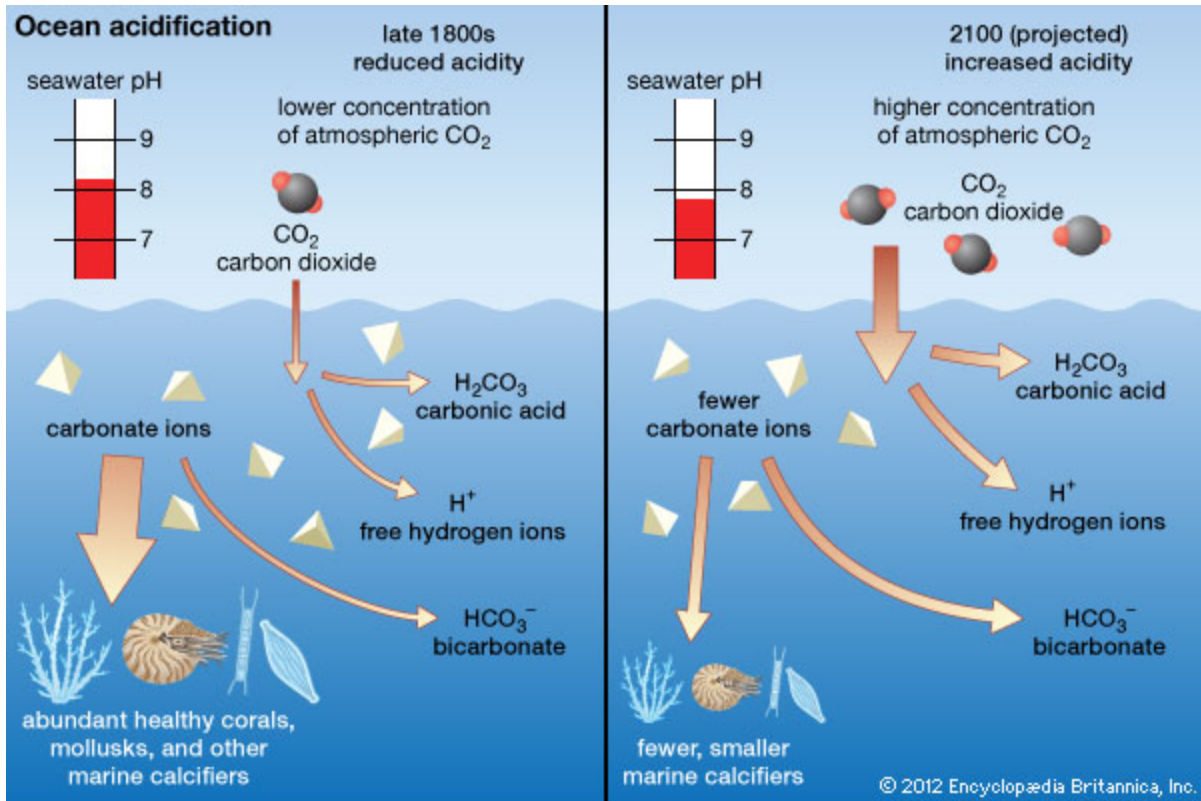
Figure 1.1. There is a fine balance between CO_2, carbonic acid, bicarbonate and carbonate ions in seawater because all of these factors co-vary in the ocean carbonate system. The dissolved CO_2 reacts with seawater to form carbonic acid, which ionizes and forms bicarbonate and carbonate ions. Carbonate ions combine with the hydrogen ions to form bicarbonate, reducing the availability of carbonate for marine calcifiers (Encyclopaedia Britannica, 2012).

2012; Wu et al., 2012). This warming is associated with climate driven strengthening in the poleward flow of the East Australian Current (EAC), which has led to warmer waters penetrating further south into the Tasman Sea (CSIRO, 2007; Ridgway, 2007; Wu et al., 2012). Sea surface temperature in this region has already increased by 2°C in the past 100 years and is predicted rise in SST of 2 to 4°C and drop in pH by 0.2 to 0.5 units by 2100 (Poloczanska et al., 2007; Hobday et al., 2006; Figueira and Booth 2010; Poloczanska et al., 2012; IPCC, 2013). In addition to aerial warming and more frequent heat waves, coastal marine invertebrates inhabit high-stress environments which are only expected to intensify with climate change (Harley et al., 2006; Somero, 2010).

Due to recent poleward movement of the East Australia Current (EAC), many species have shown southward movement of their distribution (Figueira and Booth, 2010; Poloczanska et al., 2013). With continued strengthening of the EAC in conjunction with global warming, many marine species, especially cold-temperate species, will be highly affected with a major concern for the fauna in Tasmania (Hobday et al., 2006; Booth et al., 2007; Ling et al., 2009; Figueira and Booth, 2010). The deficiency of substitute land mass between southern Australia and Antarctica means that southern temperate species will have no suitable habitat and are likely to be lost and thus Antarctic fauna are also of particular concern (Hobday and Lough, 2011). Eastern Australia as a climate change hot spot has major implications for Australian marine systems (Hobday and Lough, 2011; Poloczanska et al., 2012; Wu et al., 2012; Poloczanska et al., 2013).

1.3 Impacts of Ocean Change Stressors on the Gametes and Fertilisation of Free Spawning Marine Invertebrates

For many marine invertebrates, the life cycle involves broadcast spawning where a large number of eggs and sperm are released and fertilised in the water column. Spawning often takes place synchronously within the population, usually triggered by water temperature, photoperiod or other environmental factors (Byrne, 2001; Arnone et al., 2015). Marine invertebrates possess a characteristic planktonic larval stage with many echinoderms having pelagic, feeding larvae, a stage where skeletogenesis may begin (Komatsu and Shosaku, 1993; Young, 2002). The larvae can spend anywhere from hours to months in the water column before metamorphosing into a juvenile. Thus, larvae represent an important dispersal stage before settlement as a benthic adult

(Kurihara, 2008; Figure 1.2). Developmental stages are key to assessment of species vulnerability to ocean change stressors as they represent the most sensitive stage of their life history (Thorson, 1950; Pechenik, 1987).

Marine invertebrates will experience simultaneous exposure to ocean change stressors likely to have interactive effects. The combined effects of multiple stressors can be greater than individual effects hence the need for multistressor studies to represent real life future scenarios (Byrne 2012; Przeslawski et al., 2015). A major group of invertebrates likely to be impacted by changing ocean conditions include echinoderms, the phylum of focus in this thesis. A meta-analysis of multistressor studies found echinoderms to be one of the most vulnerable phyla to ocean stressors (Kroeker et al., 2013; Przeslawski et al., 2015). Generally, studies have found that early developing embryos (fertilisation, blastulae, gastrulae) are more resilient than later embryonic stages (larvae) to ocean warming and acidification (Byrne et al., 2009; Ericson et al., 2012).

1.3.1 Impacts on Spermatozoa

As the swimming behaviour of sperm is key to fertilisation success, many studies investigate effects of ocean change stressors on the behaviour of spermatozoa (Table 1). Increased temperature has been shown to stimulate sperm metabolism, facilitate the acrosome reaction and increase swimming speed and motility, thereby enhancing fertilisation success (Kupriyanova and Havenhand 2005; Byrne, 2011). In more recent studies, increased temperature enhances sperm swimming speeds in the sea urchin *Psammechinus miliaris* but not the percentage of motile (i.e. moving) sperm (Caldwell et al., 2011; Table 1). As sperm have a limited activity window that decreases with increased temperature, projected increases in SST may decrease the longevity of sperm, as shown for spermatozoa from sea urchins exposed to warm conditions (+4-6°C) (Christen et al., 1986; Binet and Doyle, 2013; Table 1).

Incubation of sperm of the sea urchin *Heliocidaris tuberculata* for several hours at +4 and 6°C reduced their longevity by > 60% as determined through fertilisation success (Binet and Doyle, 2013). The spermatozoa lost their ability to fertilise before their mitochondria stopped functioning (Binet and Doyle, 2013). As active mitrochondrion were present in non-viable sperm, the mechanisms underlying loss of function was not clear. The sperm of some sea urchin species can fertilise eggs several hours after release (Williams and Bentley 2002; Johnson and
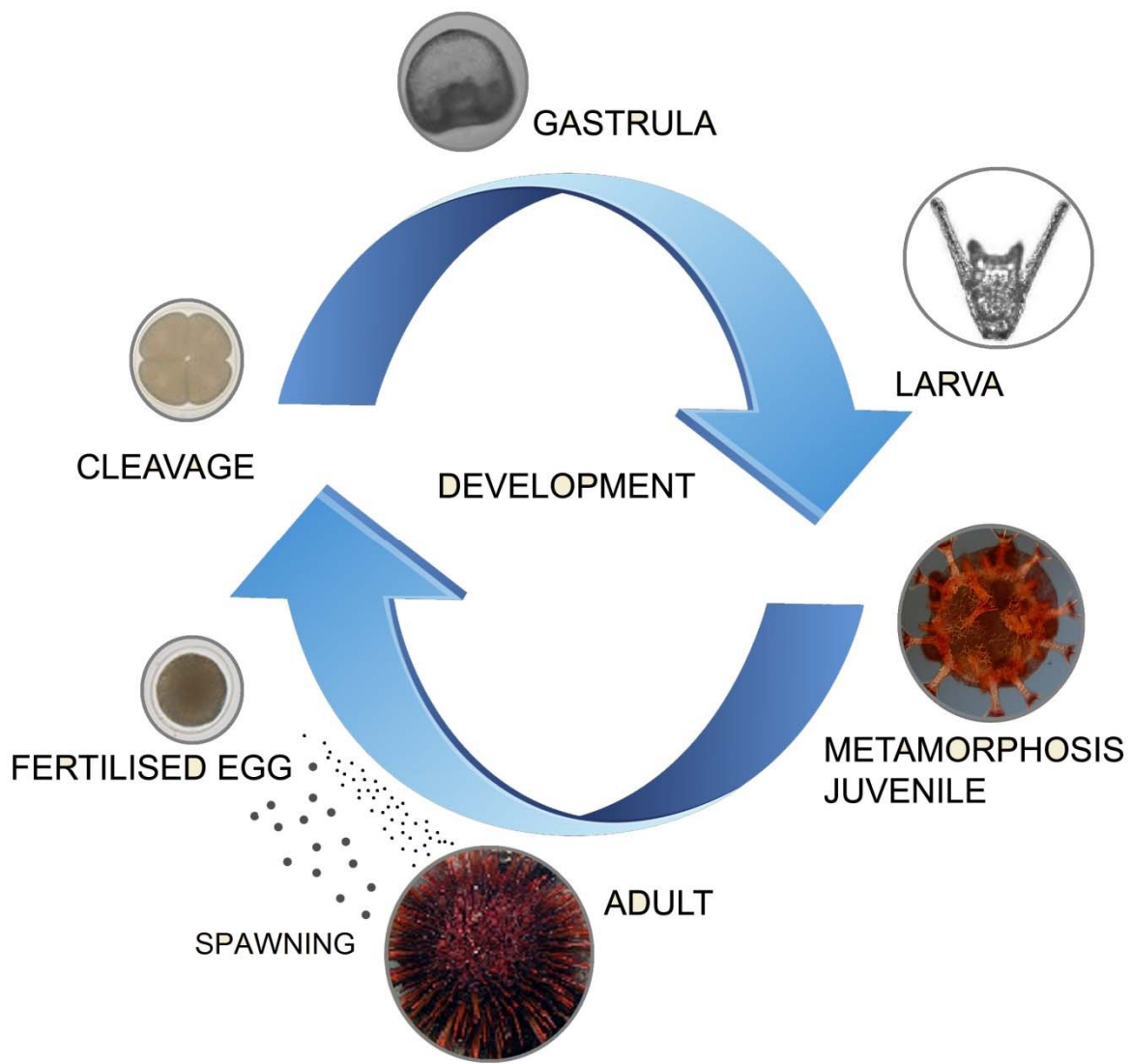
Figure 1.2. Development of a typical marine invertebrate. Eggs and sperm are released into the water column. The fertilised embryos develop through a planktonic larval stage and then metamorphose into a juvenile, where settlement occurs.

Yund 2004; Lauzon-Guay and Scheibling, 2007). Sperm longevity is an important factor in determining fertilisation success and may vary between species.

Low pH and hypercapnia are known to narcotize sperm (Melzner et al., 2009). In vivo, low gonad pH maintains the sperm in a dormant state, inhibiting respiration and motility keeping sperm quiescent prior to spawning (Johnson et al., 1983; Ward et al., 1985; Brokaw 1990). Release of sperm into seawater results in the uptake of sodium into the cell, triggering a release of hydrogen ions. This causes an increase in internal pH, which activates mitochondria and sperm motility (Christen et al., 1986; Hamamah and Gatti, 1998). It is long known that sperm require an elevation in pH for activation, as shown in studies that use various agents to increase pH to activate sperm of asteroids (e.g. Mortensen, 1921; Nakajima et al., 2005; Uthicke et al., 2013) and echinoids (Christen et al., 1986).

Most studies focus on the impacts of ocean acidification at near and far future projected levels (pH 7.6 to pH 8.1; IPCC 2013) on sperm behaviour (Table 1). Some studies also include extreme pH levels (< pH 7.4) (e.g. Lewis et al., 2013; Campbell et al., 2014). The behaviour of sperm from a diversity of marine invertebrates has been investigated, including sea urchins (Caldwell et al., 2011; Havenhand et al., 2008; Schlegel et al., 2012; Schelgel et al., 2015), sea stars (Uthicke et al., 2013), oysters (Havenhand and Schlegel, 2009), corals (Morita et al., 2010; Nakamura and Morita, 2012) and polychaetes (Lewis et al., 2013; Schlegel et al., 2014). Across these studies, ocean acidification is often reported to cause a decrease in the percentage of motile sperm and to decrease sperm swimming speed. However, for species such as the sea urchins *Hemicentrotus pucherrimus* and *Strongylocentrotus nudus*, there was no impact of this stressor on sperm behaviour (Sung et al., 2014). For two other sea urchin species, an increased sperm speed in ocean acidification scenarios was reported (Graham et al., 2015; Caldwell et al., 2011; Table 1). The metabolism of sperm is driven by mitochondrial respiration (Schlegel et al., 2015). Reduction in the mitochondrial membrane potential as reported for the sea urchin *Centrostephanus rodgersii* in response to ocean acidification conditions (-0.3-0.5 pH units) is a mechanism suggested to contribute to the reduction in sperm swimming speed reported for several urchins (Schlegel et al., 2015; Table 1).

At very low pH (7.47), the DNA of sperm from the polychaete *Arenicola marina* was damaged, as determined by the comet assay (Campbell et al., 2014). Although this low pH does

not reflect a near future ocean scenario, fertilisation in this worm occurs in the burrow. The actual pH for fertilisation in nature is not known.

A recent study highlights the importance of investigating multistressor effects on gametes. In a study of the impacts of decreased pH and oxygen (hypoxia) on sperm in the sea urchin *Paracentrotus lividus*, low pH increased sperm velocity while hypoxia deceased sperm velocity. Thus in the combined stressor scenario, the antagonistic effects equaled each other and sperm swimming speeds did not differ from controls (Graham et al., 2015).

Several studies show that there are striking differences between the performance of the sperm of individual male sea urchins, tunicates and polychaetes, where some males show decreased fertilisation success in an acidifying ocean while the performance of others is enhanced (Table 1). These differences may be due to gamete quality which can vary with collection time and parental physiological history (Crean et al., 2013). Differences in the environment of the parent can affect performance of their gametes as shown for the ascidian *Styela plicata* (Crean et al., 2013). For sea urchins, the impacts of stressors on individual males show that not all sperm are equal and not all males have similar fertilisation success (Schlegel et al., 2012; Foo et al., 2014; Sewell et al., 2014). This is likely to contribute to the contrasting results for the sperm swimming speed between semen samples from different males of the sea urchin *H. erythrogramma* in response to decreased pH (Havenhand et al., 2008; Schelgel et al., 2012). If these differences are heritable, ocean acidification may provide a source of selection against the susceptible phenotypes (Hoffmann and Parsons, 1991).

Sperm motility and swimming speed can be analysed using computer assisted sperm analysis (CASA) programs (Figure 1.3) (e.g. Caldwell et al., 2011; Campbell et al., 2014; Graham et al., 2015). CASA is a free plugin available for the Image J software (Wilson-Leedy and Ingermann, 2007) and provides measurements of many parameters. Sperm curvilinear velocity and average path velocity can precisely be identified. This enhances the accuracy of the data in comparison to studies which involve observation of sperm under the microscope and scoring by eye (e.g. Barros et al., 2013).
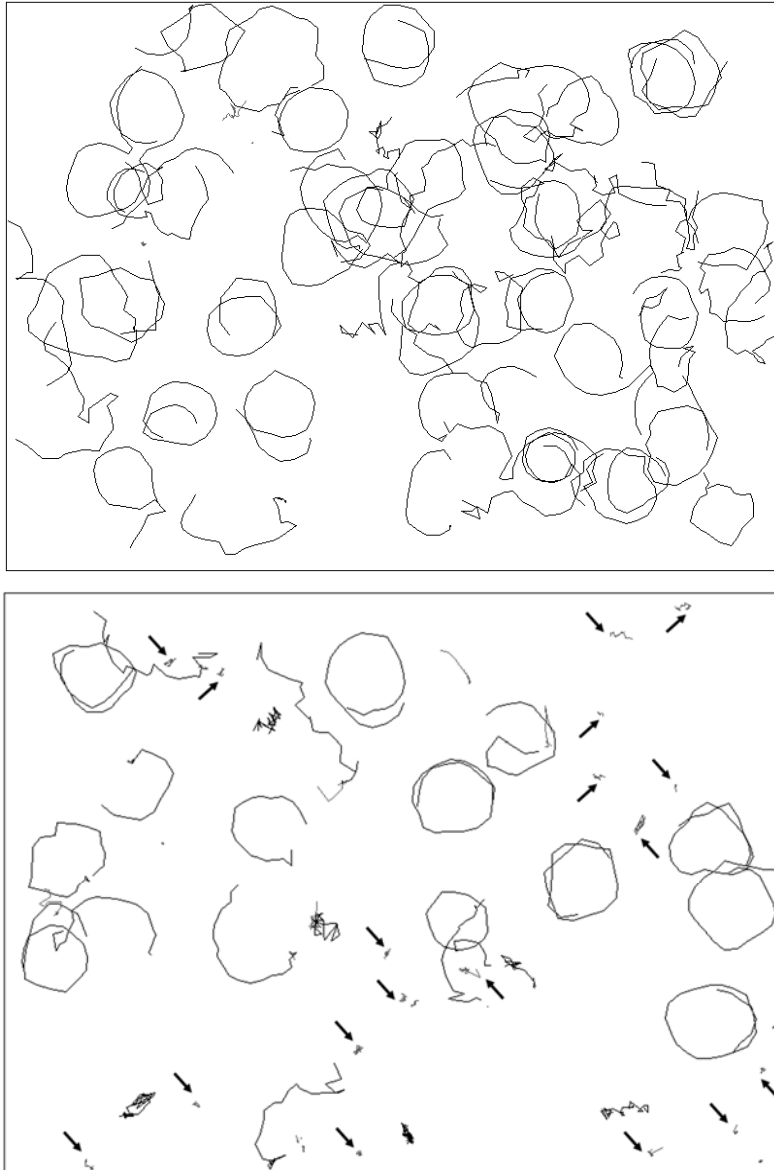
Figure 1.3. Sperm movement patterns of the sea star *Patiriella regularis* provided by the computer assisted sperm analysis (CASA) plug in for Image J. CASA can be used to quantify many sperm traits to determine how they change when exposed to stressors. The top panel shows sperm in control pH seawater conditions. Note the circular pattern of movement, characteristic of echinoderm sperm (Miller, 1985). The bottom panel shows sperm in pH 7.6 conditions. An increase in the number of immotile sperm (arrows) is evident at decreased pH (from Foo, in prep).

Table 1. Effects of ocean change stressors on the behaviour of marine invertebrate spermatozoa in response to near and far future, and extreme pH and temperature levels (IPCC, 2013). The pH levels are those provided by the study and are all pH_{NIST}. pCO_2 values are given when provided by the study.

Species	Stressor	Trait assessed	Result	Reference
Echinodermata				
Paracentrotus lividus	pH (8.08, 7.93/380, 750ppm) AND hypoxic conditions	Sperm motility Sperm velocity	Sperm swimming speed increased in pH 7.93 and decreased under hypoxic conditions, where the combined treatment resulted in swimming speeds similar to the control. For sperm motility, there was a combined negative effect of stressors on the percentage of motile sperm.	Graham et al., 2015
Centrostephanus rodgersii	pH (8.1, 7.8, 7.6/ 435, 950, 1558ppm)	Sperm mitochondrial membrane potential Sperm motility Sperm velocity	Sperm MMP was significantly reduced in pH 7.8 and further reduced in pH 7.6. Sperm motility and speed increased in 7.8, but were decreased in 7.6 treatments. Substantial inter-individual variation in responses of sperm characteristics to ocean acidification found in this study may increase the possibility for selection of resilient phenotypes.	Schlegel et al., 2015
Hemicentrotus pulcherrimus and *Strongylocentrotus nudus*	pH (7.99, 7.96, 7.92, 7.78, 7.69, 7.59/380, 450, 550, 750, 1000, 1500ppm)	Sperm motility Sperm velocity	Swimming speed and motility were unchanged under all pH levels tested.	Sung et al., 2014
Heliocidaris tuberculata	Temperature (20, 24, 26°C)	Sperm longevity Mitochondrial activity	Decreased sperm longevity in both increased temperature levels. Presence of active mitochondria even in non-viable sperm.	Binet and Doyle, 2013
Psammechinus miliaris	pH (8.06, 7.95, 7.82, 7.67) AND temperature (14, 17, 20°C)	Sperm velocity Sperm motility	Decreased pH levels of 7.95 and 7.67 increased swimming speed and motility. Increased temperature increased swimming speed with no effect on % motile	Caldwell et al., 2011
Heliocidaris erythrogramma	pH	Sperm velocity	Decreased pH caused a decrease in sperm	Havenhand et al., 2008

	(7.7/1000ppm)	Sperm motility	swimming speed and % motile	
Heliocidaris erythrogramma	pH (8.1, 7.8, 7.6 /970, 1600ppm)	Sperm velocity Sperm motility	No effect of decreased pH on swimming speeds. pH levels of 7.8 caused a reduction in % motile with a further reduction in % motile at pH 7.6.	Schlegel et al., 2012
Holothuria spp.	pH (8.03, 7.77., 7.69, 7.64, 7.31, 6.55/400-475, 775-1005, 930-1260, 905-1660, 2115-3585, 12600-21100ppm)	Sperm motility	pH levels of 7.69 and below caused a decreased in the % motile.	Morita et al., 2010
Acanthaster planci	pH (8.1, 7.9, 7.7 /520, 877, 1658ppm)	Sperm velocity Sperm motility	pH levels of 7.9 and 7.7 decreased swimming speed and the % motile.	Uthicke et al., 2013
Annelida				
Arenicola marina	pH (7.77, 7.47/ 1400, 3000ppm) AND copper toxicity	Sperm motility Sperm velocity	Sperm motility was slightly enhanced in pH 7.77 and slightly reduced in pH 7.47. Sperm velocity was decreased in pH 7.77 and 7.47. Copper decreased both motility and velocity. Furthermore pH 7.47 and exposure to copper induced significant sperm DNA damage.	Campbell et al., 2014
Galeolaria caespitosa	pH (8.1, 7.8, 7.6/427, 971, 1597ppm)	Sperm velocity Sperm motility	Sperm motility and sperm swimming speeds significantly decreased at pH 7.8 with a further decrease at pH 7.6. Resilient sperm may increase the possibility for selection of resilient phenotypes under decreasing pH.	Schlegel et al., 2014
Pomatoceros lamarckii	pH (range 8.1–7.2/302 - 3781ppm) AND copper toxicity	Sperm velocity Sperm motility	% of motile sperm and sperm velocity were significantly reduced in the more extreme treatments of pH levels < 7.4.	Lewis et al., 2013
Cnidaria				
Acropora digitifera	pH (8.15,8.05,	Sperm motility	Sperm motility decreased in pH 8.05 and	Nakamura and Morita

	7.74/300, 400, 1000 ppm)		further decreased in pH 7.74.	2012
Mollusca				
Crassostrea gigas	pH (8.2, 7.87, 7.48/580, 1386, 3573)	Sperm motility	Sperm motility was observed to decrease in pH 7.87, and to further decrease in pH 7.48.	Barros et al., 2013
Mytilus galloprovincialis	pH (8, 7.6/380, 1000ppm)	Sperm velocity Sperm motility	Sperm motility and velocity decreased in low pH.	Vihtakari et al., 2013

1.3.2 Impacts on Eggs

Few studies investigate the effects of ocean warming and ocean acidification on the egg, often the biggest cell produced by marine invertebrates (Table 2). Of those that do, most examine the effects of temperature on egg size (e.g. Dugan et al., 1991; Simonini and Prevedelli, 2003; Steer et al., 2004; Table 2). For studies on molluscs, annelids and crustaceans, results are species specific where animals reared in increased temperature produced either larger (Lacoue-Labarthe et al., 2009) or smaller (Simonini and Prevedelli, 2003) eggs or there was no difference in size (Dugan et al., 1991; Steer et al., 2004).

An early study on the heat resistance of the eggs of several echinoderms and molluscs determining the heat stress level causing impaired cleavage showed that heat resistance depended on the temperature conditions that the female experienced during oogenesis and the temperature that the species normally experience (Andronikov, 1975). This study showed that the environmental history of the female influenced the thermal stress resistance of the egg. This phenomenon appears common in nature where maternal environmental history can influence the size of the egg (Moran and McAlister, 2009) and the tolerance of fertilisation and development to increased temperature (Byrne et al., 2009; Byrne et al., 2011).

For studies which investigated effects of temperature on the egg in the context of ocean warming, exposure of eggs of the sea urchin *Heliocidaris tuberculata* for three hours to temperatures of 4 and 6°C above ambient did not affect egg viability for fertilisation (Binet and Doyle, 2013).

For studies that consider the effects of decreased pH on eggs, acclimatisation of females in low pH treatments for seven weeks had no effect on oocyte size for the sea urchin *Echinometra mathaei* (Uthicke et al., 2013). For the sea urchin *Sterechinus neumayeri*, after 6 months raised in low pH and increased temperature treatments, eggs in the control treatment were the largest. However after 17 months, the largest eggs produced belonged to the lowest pH treatment (Suckling et al., 2015).

In response to immersion in pH 7.6–7.84 seawater, cuttlefish eggs (*Sepia officinalis*) swell resulting in an increase in egg size (Dorey et al., 2013). It is not known what causes the swelling but may indicate damage to eggs. The outcome for the eggs with respect to performance is not known.

Another factor which would decrease fertilisation success of the eggs would be changes in the efficiency of the block to polyspermy, which has been found to decrease in eggs exposed to pH 7.55 for the sea urchin *S. franciscanus* (Reuter et al., 2011).

Egg and jelly coat derived compounds are important factors contributing to fertilisation success and are best studied in echinoderms, especially sea urchins (Podolsky, 2002). For echinoderms, egg-derived compounds have been shown to activate sperm motility and chemotaxis towards the egg (Morita et al., 2009). The egg jelly coat surrounding the egg has long been known to be sensitive and even removed by low pH water (Podolsky, 2002). The jelly coat is an extracellular structure formed during oogenesis that surrounds the eggs of many marine invertebrates and consists of several layers of polysaccharide fiber networks embedded in a glycoprotein matrix (Kidd, 1978; Suzuki, 1995; Bonnell et al., 1993; Bonnell et al., 1994; Farley and Levitan, 2001). The material present in the jelly coat perform a number of functions before and during fertilisation. Compounds present in the egg and jelly coat increase sperm motility and speed (Kopf et al., 1979, Hansbrough and Garbers, 1981; Suzuki et al., 1995; Nishigaki et al., 2004; Inamdar et al., 2007), stimulate specifies-specific recognition of gametes and prevent polyspermy (Hagstrom 1959; Vilela-Silva et al., 2002; Sung et al., 2014). The jelly coat also protects eggs from shear forces during spawning (Thomas et al., 1999; Bolton et al., 2000). Thus the egg jelly coat is essential for egg function.

Egg jelly coat thickness varies among species of sea urchin (Kanatani and Nagahama, 1983) and its sensitivity to decreased pH also varies. Decreased pH affects the size of the jelly coat of echinoderm eggs and this differs between species. As the jelly coat increases target size of the egg for sperm, thereby facilitating fertilisation (Farley and Levitan, 2001; Podolsky, 2002), a reduction in jelly coat size would be expected to cause a decrease in fertilisation success. For broadcast spawning invertebrates, both laboratory and field studies show that bigger eggs within species have a higher percentage fertilisation, especially under sperm limiting conditions (Coma and Lasker 1997; Levitan 1998). In the sea urchin *Lytechinus variegatus*, the jelly coat increases the egg target size by four times increasing the collision frequency with sperm by two times, thereby resulting in a significant increase in fertilisation (Farley and Levitan, 2001). If there are different responses of the jelly coat to decreased pH across species, egg incubation time may be a previously unappreciated source of variance in OA fertilisation studies.

The internal pH of eggs (pH$_i$) is important for protein synthesis, protein phosphorylation and for activation of the cells after fertilisation (Grainger et al., 1979). The intracellular pH of sea urchin eggs increases ~ 0.3 pH units after fertilisation, a requirement for initiating embryonic development (Johnson et al., 1976; Lopo and Vacquier, 1977). Intracellular pH (pH$_i$) is crucial in determining cell cycle progression with alteration in the external HCO$_3^-$ environment having potential impacts on the Na/H$^+$ exchangers in the egg responsible for controlling pH$_i$. For the sea urchin *P. lividus*, removal of external HCO$_3$ resulted in an alteration in pH$_i$ and embryos not being able to divide (Ciapa and Philippe, 2013).

For eggs of *Strongylocentrotus droebachiensis*, a low seawater pH of 7.63 significantly decreased egg intracellular pH although the exact change in pH was not measured (Bogner et al., 2014). pH levels below 7.6 may be beyond the ability of the egg to compensate their intracellular pH and buffering the effects of internal pH changes in ocean acidification scenarios may compromise the embryos energy budget (Bogner et al., 2014).

These studies demonstrate that the egg is susceptible to both increases in temperature and decreases in pH, although the degree of the effect is species specific and dependent on the length of exposure. Furthermore, significant variation in the response of the extracellular jelly coat to decreased pH has been observed. Studies on the effects of stressors on eggs are lacking, with a need for multistressor examinations on various egg characteristics. It is evident that the effect of ocean change stressors on the egg is an important consideration in climate change studies, one with consequent effects on fertilisation.

1.3.3 Impacts on Fertilisation

Many studies report that fertilisation in marine invertebrates is robust to ocean warming and acidification scenarios predicted for 2100 (Table 3). For species such as the sea urchins *Heliocidaris erythrogramma*, *Centrostephanus rodgersii* and the sea star *Patiriella regularis*, the resilience of fertilisation to decreased pH and increased temperature may be due to acclimatisation or adaptation to the shallow water and intertidal environments that they live in, which has fluctuations in temperature and pH levels (Byrne, 2012). The tide pools inhabited by *H. erythrogramma* can vary throughout the day from pH 7.54 to 8.91 with annual temperatures ranging from 10 to 24˚C (Nguyen et al., 2014). Thus fertilisation in this species may be preadapted to near-future ocean change conditions, as also suggested for *Paracentrotus lividus*

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and other species (Moulin et al., 2011). On the other hand, fertilisation in the intertidal sand flat sand dollar *Arachnoides placenta*, is negatively impacted by pH levels ≤ 7.8 across a range of sperm:egg ratios (Gonzalez-Bernat et al., 2013a).

Fertilisation in species such as the Antarctic sea urchin *Sterechinus neumayeri* and sea star *Odontaster validus*, which inhabit very stable environments, is negatively affected by extreme pH levels (pH 7.5) or only at extremely low sperm concentrations (Ericson et al., 2010, 2012; Gonzalez-Bernat et al., 2013b). For these species the lack of sensitivity of fertilisation to near future warming and acidification may be due to an inherent resilience of the individual gametes.

As species from variable environments have been shown to be resilient while others are susceptible to ocean change stressors and species that inhabit stable environments also have a robust response to near future ocean change, there is no clear trend of the influence of the stability (or lack of) habitat pH and temperature conditions to fertilisation success.

Thus the response of fertilisation to increased acidification and temperature are mixed and this appears to be influenced by different experimental designs incorporating multiple male and female parents (spawner population approach) or individual male-female pairs (Table 3). Experimental designs that pool multiple males and females often find that fertilisation is fairly robust to increased acidification and warming (Byrne, 2011, 2012). Results with single male-female crosses often detect sensitivity in some pairs but not others (Table 3; Foo et al., 2012; Schlegel and Havenhand 2012; Foo et al., 2014; Sewell et al., 2014).

1.3.3.1 Effects of ocean stressors on individual male-female pairs
Four studies, all involving sea urchin species, investigate the effects of warming and acidification or both stressors on fertilisation using individual male-female pairs (Havenhand et al., 2008; Schlegel and Havenhand 2012; Foo et al., 2014; Sewell et al., 2014). In these studies, some pairs are greatly affected by stressors while others are unaffected, and some perform better (Table 3; Schlegel and Havenhand 2012; Foo et al., 2014; Sewell et al., 2014). The pairs that remain unaffected or perform better in ocean change scenarios would be expected to seed future populations (Foo et al., 2014). Thus, not all matings are equal, a potentially important source of genetic variation.

Table 2. Effects of ocean change stressors on marine invertebrate eggs in response to near and far future, and extreme pH and temperature levels (IPCC, 2013). The pH levels are those provided by the study and are all pH$_{NIST}$. pCO$_2$ values are given when provided by the study.

Species	Stressor	Trait assessed	Result	Reference
Echinodermata				
Sterechinus neumayeri	pH (7.99, 7.70, 7.54) AND temperature (-0.3, 1.8°C)	Egg size	After 6 months acclimatisation of females in treatments, egg produced in the control were larger compared to all other treatments however after 17 months, the largest eggs were produced under the lowest pH (7.54) conditions.	Suckling et al., 2015
Strongylocentrotus droebachiensis	pH (8.13, 8.05, 7.63, 7.58, 7.20/192, 397, 770, 980, 2110)	Egg intracellular pH	pH levels of 7.6 and below caused a significant decrease in egg intracellular pH	Bogner et al., 2014
Heliocidaris tuberculata	Temperature (20, 24, 26˚C)	Egg viability	Exposure of eggs for 3 hours to increased temperature did not impact fertilisation success	Binet and Doyle, 2013
Echinometra mathaei	pH (7.5–8.1/485–1770ppm)	Oocyte size	No effect of acclimation of adults for 7 weeks on oocyte size	Uthicke et al., 2013
Strongylocentrotus franciscanus	pH (8.04, 7.81, 7.55/400, 800, 1800ppm)	Block to polyspermy	The efficiency of the egg block to polyspermy decreased in pH 7.55.	Reuter et al., 2011
Strongylocentrotus droebachiensis, Strongylocentrotus intermedius, Strongylocentrotus nudus, Paracentrotus lividus, Arbacia lixula	Temperature (up until a maximum)	Heat resistance through loss of cleavage capacity	Temperature limits for each species: *S. intermedius* - 32˚C *S. droebachiensis.*- 34˚C *S. nudus* - 34˚C *P. lividus* - 36 ˚C *A. lixula* - 38˚C	Andronikov, 1975
Mollusca				
Sepia officinalis	pH (8.10, 7.84, 7.60/378, 775, 1433ppm) AND	Egg swelling	Egg swelling increased in response to both pH 7.84 and 7.60, and warming which led to an increase in egg surface	Dorey et al., 2013

	temperature (16, 19˚C)			
Annelida				
Dinophilus gyrociliatus	Temperature (6, 12, 18, 24, 30˚C)	Egg size	The smallest eggs were produced by animals grown at 30˚C with the biggest eggs produced by animals grown at 12˚C	Simonini and Prevedelli, 2003
Mollusca				
Sepia officinalis	pH (8.1, 7.85, 7.6/400, 900, 1400ppm) AND temperature (16, 19˚C)	Egg weight	Eggs increased in weight when adults were raised at pH 7.85 and pH 7.6. Increased temperature led to an increase in egg weight but there was no interactive effect of both pH and temperature	Lacoue-Labarthe et al., 2009
Euprymna tasmanica	Temperature (11, 18˚C)	Egg size	Rearing adults in temperatures representative of tidal sandflat temperatures in summer (18°C) and winter (11°C) did not affect egg size	Steer et al., 2004
Collisella radiata, Mytilus galloprovincialis, Acanthodoris pilosa, Onchidoris muricata	Temperature (up until a maximum)	Heat resistance through loss of cleavage capacity	Temperature limits for each species: *A. pilosa* - 32˚C *C. radiata.*- 34˚C *O. muricata* - 34˚C *M. galloprovincialis* - 38˚C	Andronikov, 1975
Crustacea				
Emerita analoga	Temperature (12 - 21˚C) from southern to northern range of species	Egg size	No correlation between egg size and water temperature among northern and southern populations	Dugan et al., 1991

The multiple spawner approach may mask individual differences in male-female compatibility which are important in determining fertilisation success (Palumbi, 1999). These interactions mediate the benefits of polyandry in sea urchins where sperm competition is high. For *H. erythrogramma*, eggs were either exposed separately to the sperm of different males or simultaneously with all males and subsequent fertilisation rates were determined. Since the fertilisation rate for the most successful male in the single-male experiment was similar to the fertilisation rate in the simultaneous fertilisation experiment, most fertilisations could be attributed to the most compatible male (Evans and Marshall, 2005). Subsequent development was improved and this was attributed to a reduction in incompatible matings.

In echinoderms, as shown for sea urchins and sea stars, fertilisation is mediated by the gamete recognition protein bindin which controls sperm binding to the egg bindin receptor on the egg membrane (Vacquier and Moy 1977; Hart, 2013; Popovic et al., 2014; Jagadeeshan et al., 2015). In urchin species, eggs show strong discrimination in mate choice depending on male bindin genotype, mating most successfully with sperm having a similar bindin genotype to the egg (Palumbi 1999; Zigler et al., 2008; Evans and Sherman 2013). Male x female interactions have been shown to greatly influence fertilisation success (Evans and Marshall, 2005; Foo et al., 2012; Sewell et al., 2014; Foo et al., 2014). Thus, the response to climate change stressors in single paired matings will be influenced by inherent gamete compatibility (Evans and Marshall, 2005).

1.3.3.2 Effects of ocean stressors on a spawning population as determined with multiple males and females

The majority of experiments which investigate the effects of marine climate change stressors on fertilisation have pooled gametes from multiple males and females (Table 3). These studies show that the responses of species vary. Approximately half of the studies show that fertilisation is robust to increases in temperature and decreases in pH while the other half show that fertilisation is sensitive (Table 3; Byrne, 2011; 2012; Byrne and Przeslawski, 2013). Six of these studies find that species are only vulnerable to extreme pH levels (Table 3).

The response of fertilisation to climate change stressors may be species-specific although different fertilisation conditions, sperm-egg contact time, sperm concentrations and the vials used can influence results (Byrne, 2012). Even the length of time that the eggs are exposed to

experimental water prior to addition of sperm can influence fertilisation. Thus species-specific responses may also be influenced by variable methodological approaches.

A pattern emerging from Table 3 is that sperm concentration can affect the outcome of climate change fertilisation experiments. For the coral *Acropora tenuis*, there were no effects of stressors when optimal sperm concentrations were used (Chua et al., 2013). However, the sperm concentration required to obtain 50% of maximum fertilisation increased 6 to 8 fold with the addition of a single stressor (pH or temperature) and 50 fold when both factors interacted. Thus near-future changes in pH and temperature narrow the range of sperm concentrations that are capable of yielding high fertilisation success (Albright and Mason, 2013).

Therefore, the robust response of fertilisation to ocean change stressors seen in many studies (Table 3) could be due to the use of high sperm concentrations. It has been suggested that experiments which assess fertilisation success should aim for lower fertilisation success to allow detection of both negative and positive effects (Suquet et al., 1995; Cosson et al., 2008). Most investigations use high sperm concentrations, where fertilisation in control treatments are ≥ 75% (e.g. Byrne et al., 2010; Ho et al., 2013; Sung et al., 2014). This may not be representative of broadcast spawning in the field where sperm levels are likely to be limiting (Levitan, 1998).

In field experiments conducted with synchronously spawning sea urchins, fertilisation was low at distances over 10 cm from the spawning male, similar to laboratory experiments with low sperm concentrations (Pennington, 1985). In experiments with *Strongylocentrotus droebachiensis*, solutions with more than 10^6 sperm/ml (30-40 sperm per egg) continually resulted in 80% fertilisation (Pennington, 1985), likely due to the kinetics of sperm to egg encounters (Rothschild and Swann, 1951). Therefore the use of high sperm concentrations in climate change fertilisation experiments may be masking the effects of stressors which might be more evident at lower sperm concentrations.

1.3.3.3 Impacts on calcifying and non-calcifying larvae

A meta-analysis of effects of climate change stressors on marine invertebrate development found that the larvae were more vulnerable than embryos to both increased temperature and decreased pH (see Przeslawski et al., 2015 for comprehensive review). Increased temperature can decrease planktonic larval duration by stimulation of metabolism up to a thermal threshold limit (Chen and Chen, 1992; Staver and Strathman, 2002; O'Connor et al., 2007; Byrne et al., 2010). Ocean

acidification can impair or inhibit skeletogenesis in echinoderm larva (Byrne et al., 2012; Sheppard Brennand et al., 2010) and this appears to be due to hypercapnic effects of increased organism CO_2 on metabolism (Stumpp et al., 2012; Byrne and Przeslawski, 2013; Evans and Watson-Wynn, 2014).

As carbonate mineral saturation is reduced with a decrease in ocean pH, echinoderms and other marine calcifiers have been the focus of climate change studies because their $CaCO_3$ skeletons are expected to be vulnerable to dissolution in low pH water (Kleypas et al., 1999; Orr et al., 2005). This however may be influenced by the exposure of the skeleton to surrounding waters. For instance, mollusc larvae that do not have a protective cover on their skeleton may be more vulnerable to skeletal dissolution than echinoderm larvae whose skeleton is covered by epithelium (Ries et al., 2009). Skeletogenesis in echinoderms starts in late gastrulation/early prism stages (Politi et al., 2004). The calcareous endoskeletons are deposited through intracellular biomineralisation and are essential in creating the framework for the body, often defining the body plan in echinoderms (Yajima and Kiyonoto, 2006). The spicules that make up the larval skeleton in sea urchins are deposited by primary mesenchyme cells, which accumulate calcium from the seawater and secrete $CaCO_3$ (Wilt, 2002). The calcite skeleton possessed by echinoplutei is important for maintenance of swimming and feeding structures, passive orientation, as well as providing defense against predators (Pennington and Strathmann, 1990; Kurihara and Shirayama, 2004; Soars et al., 2009). Thus calcifying larval stages are considered to be highly vulnerable to ocean acidification, and decreased pH has been shown to be a greater stressor for calcifying rather than non-calcifying larvae (Przelawski et al., 2015).

Lecithotrophic larvae develop to the final stage without the need for exogenous nutrients. They receive nourishment through the nutritive reserves in the egg provided by the mother and hence generally have larger eggs. It has been suggested that lecithotrophic larvae may be better suited to deal with climate change effects as compared to planktotrophic larvae (Dupont et al., 2010; Hardy et al., 2014) with an evolutionary shift towards this larval type in echinoderms evident through past climate change and extinction events (Uthicke et al., 2009).

Table 3. Effects of ocean change stressors on fertilisation in echinoderms in response to near and far future, and extreme pH and temperature levels (IPCC, 2013). The pH levels are those provided by the study and represent pH_{NIST}. pCO_2 values are given when provided in the study. Studies are separated into those that used a 'spawner population approach' where gametes of multiple males and females were pooled, or those that followed 'individual pair responses' where single male-female crosses were used.

Species	Stressor	Results	Reference
Individual pair responses			
Echinodermata			
Sterechinus neumayerii	pH (8.052, 7.967, 7.83/384,473,666ppm)	Pairs showed varying responses to pH levels of 7.83 with some pairs showing positive responses to decreased pH	Sewell et al., 2014
Pseudoboletia Indiana	pH (8.08, 7.88, 7.71/348, 617, 924ppm) AND temperature (22, 25˚C)	pH levels of 7.71 decreased fertilisation with effects alleviated by +3˚C. Responses varied between pairs	Foo et al., 2014
Heliocidaris erythrogramma	pH (8.1, 7.8, 7.6 /970, 1600ppm)	Overall effects of pH levels up to pH 7.6 not significant however responses of pairs ranged from negative to positive effects	Schlegel et al., 2012
Heliocidaris erythrogramma	pH (7.7/1000ppm)	Fertilisation decreased with pH levels of 7.7	Havenhand et al., 2008
Spawner population approach			
Echinodermata			
Paracentrotus lividus	pH (8.08, 7.93/380, 750ppm) AND hypoxic conditions	There was a negative effect of low pH on fertilisation success and when combined with hypoxia, the effect was even greater.	Graham et al., 2015
Strongylocentrotus droebachiensis	pH (8.13, 8.05, 7.63, 7.58, 7.20/192, 397, 770, 980, 2110ppm)	At pH levels of 7.63 and below, the fertilisation success was greatly decreased.	Bogner et al., 2014
Evechinus Chloroticus	Temperature (+3°C) AND low salinity	No effect of temperature on fertilisation with salinities of 29pp and below causing a decrease in fertilisation success.	Delorme and Sewell, 2014
Strongylocentrotus purpuratus and *S. franciscanus*	pH (8, ~7.5/495, ~1427ppm)	Low pH decreased fertilisation however this was specific to the sperm-egg ratio used. Fertilisation of *S. purpuratus* was largely robust to pH across a wide ranges of sperm:egg ratios while *S. franciscanus* was very sensitive to decreases in pH.	Frieder et al., 2014

Hemicentrotus pulcherrimus and *Strongylocentrotus nudus*	pH (7.99, 7.96, 7.92, 7.78/380, 450, 550, 750, 1000, 1500ppm)	Fertilisation was not significantly decreased in any of the pH levels tested	Sung et al., 2014
Patiriella regularis	pH (8.15, 7.8, 7.6/423, 1058, 1738ppm)	No effect of decreased pH on fertilisation	Byrne et al., 2013
Arbacia lixula	pH (8.2, 7.9/498, ~1100ppm) AND temperature (20, 24, 26, 27°C)	Fertilisation success was significantly decreased at temperature treatments of 27°C. Temperature and pH had no significant effect on fertilisation at temperature <27°C.	Gianguzza et al., 2013
Arachnoides placenta	pH (8.4, 7.79, 7.65, 7.12/526 1301, 1892, 6784ppm)	Fertilisation decreased significantly with pH levels of 7.79 and below across a range of sperm:egg ratios (4:1 to 4000:1).	Gonzalez-Bernat et al., 2013a
Odontaster validus	pH (8.1, 7.8, 7.6, 7/327, 691, 1130, 4604ppm)	At near-future pH ranges (pH 7.8 and 7.6), fertilisation was not significantly decreased, except at the lowest sperm concentration (10^3 sperm/ml) where fertilisation was reduced to 60 and 61% in pH 7.6 and 7.8, respectively.	Gonzalez-Bernat et al., 2013b
Sterechinus neumayerii	pH (8.12, 7.87, 7.69/433, 927, 1417ppm) AND temperature (1, 3, 5 °C)	Fertilisation resilient to pH levels tested and temperature increases of +4°C	Ho et al., 2013
Centrostephanus rodgersii	pH (8.1, 7.8, 7.6, 7.04/469, 1011, 1652, 6238ppm) AND temperature (+3°C)	Fertilisation was robust to pH levels of 7.6 and only slightly reduced at pH 7.04 with no effects of temperature.	Pecorino et al., 2013
Acanthaster planci	pH (8.1, 7.9, 7.7 /520, 877, 1658ppm)	Fertilisation rates were decreased in pH 7.9 and further reduced in pH 7.7, likely due to decreases in sperm motility	Uthicke et al., 2013
Paracentrotus lividus	pH_T (8, 7.8, 7.6, 7.4, 7.2, 7., 6.8/311,697,1037,1690,2686,4292, 8108ppm)	Fertilisation decreased with pH levels of 7.6 and below	Moulin et al., 2011
Strongylocentrotus franciscanus	pH (8.04, 7.81, 7.55/464,828, 1578ppm)	Fertilisation decreased with pH levels of 7.8 and below	Reuter et al., 2011
Sterechinus neumayerii	pH (8, 7.7, 7.5) AND temperature (+1.5 °C and 3°C)	At elevated temperatures, there was a negative interactive effect of temperature and pH 7.5 on fertilisation success	Ericson et al., 2012
Heliocidaris erythrogramma,	pH (8.2, 7.9, 7.8, 7.6/ 327-335 814-851 1051-1104 1729-1828ppm) and	No effects of pH or temperature on fertilisation success	Byrne et al., 2010

H. tuberculata, Tripneustes gratilla, Centrostephanus rodgersii and *Patiriella regularis*	temperature (18, 20, 22, 24, 26˚C)		
Hemicentrotus pulcherrimus and *Echinometra mathaei*	pH (8.01, 7.77, 7.61, 7.38, 7.03, 6.83/360, 860, 1360, 2360, 5360, 10360ppm)	Fertilisation decreased with pH levels of 7.4 and below	Kurihara and Shirayama, 2004
Annelida			
Arenicola marina	pH (7.77, 7.47/1400, 3000ppm) AND copper toxicity	Fertilisation success was negatively affected by both pH 7.77 and copper individually, but no additive effects when exposed as combined stressors.	Campbell et al., 2014
Pomatoceros lamarckii	pH (range 8.1–7.2/302 -3781ppm) AND copper toxicity	Fertilisation success was slightly but significantly reduced at the 7.6 and 7.4 pH treatments with no additional impact of copper	Lewis et al., 2013
Cnidaria			
Acropora digitifera	pH (8.1, 7.79/438, 990ppm) AND temperature (+4°C)	Fertilisation success decreased in response to increased temperature. In contrast, fertilisation was not affected by decreased pH.	Iguchi et al., 2014
Acropora tenuis	pH (8.01, 7.78/400, 800ppm) AND temperature (+3°C)	The sperm concentration required to obtain 50% of maximum fertilisation increased 6 to 8 fold with the addition of a single stressor (pH or temperature) and 50 fold when both factors interacted.	Albright and Mason, 2013
Acropora millepora and *A. tenuis*	pH (8.16, 8.03/~421, ~655ppm) AND temperature +(2°C)	No effects on fertilisation for either species	Chua et al., 2013
Mollusca			
Haliotis diversicolor and *H. discus hannai Crassostrea angulata*	pH (8.15, 7.94, 7.71, 7.61, 7.43/448, 784, 1401, 1794, 2780ppm)	For all species, fertilisation significantly decreased in pH levels of 7.43.	Guo et al., 2015
Mimachlamys asperrima	pH (8.2, 7.89, 7.81, 7.69/390, 600, 750, 1000ppm)	Fertilisation decreased at pH 7.81 and below	Scanes et al., 2014
Crassostrea gigas	pH (8.2, 7.87, 7.48/580, 1386, 3573ppm)	Fertilisation was reduced in pH 7.87 and further reduced in pH 7.48.	Barros et al., 2013
Macoma balthica	pH (8.1, 7.8, 7.5/601, 1455, 2128ppm)	Fertilisation greatly declined in pH 7.5.	Van Colen et al., 2012
Mytilus edulis	pH (8.06, 7.62/419–469, 1388–	There was no effect of decreased pH on	Bechmann et al., 2011

	1493ppm)	fertilisation success	
Haliotis discus hannai	pH (7.94 7.68 7.49 7.41/500, 1100, 1650, 2150ppm)	Fertilisation success was only decreased when exposed to pH levels of 7.49 and below.	Kimura et al., 2011

1.4 Identifying gaps in ocean change studies

Investigation of the effects of ocean acidification and ocean warming on marine invertebrates has involved multistressor studies of larvae, fertilisation and the sperm cell. There have been contrasting results among species and stages of development. However, both gametes are important and the egg may be negatively affected by ocean change stressors. Research on the egg is lacking. This is particularly important to address because empirical data and fertilisation models in flow indicate that egg target size and turbulent mixing determine collision frequency, rather than the swimming ability of the sperm (Denny and Shibata 1989).

The egg cell is often the biggest cell produced by marine invertebrates and yet there is a great knowledge gap on effects of ocean change stressors on the egg cell with only one study on the effects of low pH on egg intracellular pH, and no existing studies on the effect of stressors on the jelly coat. This is a focus in this thesis.

This thesis aims to fill current knowledge gaps effects of ocean acidification on the egg cell through investigation of the effects of ocean acidification on the size of the jelly coat, comparing responses of the eggs of four echinoids (*Heliocidaris erythrogramma*, *H. tuberculata*, *Centrostephanus rodgersii*, and *Echinometra mathaei*).

1.5 The Potential to Persist in the Face of Ocean Change; Acclimatisation and Adaptation

Environmental stressors, such as those associated with climate change, are a significant evolutionary force in nature and will influence the shape of marine communities now and into the future through selection (Hoffmann and Merilä, 1999). In the face of a changing ocean, the adaptive capacity of marine species will include a mixture of organism plasticity, shifts in species range and genetic evolution. The pace of adaptation will be greatly influenced by stress tolerance, dispersal ability, the latitudinal range the species inhabits and potential for genetic change (Bernhardt and Leslie, 2013; Williams et al., 2008).

The four main outcomes for populations subjected to global change stressors are (1) acclimatisation, (2) shifts in distribution, (3) microevolution/adaptation and (4) extinction (Hoffmann and Parsons, 1991). Table 4 provides a glossary of evolutionary biology and quantitative genetic terms. Although animals can evolve on rapid ecological timescales, relatively few studies consider adaptive capacity of marine species to climate change (Merilä and Hendry 2014). Thus far, most climate change stressor studies have involved placing the life

stages of marine species directly into future scenarios. Although this approach provides some insight into the tolerance of species to ocean change, it represents a "future shock" approach and is likely to overestimate the sensitivity to stressors and their impacts (Byrne, 2012; Dupont et al., 2013). Furthermore, the life stage (e.g. fertilised eggs, embryos, larvae, juveniles and adults) at which species are introduced to environmental conditions has differed greatly among studies (Byrne, 2012). We need to go beyond the limited life stage approach to complete the lifecycle of species, where possible to incorporate acclimatisation and adaptation, important considerations as climate and ocean change is much more gradual than in experimental conditions (Gaylord et al., 2014; Munday et al., 2013; Somero et al., 2012; Stillman and Paganini, 2015).

More recent studies have involved longer term incubations (months to years) with the aim to achieve acclimation of embryos and adults to the new environment (e.g. Suckling et al., 2015; Thor and Dupont, 2015). The rate at which stressors are introduced is also important and can influence experimental outcomes (Suckling et al., 2014b). To avoid the acute approach, stressors can be introduced gradually over weeks until the target experimental levels are reached and then the animals are held in conditions to achieve acclimation. Furthermore, the length of the incubation/acclimation period also varies and has been shown influence whether the effect of acclimation is neutral, positive or negative (Suckling et al., 2014a). For example, for the sea urchin *S. droebachiensis*, female fecundity decreased when acclimated to low pH for 4 months with no difference observed for females acclimated for 16 months (Dupont et al., 2013). A key consideration is the physiological state and season that the incubations begin. For instance, it took one year to retrain the gametogenic cycle and reprogram physiology of sea urchins in experiments where photoperiod was manipulated (Bay-Schmith and Pearse, 1987).

1.5.1 Acclimatisation

Marine animals, such as those that reside in the intertidal may be adapted to tolerate fluctuating environments (Byrne, 2011; Melzner et al., 2009; Sanford and Kelly, 2011). Intertidal habitats can fluctuate more than 0.5 pH units daily (Duarte et al., 2013; Wootton et al., 2008), a phenomenon also noted for coral reefs during day-night cycles (Birkeland et al., 2008). Populations of the seastar *Parvulastra exigua* can experience pH levels from 7.54 to 8.91, and temperatures from 10 to 24°C across a 24 hour cycle in the tide pools it inhabits (Nguyen et al., 2014). Juveniles from these populations are resilient to conditions well beyond near future ocean

Table 4: Glossary of evolutionary biology and quantitative genetic terms

Term	Definition
Acclimation	Species adjust to experimental conditions without an adjustment in their genetics. Effects are therefore reversible
Acclimatisation	Similar to acclimation, however the term used when the effect is induced by natural environmental changes
Adaptive evolution	Genetic change in a population over many generations to adjust the organism to its environment. It is maintained by natural selection.
Additive genetic variation	Primary cause of resemblance between relatives and primary determinant of observable genetic properties of the population, and of the population response to selection
Allele	Alternative forms of a gene found at the same location on a chromosome
Broad sense heritability	Broad-sense heritability of a trait describes the proportion of phenotypic variation due to genetic effects, and thus may also include dominance and epistasis effects
Dam	Female parent
Dominance	The connection between alleles in one gene where the effect of one allele on phenotype masks the contribution of the second allele
Epigenetics	Heritable modification of gene expression without change to DNA sequences. DNA methylation, modification of histones and non-coding RNA associated gene silencing are all systems which can initiate epigenetic change
Epistasis	Interactions within or between genes
Environmental stressor	A situation that lies outside the organism's optimal conditions, causing an impact on Darwinian fitness, and can be an influential evolutionary force
Evolutionary rescue	The genetic adaptation of a population allowing persistence through environmentally induced effects which would have otherwise caused extinction
Fitness	The potential of a certain genotype to pass on genes to future generations that influence reproductive success
Genetic assimilation	The process where phenotypes induced by an environmental signal become genetically fixed via natural selection, i.e. the environmental signal is no longer required for expression of that phenotype
Genetic correlation	Proportion of variance that two genetic traits share
Genetic rescue	Increased population fitness and genetic diversity through immigration of new alleles
Genotype x Environment (G x E) interactions	The differing responses of individual genotypes under changes in the environment
Heritability	The proportion of observed differences of a trait among individuals due to genetic differences
Narrow sense heritability	Narrow-sense heritability is the proportion of genetic variation that is due to additive genetic effects only and describes the degree of resemblance between relatives
Non-additive genetic	The proportion of phenotypic variance which is due to epistatic interactions

variation	and dominance deviations
North Carolina II	A breeding design involving individual mating of N_{sires} and N_{dams} to allow partitioning of the phenotypic variance of offspring of known relatedness into genetic and environmental components
Phenotypic plasticity	The ability of an organism of one genotype to produce more than one phenotype when exposed to different environments. Plasticity can be adaptive (promotes persistence in new environment) or non-adaptive (response is away from favoured optimum)
Quantitative genetics	The study of the effects that heredity and environment have on traits that can be quantitatively measured
Reaction norm	Also known as interaction plot, shows the pattern of phenotypic expression of specific genotypes over certain environments
Selection	Where the environment or genetics determine which types of organism succeed
Selective breeding	Also known as artificial selection, is the process where animals and/or plants are bred for particular traits
Sire	Male parent
Transgenerational effects	Effects on offspring phenotype and patterns of gene expression that are passed from one generation to the next that cannot be explained by changes to the DNA sequence
Transgenerational plasticity	The transmission of information from one generation to the next resulting in an alteration of traits without an alteration to DNA. Transgenerational effects can be adaptive, resulting in pre-adapted offspring that exhibit traits associated with increased fitness in environmental conditions experienced by their parents

change conditions. The adults also showed high metabolic resilience to ocean warming and acidification, levels well beyond projected climate change (McElroy et al., 2012). In contrast, the mussel *Mytilus calfornicanus* which experiences fluctuations in pH due to seasonal upwelling was less tolerant to acidification than its congener *M. galloprovincialis* which occurs in a more stable pH environment (Waldbusser et al., 2015). Both species showed a very similar response in shell growth when exposed to low pH.

Although many shallow water and intertidal species inhabit environments where temperature and pH can fluctuate markedly, laboratory studies rarely incorporate fluctuating conditions and therefore the treatments may not be realistic. A study which compared the response of the calcifying macroalga *Arthrocardia corymbosa* to either a constant low pH treatment or a treatment which incorporated natural diurnal pH fluctuations found different responses in growth dependent on whether pH was constant or fluctuating (Cornwall et al., 2013). Moreover, fluctuating pH conditions reduced the negative effect of acidification on corals (Dufault et al. 2012).

1.5.1.1 Acclimatisation and Thermal Tolerance Limits

Species with broad latitudinal distributions across thermal regimes may have an in-built capacity to persist in warming oceans (Bradshaw and Holzapfel, 2001). It is often found that progeny of parents from cooler climates are less thermotolerant than those from the warmer regions of their range, likely due to adult thermal acclimatisation (Byrne et al., 2010; Visser, 2008; Zippay and Hofmann, 2010). For the sea urchin *Heliocidaris erythrogramma*, Northern (warmer) populations show significantly higher warm thermal tolerance than Southern (cooler) populations, providing the possibility that these populations could persist through poleward migration of thermotolerant propagules with southward flow of the East Australian Current (Byrne et al., 2010). Similar results have been seen for the snail *Littorina littorea* and the mussel *Mytilus edulis* where warmer populations have a higher thermal tolerance (Sorte et al., 2011). This phenomenon of poleward migration is a global phenomenon due to global climate warming (Sunday et al., 2015).

Temperature acclimatisation capacities differ greatly between marine invertebrates with limits set by their physiological systems (Somero, 2010). Animals can alter their physiology in response to various environmental factors without a change in genetics and thereby reducing

their sensitivity to temperature change (Chevin et al., 2010; Hoffmann and Sgrò, 2011; Seebacher et al., 2014; Stillman and Paganini, 2015). Although physiological acclimatisation to a changing climate is a feature of many species, a recent meta-analysis shows physiological rates have increased ~20% in the past 20 years (Seebacher et al., 2014). It is not known whether this increase is detrimental or has the potential to compromise normal physiological functioning but may explain the differences in acclimatisation potential between different species (Seebacher et al., 2014).

Phenotypic plasticity of many marine invertebrates has been investigated through laboratory based studies on thermal tolerance limits and acute heat stress tests because temperature is a key factor determining the biogeography and distribution of marine species (Monaco and Helmuth, 2011; Somero et al., 2010; Sunday et al., 2014; Terblanche et al., 2011; Tomanek, 2010). For example, large thermal envelopes for development have allowed expansion of invasive species in a warming ocean, as shown for the sea urchin *C. rodgersii* which has a 9°C thermal envelope (13–22°C) for development. This influenced expansion of this species over 1000km in ~60 years (Ling et al., 2008). The lower thermal limit for successful development of *C. rodgersii* corresponds to the maximum winter temperature in Tasmania thus allowing local populations to reproduce (Hardy et al., 2013; Ling et al., 2009). Similarly, for *C. rodgersii* in New Zealand, the thermal window for early development is likely to contribute to its current distribution where the current southern limit of distribution coincides with the lower limit of their larval thermal window (Pecorino et al., 2013).

Although species may display broad developmental thermal envelopes, it does not necessarily mean that this envelope is reflected in their latitudinal distribution (Garcia Molinos et al., 2015; Hardy et al., 2013). The tropical echinoid *Arachnoides placenta* displays a broader envelope (17–31°C) than *C. rodgersii* (13–22°C) but has not expanded its distribution likely due to limited habitat (Hardy et al., 2013). For both species, future warming of their habitat is likely to lead to contractions at their warm range edge as they are currently living near the upper thermal tolerance limits. Therefore in this case, a broader thermal envelope will not necessarily be beneficial for the resilience of the species to ocean warming (Hardy et al., 2013). The shifts in marine distribution currently underway are creating novel communities and novel species interactions (Burrows et al., 2014; Sunday et al., 2015).

Many marine species appear to be currently operating at the edge of their thermal tolerance (Sunday et al., 2014) and so further acclimatisation to warming may be limited, especially for some polar species (Peck et al., 2009; Peck, 2015). The ecologically dominant asteroid *Odontaster validus* is one of the most thermotolerant of Antarctic marine species studied to date and so may be resilient to habitat warming (Peck et al., 2008). A study on the porcelain crab *Petrolisthes cinctipes* shows that in the short term, increased tolerance to warmer temperatures is beneficial, but in the long term the effects are detrimental due to a reduction in overall energy (Paganini et al., 2014).

It is important to combine information on the thermal tolerance of adults and progeny with other traits such as metabolic performance, swimming ability and sublethal responses (e.g. reproduction, growth) to more fully understand species' vulnerability and to forecast individual responses to ocean change (Chan et al., 2015; Chown et al., 2010; Dawson et al., 2011; Francis Pan et al., 2015; Magozzi and Calosi, 2015; Stumpp et al., 2011; Sunday et al., 2015). In response to ocean warming, based on climate velocity trajectories and species' thermal tolerance, current biodiversity is likely to be redistributed with the ability of marine ectotherms to reflect their thermal niche dependent on suitable colonisation conditions (Burrows et al., 2014; Garcia Molinos et al., 2015; Sunday et al., 2015).

Marine organisms live in a multistressor world and, while understanding thermal tolerance is important, other stressors need to be considered and in combination. The addition of a second stressor (e.g. ocean acidification) may reduce thermal tolerance breadth (Pörtner, 2008; Pörtner, 2010). This is seen in larvae of the sea urchin *Strongylocentrotus purpuratus* where increased temperature and low pH had additive effects that exceeded thresholds for optimal physiological performance as revealed by significant reductions in larval metabolism and downregulation of histone encoding genes (Padilla-Gamino et al., 2013). For the sea urchin *Sterechinus neumayeri*, blastulae raised in control conditions were able to survive heat shocks up to +20°C, 5°C higher than embryos raised in low pH conditions (Kapsenberg et al., 2014).

1.5.1.2 Phenotypic Plasticity and Genetic Assimilation

When phenotypes induced by environmental conditions become genetically fixed through natural selection, even when the environmental signal is no longer required for expression of that phenotype, genetic assimilation has occurred in the population (Collins et al., 2013; Pigliucci et

al., 2006). Genetic assimilation can facilitate phenotypic evolution and can thus alter natural selection (Pigliucci et al., 2006). However, it is often contested whether phenotypic plasticity facilitates or hinders genetic evolution (Chevin and Lande, 2010; Merilä, 2015). A recent study on guppies provides evidence that adaptive phenotypic plasticity can weaken the strength of directional selection which in turn reduces the rate of genetic adaptation (Ghalambor et al., 2015). However, these authors also found that 89% of the genes expressed changed in the opposite direction to that of phenotypic plasticity. This inverse relationship can facilitate evolution by increasing directional selection (Ghalambor et al., 2015).

Thus, in the short term, acclimatisation can allow adjustment to changing conditions in some species and may help buy species time to allow for genetic adaptation to occur. However, plasticity has limits in its potential to buffer marine ectotherms to increased temperature and acidification (Gunderson and Stillman, 2015). Therefore in the long term, adaptation will be required for population persistence (Hoffmann and Parsons, 1991).

1.5.2 Adaptation

Adaptation or micro-evolution (Table 4) occurs over many generations and is a heritable, genetic change in response to environmental selection (Hoffmann and Merilä, 1999; Hoffmann and Parsons, 1991). It involves a change in gene frequency within a population with natural selection playing a primary role (Hoffmann and Parsons, 1991). It is important to be able to determine whether responses are genetic (evolutionary) or phenotypic (plastic, non-genetic) to identify the role of each in resiliency to ocean change (Gienapp et al., 2008). Clear-cut evidence of species genetic adaptation to global warming is scarce (Gienapp et al., 2008).

The rate of adaptation is influenced by generational turnover time with short-lived species, and those with fast generation likely to show greater potential evolutionary adaption (Byrne, 2011; Dam, 2013). For example, the coccolithophore *Emiliania huxleyi* was exposed to low pH over 500 asexual generations and it was found that growth and calcification in the selected clones was eventually restored (Lohbeck et al., 2012). Similar results were seen with sexual reproduction in the the copepod *Tisbe battagliai* after several generations in low pH (Fitzer et al., 2012).

The ability to adapt to future changes in environmental conditions depends on the existence of additive genetic variances within populations (Table 4), the proportion of genetic

variation that responds to natural selection (Billington and Pelham, 1991). Selection will favour the individuals with more advantageous traits where the genetic basis of these phenotypes will become more common in a population, eventually resulting in macroevolution (Gassmann et al., 2009; Hoffmann and Parsons, 1991). The types of genetic variance include additive and non-additive, with the latter being interactions between parental haplotypes. Additive genetic variance is considered to be the intrinsic genetic quality of the male and female parent (Neff and Pitcher, 2005). Natural selection favours the traits that facilitate success of following generations. Thus the potential to adapt to the pressures exerted by climate change depends on the rate that these climate change stressors are being altered and the amount of additive genetic variation in fitness related traits within populations (Billington and Pelham, 1991). Fitness related traits are those that contribute to species' ability to survive and produce viable offspring. If there is a selection gradient, traits are heritable, and if genetic variance is present in the population, adaptation to ocean stressors will proceed (Dam, 2013).

1.5.2.1 Evidence of Standing Genetic Variation

Standing genetic variation, i.e. genetic variation already present in current populations (Table 4), could provide a reservoir of resilience to ocean change (Anttila et al., 2013; Hoffmann and Sgrò, 2011; Kelly et al., 2013; Pespeni et al., 2013a, 2013b). For Atlantic salmon, high phenotypic variation between families and great similarity between siblings indicates the presence of standing genetic variation in the response to increased temperature, providing increased resilience to ocean warming (Anttila et al., 2013). Garfield et al., 2013, found extensive variation in gene expression in the gene regulatory network (GRN) for the sea urchin *Strongylocentrotus purpuratus*, and this was associated with measureable variation in larval skeleton morphology. As the expression of most genes were attributed to significant paternal effects, this variation is likely to be heritable and shows that the larval skeleton is a trait that can be targeted by natural selection (Garfield et al., 2013). Latitudinal temperature gradients appear to have generated local genetic adaptation in *S. purpuratus* along the coast of North America, despite having an open population with a recruitment regime dependent on larvae. Larvae generated from the gametes of adults from six populations of *S. purpuratus* spanning regions of different temperature, showed differences in gene regulation related to biomineralization and ion transport (Pespeni et al., 2013a).

Studies of local adaptation can provide important information on the potential for natural selection giving insight into the ecological and genetic factors that influence evolution (Kawecki and Ebert, 2004). A further study by Pespeni et al., (2013b) cultured larvae from different populations of *S. purpuratus* under near future ocean acidification scenarios. These populations included the progeny of adults that lived in different pH environments due to exposure to variable upwelling conditions. Although there was little observable difference in performance of larvae between low pH and control conditions, larvae from different populations showed significant differences in over 40 functional groups of proteins, including genes for lipid metabolism and biomineralization (Pespeni et al., 2013b).

For the coral *Acropora hyacinthus*, populations that are living in naturally high temperature environments were more resistant to bleaching, likely due to a reservoir of alleles that were pre-adapted to high temperature (Bay and Palumbi, 2014; Palumbi et al., 2014). Furthermore, constitutive frontloading of transcripts related to heat shock proteins and antioxidant enzymes enabled corals to maintain physiological homeostasis during periods of temperature stress (Barshis et al., 2013).

These case studies demonstrate that for sea urchins and corals, the capacity for rapid evolution to ocean acidification is likely to occur due to standing genetic variation present in current populations.

1.5.2.2. Evolutionary Rescue

Evolutionary rescue allows populations to survive a rapidly changing climate where adaptive evolutionary change restores positive growth to the population thus preventing extinction (Carlson et al., 2014; Gonzalez et al., 2013; Whiteley et al., 2015). For evolutionary rescue in the face of a warming and acidifying ocean, factors such as population size, organism life span and the amount of genetic variance for the required traits greatly determine the success of a species (Bell and Gonzalez, 2009; Hoffmann and Sgrò, 2011; Willi et al., 2006). Thus, determining the existence of genetic variation in populations will help to determine whether evolutionary rescue is possible as the ocean continues to warm and acidify.

For the porcelain crab species, some larvae and juveniles show increased tolerance to decreased pH. For the resilient subset, enhanced acid-base regulation prevented a decrease in metabolism, a phenomenon observed in other ectotherms in response to decreased pH (Carter et

al., 2013). These variable responses suggest potential for this species to adapt to ocean acidification. Studies which track specific genotypes' performance across environmental conditions have shown genetic variation in response to ocean acidification (Sunday et al., 2011; Kelly et al., 2013), ocean warming (Pistevos et al., 2011) and both (Foo et al., 2012, Foo et al., 2014) which would suggest the potential for evolutionary rescue of these species in a changing ocean.

1.5.2.3 Human Assisted Evolution

The genetic enhancement of corals with enhanced stress tolerance through human assisted evolution is currently being investigated. Coral nurseries are used to grow coral to a size that allows them to survive transplantation and seeding of degraded reefs (Amar and Rinkevich, 2007; Guest et al., 2014). This idea can be expanded through assistance of the genetic adaptation of the symbionts that are essential for coral survival. These symbionts are subjected to ocean change stressors in the lab to identify those with enhanced tolerance. Larval and juvenile corals can then be inoculated with the stress-tolerant symbionts (Van Oppen et al., 2015).

A similar concept to human assisted evolution is genetic rescue, where the immigration of new alleles into a population restores growth. The main purposes of genetic rescue are to restore genetic diversity in populations that are small and isolated (Whiteley et al., 2015). Genetic improvement of many plants and animals has been utilised for many years, and could help augment the capacity of corals and other ecologically and economically significant species to endure a changing climate (Van Oppen et al., 2015). For the oyster *Saccostrea glomerata*, the progeny of adult lines selected for disease resistance or growth rate (Parker et al., 2011) were more resilient to low pH than wild larvae. When larvae/juveniles generated under low pH were outplanted into ambient conditions until reproductive maturity, positive carry over effects were still evident in the F2 generation. This emphasizes the importance of human assisted evolution through laboratory selection and outplanting in generating resilient genotypes for the aquaculture industry (Parker et al., 2015). A recent genomic study shows that genetic rescue of reef-building corals is possible where the survival of coral larvae under high temperatures increased tenfold when parents came from a warmer location from a different latitude (Dixon et al., 2015).

1.5.3 Reaction Norms and Visualization of Genotype by Environment Interactions

When phenotypic variation occurs as a result of exposure to different environmental conditions, this is indicative of interactions between genes and the environment (G x E interaction; Figure 1.4; Neff and Pitcher, 2005). Reaction norms show the response of a specific genotype across a range of environments and allow visualization of G x E interactions (Lynch and Walsh, 1998). There are four main environmental response patterns (reaction norms) that can occur (Figure 1.4a-d): (1) different genotypes display a similar response to a range of environments due to previously strong selection and (2) genotypes display parallel responses. Both of these examples do not show evidence of genetic variation. The other two cases which are more common, are indicative of G x E interactions: (3) non-parallel reaction norms or trait expression across environments with genotypes responding similarly in some environments but differently in others, affecting selective outcomes and (4) the rank order of the genotypes for a trait varies depending on the environment (Neff and Pitcher, 2005; Sultan, 2007). Thus a G x E interaction could be summarized as a genotype that performs well in one environment but not as well in a second environment (Eisen and Saxton, 1983).

1.6 Assessing Evolutionary Potential in a Changing Ocean

To assess whether a population can respond to ocean change stressors, studies investigate the magnitude of genetic variance and/or presence of different genotypes for tolerance of the different traits in environments that differ in stressor levels (Tables 5-7). Experimental designs can replicate genotypes in different ways including as full-sib or half-sib families, or clones (Tables 5-7). Performance of the different genotypes is assessed across various environmental conditions allowing an interaction between genotype and the stressor to be detected. These interactions are indicative of genetic variation in stress tolerance, and from this, the heritability of stress tolerance can also be estimated (Shaw and Etterson, 2012).

1.6.1 Use of Quantitative Genetic Designs with Free Spawning Marine Invertebrates

For free spawning invertebrates, male and female gametes can be isolated for experimental matings for application in evolution studies (such as quantitative genetic designs) which follow the success of the offspring of individual sets of parents. They provide a tractable and
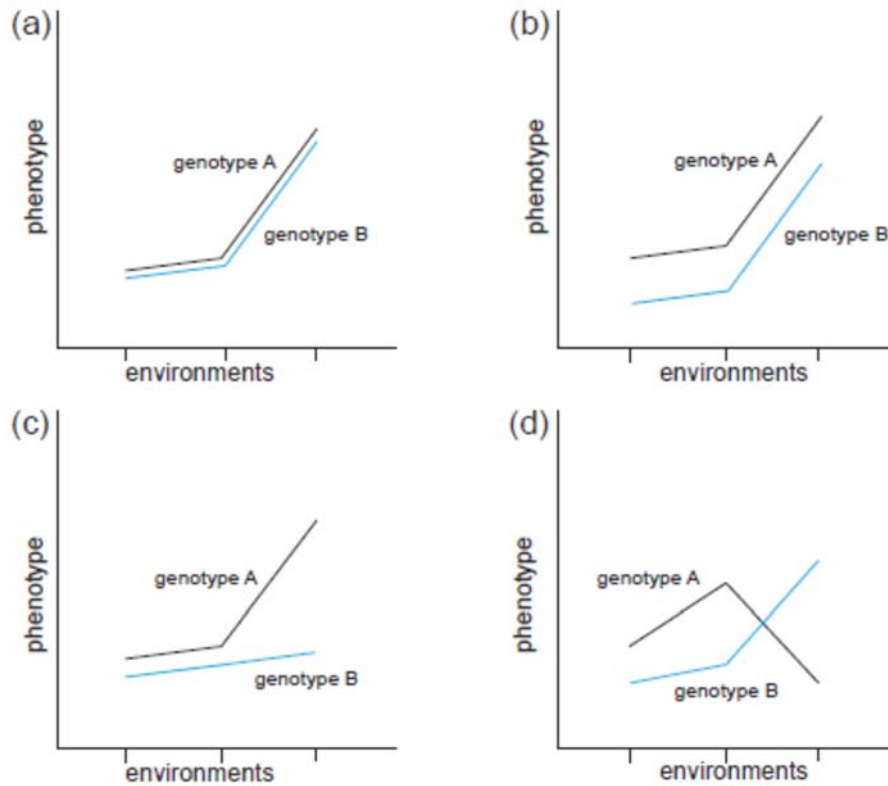
Figure 1.4. The pattern of phenotypes produced by given genotypes under different environmental conditions (reaction norms). The variation in the fitness of a genotype and the expressed phenotype across multiple environments is due to genetic effects, environmental effects and genetic x environmental effects (G x E). Within a population, genotypes can show (a) almost identical responses across a range of environments or (b) differ consistently across environments. When the genotypic difference varies from one environment to another (non-parallel reaction norms), this is indicative of G x E interactions and is shown by a difference in the genotype's magnitude of response to environment by (c) expression of different genotypes only in some environments or (d) the rank order of the genotype varies depending on environment, i.e. there is a G x E effect with a change in the fitness rank of the two genotypes. This can promote selective diversification among distinct environments.

controllable model system for quantifying the contribution of heritable genetic variation to the overall phenotypic variation (Neff and Pitcher, 2005).

The North Carolina II (NCII) quantitative breeding design allows variance of a population among relatives of known relatedness to be partitioned into additive, maternal, interactive and environmental components (Lynch and Walsh, 1998). It involves mating a set of N_S sires with N_D dams in all combinations generating Ns x Nd families or genotypes, allowing genetic effects to be detected as it examines all possible crosses between individuals. Thus free spawning marine invertebrates provide an ideal model system for utilisation of the NCII design (Figure 1.5; Lynch and Walsh, 1998; Neff and Pitcher, 2005).

As the development of specific genotypes can be tracked using paired mating model systems, quantitative genetics and animal breeding designs including the NCII have been used for many years in agriculture and aquaculture to generate stress tolerant animals and crops and those most suited to a specific environment (Falconer and Mackay, 1996; Henning and Townsend, 2005; Lynch and Walsh 1998). Selective breeding of salmon has created family lines that are resistant to sea lice, a problem costly to the industry (Jones et al., 2002). The selective breeding program for Pacific oysters (*Crassostrea gigas*) selects for genetic improvement and resistance to disease (Kube et al., 2011; Ward et al., 2000). Pedigree inbred lines have shown the potential of *C. gigas* to redistribute energy in response to ocean change stressors (Applebaum et al., 2014). Pedigree lines provide genotypes that can be re-tested, allowing non-additive genetic components of phenotypic variance to be captured (Applebaum et al., 2014; Pace et al. 2006).

The NCII design has also been used to estimate the amount of variation in the size of the tropical abalone *Haliotis asinina* attributable to additive genetic effects in selective breeding programs (Lucas et al., 2006). Animal breeding design experiments with the ascidian *Styela plicata*, show a difference in resistance to differing copper concentrations between different male-female crosses (Galletly et al., 2007). This suggested that this population used different genetic mechanisms to adapt to different pollution levels. However only recently has this design been utilised for marine species to assess adaptive capacity to ocean change stressors (Clark et al., 2013; Foo et al., 2012; Munday et al., 2013 Sunday et al., 2011;). As changing ocean conditions are creating intense selection pressure on many marine organisms, studies which demonstrate genetic adaptation to climate change are essential but scarce (Merilä and Hendry, 2014). The studies using this design in a climate change context are listed in Table 5.
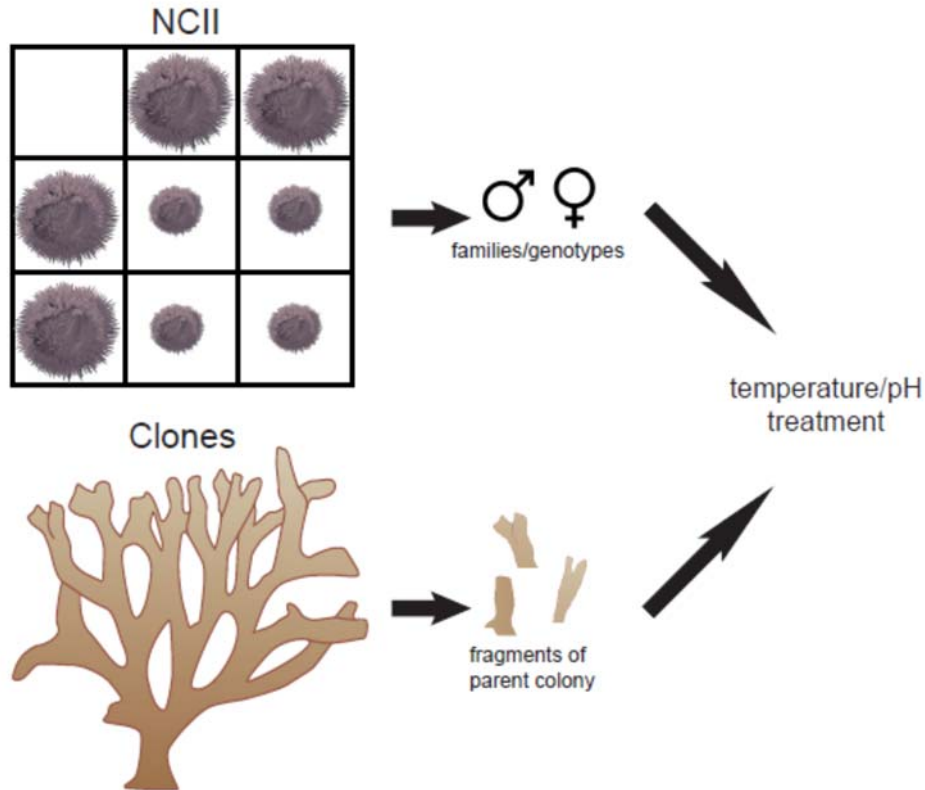
Figure 1.5. The North Carolina II design with free spawning marine invertebrates. The design involves mating N_S sires with N_D dams in all combinations allowing genetic effects to be partitioned into additive, maternal, interactive and environmental components. The example on the top shows that fully crossing two dams with two sires results in four possible genotypes. For a tractable and robust design, this needs to be repeated in blocks to create large numbers of genotypes for analysis. Clonal studies replicate genotypes by using clones of colonial organisms such as corals and bryozoans. Clones of the same genotype can be placed across various treatments and performance contrasted. Sea urchin and coral symbols courtesy of the Integration and Application Network, University of Maryland Center for Environmental Science (ian.umces.edu/symbols/).

As the ability to adapt to future changes in the environment depends on the existence of additive genetic variance within populations and DNA is considered to be the only contribution from the father to offspring, determining paternal variance is a good way to estimate genetic quality and determine species' adaptive potential. A male possessing good genes will produce offspring with a higher fitness regardless of female genotype (Neff and Pitcher, 2005). If species possess the adaptive capacity for ocean warming and ocean acidification, there needs to be evidence of a significant interaction between sire and either temperature or pH treatments, this would indicate existing genetic variation in the species response to climate change stressors. A significant dam and temperature interaction would indicate effects of both maternal provisioning (environmental, phenotypic) and genetic variation. Maternal effects are also important in population evolutionary dynamics as they impact the rate and direction of genetic change under selection (Tadros and Lipschitz, 2009). However, there are two maternal effects components: genetic and environmental, and these cannot be separated in quantitative genetic experiments (Rasanen and Kruuk, 2007). An interaction between sire and dam would indicate genetic variance due to non-additive genetic effects, i.e. the influence of the particular compatibility of the set of male and female gametes (Falconer, 1989). Although these are non-heritable effects, investigation of pair compatibility is also of interest in ocean change research and effects and individual pair results can be investigated with the NCII design.

Quantitative genetic designs are powerful tests which may reveal hidden evolutionary capacity. For the polychaete *Galeolaria caespitosa*, genetic variation was found in the species' response for two lower temperatures but not to high temperature, which may have led to the conclusion that ocean warming may eradicate this species. However using a multivariate analysis, *G. caespitosa* is likely to have high adaptive potential due to correlated responses to selection across all thermal environments (Chirgwin et al., 2015).

Studies on sea urchins, mussels and macroalgae have found significant levels of variation among genotypes, providing the potential for adaptation to ocean warming and acidification (Clark et al., 2013; Foo et al., 2012; Foo et al., 2014; Kelly et al., 2013; Lymbery and Evans, 2013; Sunday et al., 2011;). These studies have largely investigated adaptation to a single stressor (temperature: Chirgwin et al., 2015; Clark et al., 2013; Lymbery and Evans, 2013; acidification: Kelly et al., 2013; Sunday et al., 2011) with two studies investigating the response to both stressors concurrently (Foo et al., 2012; Foo et al., 2014). Although these experiments all

involve the use of the NCII design, they differ in whether the male-female crosses are fertilised in treatments, or transferred to treatments after fertilisation in control conditions. Thus far there are seven published studies using the gametes of free spawning invertebrates to investigate within-population genetic variation for tolerance to climate change stressors (including temperature and/or acidification; Table 5).

It is now understood that environmental effects can also alter sperm phenotype. There is evidence that both the pre and post-release environments of the sperm can affect offspring phenotype (Marshall et al., 2015). Within ejaculate differences in the sperm of *Styela plicata* has been shown to influence offspring fitness (Crean et al., 2012). Traditionally in quantitative genetic studies, sperm effects are assumed to be purely genetic (Lynch and Walsh, 1998) and thus it is assumed that the only source of variance contributed to offspring from the sire is genetic effects. However, as the sperm environment may also affect the offspring phenotype, estimating genetic variance from paternal lines may not be accurate (Crean and Bonduriansky, 2014). This has important implications for evolutionary studies (Bonduriansky and Day, 2009). Thus far there are six published studies using the gametes of free spawning invertebrates to investigate within-population genetic variation for tolerance to climate change stressors (including temperature and/or acidification; Table 1.4). Our understanding of multistressor interactions is limited and this is a focus in this thesis.

1.6.2 Clonal studies

Another approach to replicate genotypes without sourcing gametes for dam x sire crosses is to use clones of colonial organisms such as bryozoans and corals (Figure 1.5; Császár et al., 2010; Durrant et al., 2013; Pistevos et al., 2011). Different colonies of the same species are used to represent different genotypes. These are then divided up to form replicates or clones. A recent study by Durrant et al., (2013) compared the growth of different colonies of the bryozoan *Celleporaria nodulosa* to various temperature and pH treatments, comparing performance across the seasons. Decreased pH and increased temperature reduced the growth of the colonies with a large seasonal effect however there was no presence of G x E interactions where all genotypes (or clones) performed similarly to treatments in both seasons. This indicates little adaptive potential within populations under directional selection from climate change stressors. In contrast, another study using similar methods found that clones of the bryozoan *Celleporella hyalina* had

Table 5. Studies with marine animals and plants that use the North Carolina II design to test for within-population genetic variation in tolerance to ocean change and other anthropogenic stressors. This quantitative genetic design allows replication of different genotypes across environmental conditions and allows interactions between genotypes and stressors to be detected. The stressors tested and biotic trait scored, experimental design, fertilisation conditions and outcome are indicated. Most studies examine only one stressor and fertilise in control conditions. Only two have incorporated ocean warming and acidification scenarios and fertilised in treatment.

Species	Stressor/Trait	Experimental design	Fertilisation conditions	Outcome	Reference
		Single stressor studies			
Hormosira banksia (macroalga)	temperature/ growth, photosynthesis	NCII design, 3 sires x 3 dams x temperature, fully crossed	Crosses were fertilised and left for an hour in control conditions to let phototactic zygotes settle and then transferred to treatments	The presence of genetic variation in thermal sensitivity was found	Clark et al., 2013
Strongylocentrotus franciscanus (urchin) and *Mytilus trossulus* (mussel)	pH/ growth	NCII design, sire x dam x pH, fully crossed (*M. trossulus*: 4 dams and 10 sires, *S. franciscanus*: 10 dams and 10 sires)	Crosses were fertilised in control conditions and then transferred to treatments	The urchin showed greater genetic variation for larval size in response to ocean acidification than the mussel	Sunday et al., 2011
Strongylocentrotus purpuratus (sea urchin)	pH/ larval size	Modified NCII, 2 sires x 4 dams (where 1 male and 2 dams of each cross were from different sites)	Not stated	Incorporating heritability/adaptation into modelling showed that the low pH driven decrease in population growth rate is up to 50% smaller than that predicted by the 'no-adaptation' scenario	Kelly et al., 2013
Heliocidaris	temperature/	NCII design, 3 sires x	Crosses were fertilised	Hatching success was	Lymbery and

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erythrogramma armigera (sea urchin)	larval hatching success	3 dams x temperature, fully crossed	in control conditions where only fertilised eggs were transferred to treatments	reduced at higher temperatures, however analyses revealed significant additive genetic variance and G x E interactions underlying hatching success	Evans, 2013
Styela plicata (ascidian)	copper/ hatching success	NCII design, 3 sires x 3 dams x copper concentration, fully crossed	Fertilised in control conditions and then transferred to treatments	Significant G x E interactions in hatching success across copper concentrations	Galletly et al., 2007
Galeolaria caespitosa (polychaete)	temperature/ survival	NCII design, 2 sires x 2 dams, fully crossed	Fertilised in control conditions and then transferred to treatments after 2 hours	Significant sire x temperature interactions with correlated responses across all thermal environments	Chirgwin et al., 2015
Multistressor studies					
Centrostephanus rodgersii (sea urchin)	temperature, pH/ early development	NCII design, 3 dams x 3 sires x temperature x pH, fully crossed	Fertilised in treatments	Significant sire x stressor interactions indicate adaptive potential	Foo et al., 2012
Pseudoboletia indiana (sea urchin)	temperature, pH/ early development	NCII design, 2 dams x 4 sires x temperature x pH, fully crossed	Fertilised in treatments	Significant sire x stressor interactions, increased temperature alleviated effects of low pH	Foo et al., 2014

contrasting responses to increasing temperature and decreasing pH, thus demonstrating the existence of genetic variation which may enable future adaptation to ocean change (Table 6; Pistevos et al., 2011).

In field studies with the seagrass *Zostera marina*, increasing the genotypic diversity of communities exposed to extremely high water temperatures enhanced density and biomass production allowing normal functioning. This study highlights the importance of genetic diversity and its role in buffering against extreme climactic events (Reusch et al., 2005).

1.6.3 Laboratory selection experiments with short generation species

For taxa that have short generation times, laboratory selection experiments have been utilized to assess whether animals can adapt to environmental stressors over multiple generations. Taxa such as diatoms, phytoplankton and coccolithophores can produce multiple generations in the order of hours to days. Thus, laboratory cultures of different genotypes of these taxa can easily be established and studied over timescales where populations can evolve (Table 7; Figure 1.6; Collins et al., 2014; Dam, 2013; Kurihara and Ishimatsu 2008; Stillman and Paganini, 2015). These short generation species present unique opportunities to quantify evolutionary responses of populations to ocean change stressors. Zooplankton are well equipped for rapid evolutionary responses to ocean change due to extremely large population sizes and population genetic structure greatly linked to the particular ecological requirements of each organism (Peijnenburg et al., 2013).

The ability of the different populations to respond to selection by various stressors are tested by exposing them over multiple generations to the stressor of interest and the remaining genotypes can then be compared to controls to see if they have adapted to environmental conditions (Collins et al., 2014; Dam, 2013; Lohbeck et al., 2012). For example, the coccolithophore *Emiliania huxleyi* was exposed to low pH over 500 asexual generations and it was found that growth and calcification in the selected clones was eventually restored (Lohbeck et al., 2012). Rapid adaptation has also been noted in aquatic environments, with copepods inhabiting lakes acidified to pH 6 due to SO_2 emissions over an 8 year period (Derry and Arnott, 2007). However, not all populations have the presence of additive genetic variation in response to stressful environments. Other species present in the acidified lakes were unable to adapt as seen for the copepod and were eradicated (Derry and Arnott, 2007). For the copepod *Tigriopus californicus*, there was little adaptive potential in response to increased temperature across ten generations (Kelly et al., 2011). For the copepod, *Tisbe battagliai*, exposure of several generations to low pH conditions resulted in a

Table 6. Studies with marine animals and plants that use clones to test for within-population genetic variation in tolerance to ocean change stressors. The stressors tested and biotic trait scored, experimental design and outcome are indicated. Studies are separated into those that examine either single or multiple stressors.

Species	Stressor/Trait	Experimental design	Outcome	Reference
Single stressor studies				
Zostera marina (seagrass)	temperature/ growth rate, survival	Field experiment using genotyping to identify different clones	Increasing genotypic diversity of seagrass communities helped maintain normal functioning even with exposure to high temperature	Reusch et al., 2005
Zostera marina (seagrass)	temperature/ growth	Clone diversity x temperature	Positive effect of genotypic diversity on shoot densities of eelgrass in high temperature	Ehlers et al., 2008
Acropora millepora (coral) symbiont	temperature/ photosynthesis, gene expression, growth	Among clonal lineages with 4 pairs of branches from 20 colonies	Unlikely that thermal adaptation of the coral hosts will occur in time to match predicted rates of rapid ocean warming	Császár et al., 2010
Multistressor studies				
Celleporella hyalina. (bryozoan)	temperature, pH/ growth, reproduction	4 colonies (genotype) cut into 25 fragments (clones) and exposed to temperature x pH treatments	The presence of relevant levels of genetic variation among individuals may enable future adaptation via non-mutational natural selection to low pH and high temperature	Pistevos et al., 2011
Celleporaria nodulosa (bryozoan)	temperature, pH/growth	7 colonies cut into 18 fragments and exposed to temperature x pH treatments	In winter, low pH decreased growth. In summer, high temperature decreased growth of bryozoan colonies. The effects of decreased pH and increased temperature may be seasonally dependent and worse during summer.	Durrant et al., 2013
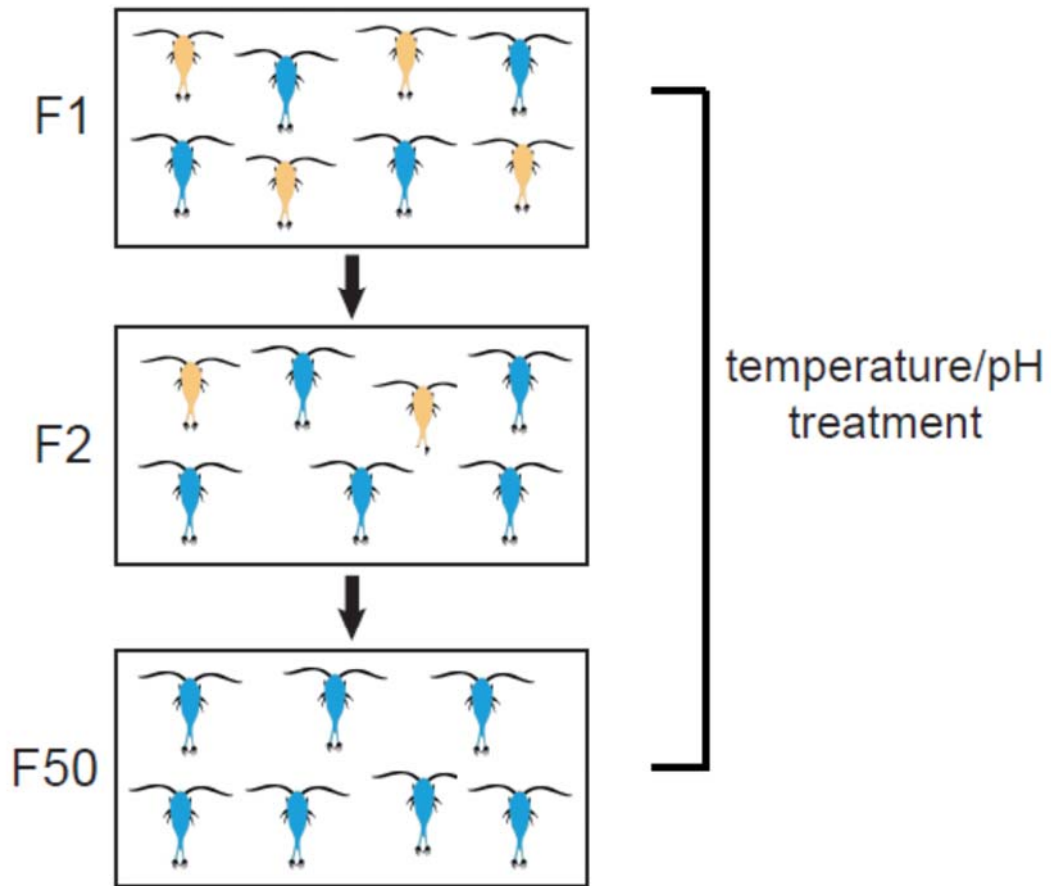
Figure 1.6. Laboratory selection experiments are used for taxa that have short generation times. Populations are exposed over multiple generations to the stressor of interest and the remaining genotypes can then be compared to controls to see if they have adapted to environmental conditions. Copepod symbol courtesy of the Integration and Application Network, University of Maryland Center for Environmental Science (ian.umces.edu/symbols/).

reallocation of energy resources to maintain reproductive output. However, this came at a cost with decrease in somatic growth (Fitzer et al., 2012).

Laboratory selection experiments have been taken one step further through identification of gene differences between the start and end of the adaptation process (Lohbeck et al., 2014). Genes related to pH regulation and carbon transport were up-regulated in low pH adapted populations which allowed restoration of growth and calcification in the coccolithophore *Emiliania huxleyi*, highlighting the underlying molecular mechanisms related to adaptation to low pH (Lohbeck et al., 2014). More recently, laboratory selection experiments have been utilized to understand transgenerational effects and their part in increasing resilience to ocean change (see below).

1.6.4 Genetic Correlations; Interactions Across Multiple Environments

Genetic correlations are useful in understanding the performance of genotypes in response to different environments and accompany identification of G x E interactions as they help understand the relationship across multiple environments (Sgrò and Blows, 2004). For example, in multistressor studies, genetic correlations can reveal whether adaptation to both stressors can occur simultaneously (Figure 1.7) (Clark et al., 2013; Foo et al., 2012; Foo et al., 2014; Sgro and Blows, 2004). A genetic correlation is the proportion of variance that two genetic traits share and is central to understanding evolutionary processes (Astles et al., 2006). A trait expressed in multiple environments is treated as two different traits and so a high genetic correlation indicates that the same set of genes influences the two traits similarly and that genotypes would be consistent across environments. For example, Clark et al., (2013) found positive genetic correlations between 120 hour old embryos of the marine alga *Homosira banksii* grown in various temperature treatments, indicating that genotypes that performed well in the control were also those who performed well in elevated temperatures. Furthermore, positive genetic correlations have been found for the embryos of the sea urchin *Centrostephanus rodgersii* where embryos that performed best in high temperature scenarios also performed best in low pH (Foo et al., 2012).

Table 7. Studies with marine animals and plants that use selection experiments with short generation species to test for adaptation to ocean change stressors. The stressors tested and biotic trait scored, experimental design and outcome are indicated.

Species	Stressor/Trait	Experimental design	Outcome	Reference
Scottolana Canadensis (copepod)	temperature/ growth	Northern and southern populations reared in different temperature scenarios over several generations	Northern populations locally adapted to grow in lower temperatures	Lonsdale and Levinton, 1985
Acartia tsuensis (copepod)	pH/ survival	The copepods were grown in low pH over two generations	No effect of low pH with copepods, with first and second generations developing from eggs to adults normally	Kurihara and Ishimatsu, 2008
Tisbe battagliai (copepod)	pH/ naupliar production, growth	The copepods were raised in low pH conditions for four generations	Copepods reallocated energy resources with great costs to somatic growth	Fitzer et al., 2012
Gephyrocapsa oceanica (coccolithophorid)	pH/ growth rate, carbon fixation	The coccolithophores were exposed to low pH over 670 generations	Selected coccolithophores were adapted to low pH conditions	Jin et al., 2013
Tigriopus californicus (isopod)	temperature/ survival	30 different lines exposed over 10 generations to increased temperature	Low adaptation potential to increased temperature	Kelly et al., 2011
Thalassiosira pseudonana (diatom)	pH/ photosynthesis	Diatoms were exposed to low pH conditions over 100 generations	No evidence of genetic variation in low pH conditions	Crawfurd et al., 2011
Emiliania huxleyi (coccolithophore)	pH/ growth, calcification	Exposed populations of clones to low pH and assessed growth over 500 asexual generations	Growth and calcification in selected clones raised in low pH was mostly restored	Lohbeck et al., 2012; 2014
Daphnia pulex (crustacean)	temperature, salinity/ metabolism	*Daphnia* were raised for six generations in various temperature and salinity treatments	The effects of temperature and salinity were reduced with each generation grown in treatment conditions	Chen and Stillman, 2012

Calanus finmarchicus (copepod)	pH/ food availability	The copepods were exposed to low pH for two generations under limited food availability	The delay in developmental rate observed in low pH in F1 disappeared in the F2 offspring	Pederson et al., 2014
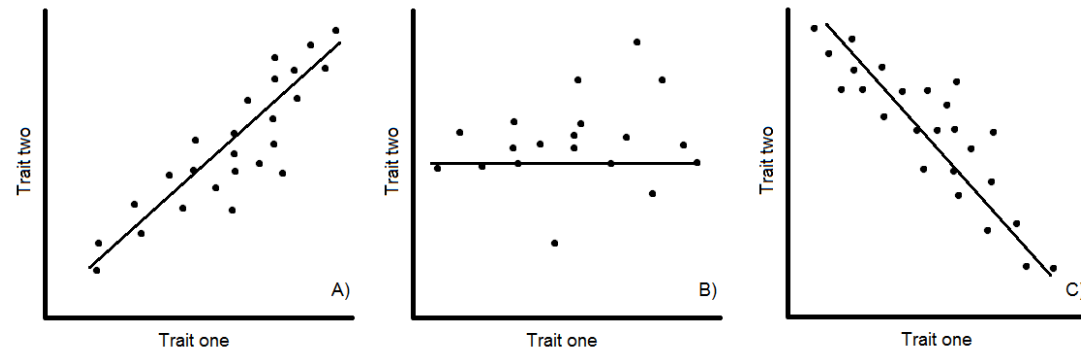
Figure 1.7. Examples of possible genetic correlations. Genetic correlations calculate the proportion of variance that two traits share. For positive genetic correlations (A), similar genes influence both traits. For a correlation of 0 (B), different sets of genes influence both traits. For negative genetic correlations (C), performance in trait one will have trade-offs with performance in trait two.

With more pronounced G x E interactions, the genetic correlation across the environments becomes lower. If the correlation is 0, the performance of a genotype in one environment does not predict its performance in another. However, negative genetic correlations indicate that performance in one environment has trade-offs with performance in the other environment, which is an important prerequisite for evolutionary specialization (Figure 1.7) (Eisen and Saxton, 1983). For example, if a negative genetic correlation was found for a species regarding performance across decreased pH and increased temperature, it is unlikely that adaptation to both stressors simultaneously could occur (Sgrò and Blows, 2004). Therefore calculating genetic correlations is important because, although genetic variance may be present, selection by ocean warming and acidification will only result in adaptation if tolerance is unconstrained by negative genetic correlations (Blows and Hoffmann, 2005).

1.6.5 Heritability

Heritability is defined as the proportion of phenotypic variation (V_P) due to variation in genetic values (V_G). Genotypes are not passed on from parents to offspring; it is the alleles at the loci that influence different traits that are inherited. The effect a particular allele has on a trait depends on that allele's frequency in the population, and the effect of each genotype that includes that allele. The additive genetic value of an individual is then the sum of the average effects of all the alleles the individual carries (Falconer and Mackay, 1996). According to Mendelian principles, one allele from each locus is present in the egg and the sperm, and it is in this way that additive genetic values are passed on from parents to offspring.

Broad-sense heritability of a trait is defined as the proportion of trait variation that is due to genetic effects, and thus includes all potential sources of genetic variation (additive, maternal, paternal, dominance and epistasis effects). For example, parents of corals from warm locations delivered greater thermotolerance to their offspring compared to parents from cooler locations, where broad-sense heritability accounted for 87% of larvae survival (Dixon et al., 2015). Narrow-sense heritability is the proportion of genetic variation due to additive genetic effects only and describes the degree of resemblance between relatives. A study with the polychaete *Hydroides elegans* calculated the narrow sense heritability of egg size to be 0.45, which indicates that there is significant potential for egg size to respond to selection pressures such as ocean change (Miles et al., 2007).

There is often no distinction made between broad and narrow sense heritability, however narrow-sense is most commonly used in animal and plant selection programs

because the response to selection depends on only additive genetic variance (Falconer and Mackay, 1996; Hill et al., 2008; Lynch and Walsh, 1998). As heritability is calculated as a ratio of variance components, the value always lies between 0 and 1, where a value of 1 is completely genetic.

Heritability is estimated by measuring the extent to which the offspring resemble the parents (Kruuk et al., 2000; Kruuk, 2004). As it is not often possible to observe similarity between two generations (parents and offspring), it is often favourable to measure heritability across one generation (Lynch and Walsh, 1998). For studies such as those involving the NCII method, the design allows variance to be separated into paternal, maternal, interaction and error effects (Lynch and Walsh, 1998). Therefore, heritability can be calculated by partitioning total phenotypic variance (V_P), i.e. the trait of interest, into genetic (V_G) and environmental variance (V_E) components obtained through analyses of variance (ANOVA). Depending on whether the study involves clones as replicates or half-sib and full-sib families, this determines which components from the ANOVA represent V_G and V_E. Then heritability can be calculated as V_G/V_P (Falconer and Mackay, 1996).

In Sunday et al., (2011), narrow sense heritability was calculated for sea urchins and mussels for larval size in a low pH environment. By incorporating the calculation into the breeder's equation to simulate the response to selection, they determined that the sea urchin was likely to have a faster evolutionary response than the mussel. Similarly, heritability was calculated for sea urchins in multistressor environments in Foo et al., (2014) where the dam contribution was much larger at fertilisation than at gastrulation, suggesting that performance at the prezygotic stage was dominated by maternal effects. Sire effects remained similar throughout both developmental stages.

For clonal studies, variation within clones gives an estimate of V_E where variation among colonies is due to $V_G + V_E$. Therefore genetic variation among colonies (V_G) can be estimated by $V_P - V_E$. For laboratory selection experiments, similarities between parents and offspring can be observed directly (Collins et al., 2014).

1.7 Transgenerational Effects

Transgenerational effects are effects on offspring phenotype determined from parental environmental history. In this case, gene expression patterns are passed from one generation to the next and cannot be explained by changes to the DNA sequence. Transgenerational effects can result in pre-adapted offspring that exhibit traits associated with increased fitness in environmental conditions experienced by their parents. This can be a type of

transgenerational plasticity (Figure 1.8; Salinas and Munch 2012) or be due to differential selection for favourable alleles in a population of larvae. Transgenerational experiments involve exposure of parents to environmental conditions during their reproductive conditioning (i.e. gamete development). These parents are used to generate gametes for the F1 population. The resultant offspring are then exposed to the environments the parents were exposed to, to determine whether parental exposure can influence effects on the offspring (Parker et al., 2015). It has been established that the stress response experienced during an early life stage can carry over to subsequent stages. For instance, juveniles of the oyster *Ostrea lurida* performed worse in low pH if the larvae were also exposed to low pH (Hettinger et al., 2012).

Several recent studies have investigated transgenerational plasticity in response to climate change stressors, especially with fish. In these studies, exposure of parents to environmental conditions reduced the negative impacts of the stressors on their offspring (Donelson and Munday, 2015; Munday, 2014; Salinas et al., 2013). Donelson et al., (2011) found that the negative effects of warming in a tropical reef fish was completely ameliorated when previous generations were exposed to the same elevated temperatures. Similar transgenerational plasticity was seen in the minnow *Cyprinodon variegatus* (Salinas and Munch, 2012). For reef fish juveniles, parental exposure to low pH increased juvenile survival in low pH conditions, much higher than juveniles whose parents were sourced from ambient conditions (Miller et al., 2012). Similar results were also seen for Atlantic silversides (Murray et al., 2014) and offspring of the fish *Gasterosteus aculeatus* (Schade et al., 2014).

Transgenerational effects can also change with season, where success of offspring of the fish *Menidia menidia* in reduced pH corresponded with pH fluctuations in the parents' habitat at different times of the year (Murray et al., 2014). More recently, the molecular processes underlying transgenerational acclimation to increased temperature were investigated in tropical reef fish. For offspring whose parents were acclimated to increased temperature, there was an upregulation of immune and stress related genes which better equipped the juveniles to cope with thermal stress (Veilleux et al., 2015).

A marine fish that has temperature-dependent sex determination was raised over several generations in various ocean warming scenarios (Donelson and Munday, 2015). For temperatures of +1.5°C, there was complete restoration of the normal offspring sex ratio of 0.5 (equal numbers of male and female offspring) after one generation. At +3°C however, there was only a limited improvement in sex ratio even after two generations of rearing in elevated temperature. In this case the proportion of female offspring was greatly reduced.

Therefore transgenerational plasticity can ameliorate effects of ocean warming on the sex ratio of the reef fish species that was investigated to some extent but may be limited as environmental conditions become more extreme (Donelson and Munday, 2015).

Climate change transgenerational experiments have also been conducted with marine invertebrates. In the Sydney rock oyster, adults exposed to decreased pH for five weeks during reproductive conditioning produced larvae with a reduction in development time and increased body size when raised in similar conditions (Parker et al., 2012). It is important to note that the time parents are acclimated has been shown to give rise to differing responses in their offspring. Adults of the sea urchin *Psammechinus miliaris* acclimated to environmental conditions for either 28, 42 or 70 days produced offspring with varying survival rates dependent on parental pre-exposure (Suckling et al., 2014a). Exposure of the sea urchin *Echinometra mathaei* to low pH for seven weeks did not improve performance of larvae in low pH (Uthicke et al., 2013). Similarly, for the sea urchin *S. droebachiensis*, fewer offspring compared to the control successfully developed into juveniles when exposed to low pH when adults were only acclimated for four months. However, when adults were acclimated for 16 months, there was no difference in larval survival in response to low pH in comparison with the control (Dupont et al., 2013). Ideally, acclimation periods for adults should cover the minimal length of time needed for negative effects of ocean stressors to disappear, which is likely to vary for different species (e.g. Sydney rock oyster: 5 weeks, Green sea urchin: 16 months; Parker et al., 2012; Dupont et al., 2013). The duration of gametogenesis is a key consideration when assessing appropriate acclimation period.

Carry over effects are not always positive (Parker et al., 2015). For the spiny damselfish, parental acclimation did not improve behavioural or sensory performance in response to decreased pH (Welch et al., 2014). Genetic adaptation will be essential for this species to overcome this negative effect of ocean acidification. This emphasizes that the type of trait assessed (e.g. temperature tolerance, calcification, sensory performance) can determine the inferences made with respect to the presence of positive or negative transgenerational effects. It is important to assess a suite of traits to better understand the effects of parental environment on performance and the fitness of offspring (Monaco and Helmuth, 2011; Stillman and Paganini, 2015).
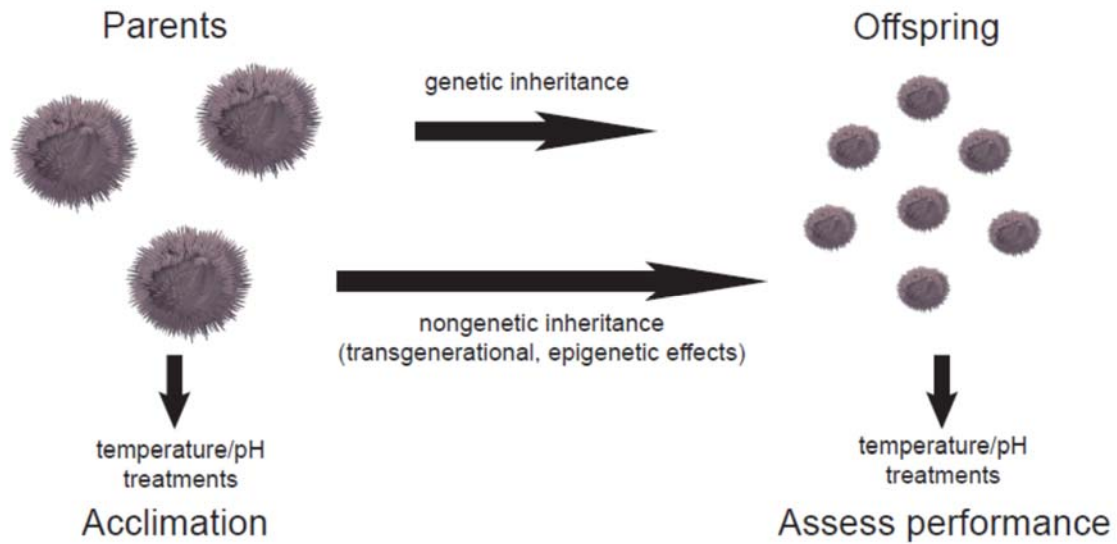
Figure 1.8 The performance of offspring in a changing ocean can be influenced by both genetic effects, and transgenerational effects when their parents have experienced a similar environment. Acclimation of parents to experimental conditions erases/reduces the influence of physiological history. The parents are conditioned to a new environment during gamete development. Performance of the offspring can then be assessed by considering morphological traits, changes in physiology and the epigenome. Sea urchin symbol courtesy of the Integration and Application Network, University of Maryland Center for Environmental Science (ian.umces.edu/symbols/).

Female multiple mating, has been identified as a bet hedging strategy that yields multigenerational fitness (Garcia-Gonzalez et al., 2015). For the sea urchin *H. erythrogramma armigera*, polyandrous females show maximized chances of offspring survival in changing ocean conditions (Garcia-Gonzalez et al., 2015). Mating with multiple males creates larvae of greater genetic diversity, increasing the chance that the optimal phenotype for survival in environmental conditions will be represented. For offspring of the tubeworm *Hydroides elegans*, performance of offspring from parents acclimated in control and low pH environments in the laboratory was mediated by parental acclimation with different influences from each gender (Lane et al., 2015). Overall, performance of offspring in low pH scenarios were similar regardless of whether the parents were acclimated to control or low pH environments. However, the growth rate for offspring of females acclimated to low pH conditions decreased in control conditions, a result not found for males (Lane et al., 2015). This research highlights that transgenerational effects can both facilitate and impede genetic evolution (Chevin and Lande, 2010; Merilä, 2015) and this may be driven by gender differences and different selection pressures (Lane et al., 2015).

1.8 Multigenerational Effects

For the majority of transgenerational studies, adults are acclimated in environmental conditions and performance of offspring are assessed to determine whether resilience can be transferred across a generation (Parker et al., 2015; Salinas and Munch, 2012). Whether these positive effects are able to persist into adulthood and further on into the next generation is less understood (Parker et al., 2015).

A study with the copepod *Pseudocalanus acuspes* raised for two generations in low pH treatments found that ocean acidification effects were alleviated. When the parental population were acclimated to low pH, subsequent generations showed a recovery in metabolism and respiration, measurements which were decreased in offspring whose parents were not exposed to low pH (Thor and Dupont, 2015). Furthermore, the fecundity of adults was significantly decreased in low pH if parents were not previously exposed to low pH (Thor and Dupont, 2015). More recently, Parker et al., (2015) examined whether transgenerational effects continued across generations where larvae of the oyster *Saccostrea glomerata* with a prior exposure to decreased pH were raised into adults and spawned, with the F2 generation also raised in low pH. Prior history of larval exposure to decreased pH carried on into adult hood and resilience of the F2 larvae and juveniles were also increased

with lower abnormality seen in decreased pH treatments in comparison to those with no prior history of low pH exposure (Parker et al., 2015).

Parental exposure to climate change can cause transgenerational changes that allow offspring to endure stressors, with carry over effects persisting over later life-history stages and multiple generations. A caveat with multigenerational experiments though, is that only the survivors of previous generations are considered. As stated in Parker et al., 2015, there was high mortality (46%) during F1 larvae development which would have resulted in a naturally selected population of adults most tolerant to low pH which were then used for the F2 generation.

1.9 Epigenetics

The outcomes of quantitative genetic breeding, multigenerational and transgenerational experiments in regimes where climate change stressors are used may be influenced by the epigenome (Vandegehucte and Janssen, 2014). For the anemone fish and green sea urchin, epigenetic inheritance was identified as the transgenerational mechanism in which parental exposure improved offspring performance to low pH (Dupont et al., 2013; Miller et al., 2012). Transgenerational effects can include epigenetic effects which are heritable changes in the genome, without alteration to DNA (Figure 1.8; Burton and Metcalfe, 2014; Turner, 2009).

Some examples of mechanisms that can produce such changes include DNA methylation and modification of histones which can alter how genes are expressed without changing the DNA sequence (Cavalli 2006; Henikoff et al., 2004). DNA methylation is well documented in mammals but there is very limited data on invertebrates. Investigation of DNA methylation in the oyster *Crassostrea gigas* revealed that methylation is common in the oyster genome (Gavery and Roberts, 2010) where categories of functional genes display significantly different levels of methylation, especially with respect to gene families involved in stress and environmental responses. DNA methylation has been identified as the mechanism in which *C. gigas* survives changing ocean conditions (Vandegehucte and Janssen, 2014). In a study with the Antarctic polychaete *Spiophanes tcherniai*, embryos were raised for one month in either control or increased temperature scenarios (+4°C). DNA methylation in the epigenome was much higher for groups of those cultured at increased temperature treatments in comparison to the control polychaetes (Marsh and Pasquelone, 2014). More research into the patterns of methylation is needed though to understand its contribution to gene regulation.

For *Drosophila* embryos exposed to heat stress, modification of the histones changed the chromatin structure of the DNA resulting in the expression of silenced genes (Seong et al., 2011). This also resulted in a phenotype of white eye colour which was often passed on to offspring suggesting these epigenetic changes were heritable (Seong et al., 2011).

It is becoming increasingly evident that environmental history of parents and even previous generations (e.g. grandparents) can play a major influence on the phenotype of offspring through epigenetic effects (Burton and Metcalfe, 2014; Daxinger and Whitelaw, 2010; Ho and Burggren, 2010; Marsh and Pasquelone, 2014). Since epigenetic inheritance can allow phenotypic plasticity to cross generations, where plastic responses in the parents can alter offspring development, plastic responses can allow population to persist in a changing environment, especially those changing too fast for genetic adaptation (Lloyd Morgan 1896; Merilä, 2012). Therefore, the phenotypic change in a population might not always involve adaptive evolution and be entirely mediated by non-genetic factors (Bonduriansky and Day, 2009). Considering only genetic variation in adaptive capacity studies might not reveal true population persistence, and effects of stressors may be overestimated (Sunday et al., 2011; Thor and Dupont, 2015).

1.10 Thesis outline

Sea urchins provide a tractable system for study of gamete and fertilisation responses. The effects of ocean acidification and ocean warming on marine invertebrate gametes have mainly focused on the sperm cell. This thesis addresses this issue with an aim to identify effects of ocean acidification on the extracellular jelly coat of the egg. We focus on four sea urchin species; *Centrostephanus rodgersii*, *Heliocidaris erythrogramma*, *Heliocidaris tuberculata*, *Echinometra mathaei*.

For free spawning invertebrates, male and female gametes can be isolated for experimental matings for application in quantitative genetic studies which follow the success of the offspring of individual sets of parents and a tractable system to investigate genetic variation to climate change stressors. This thesis investigates the genetic basis of resistance to warming (+3°C) and acidification (-0.3-0.5 pH units) in early development of sea urchins including polar, tropical and temperate species. As ocean change is gradual and animals may show potential to adapt, quantitative genetic designs are used here to investigate adaptive capacity of echinoid species to ocean acidification and warming.

The potential for an evolutionary response of early development (to blastulae) of the Antarctic sea urchin *S. neumayeri* to near future ocean warming and acidification was

investigated in multifactorial experiments using a NCII breeding design. A similar design (to gastrulae) was employed for the tropical sea urchin *P. indiana*, with incorporation of heritability estimates across the different environments tested. For the temperate sea urchin *H. erythrogramma*, genetic variance in response to ocean warming and ocean acidification was quantified across the life cycle from fertilisation to the settled juvenile, incorporating effects of treatments on calcification in the juveniles.

1.10.1 Aims

The aims and hypotheses are addressed in four data chapters:

1. Chapter Two characterises the response of the egg jelly coat in response to ocean acidification across four echinoderm species. It was predicted that acidification would reduce the size of the jelly coat as low pH water is a routine method to remove the jelly coat for developmental biology studies.

2. Chapter Three investigates the adaptive capacity of early development in the Antarctic sea urchin *Sterechinus neumayeri* to increased temperature and acidification. For this highly stenothermal species where isolation in cold waters over evolutionary timescales is associated with losses from the genetic tool kit, it was predicted that there would be a reduction in the genetic variation required for adaptation to a changing ocean.

3. Chapter Four investigates whether the tropical sea urchin *Pseudoboletia indiana* possesses heritable genetic variation in response to increased temperature and acidification. Due to recent expansion to Sydney, it was predicted that warming would facilitate development in Sydney populations without deleterious effects.

4. Chapter Five investigates genetic variation in the sea urchin *Heliocidaris erythrogramma* to ocean change stressors across the life cycle, from fertilisation to the settled juvenile. It was predicted that the magnitude of the effect of stressors across the various developmental stages would differ, and that as a lecithotrophic species with a large egg, *H. erythrogramma* may show strong maternal effects in response to ocean acidification and warming.

CHAPTER TWO: CHANGES IN THE JELLY COAT OF ECHINOID EGGS IN RESPONSE TO ACIDIFICATION, COULD THIS DRIVE VARIATION IN FERTILISATION ASSAYS?

2.1 Abstract

The egg jelly coat performs important roles in fertilisation as a source of sperm activating compounds, in gamete recognition, and in increasing the target size for sperm. The effect of ocean acidification (OA) (-0.5 pH units) on jelly coat area was investigated in four echinoids: *Echinometra mathaei*, *Heliocidaris tuberculata*, *Centrostephanus rodgersii* and *H. erythrogramma*. After 15 minutes, there was a significant reduction in jelly coat area for *E. mathaei* and *C. rodgersii* of ~50% at pH 7.6 with no effect of decreased pH for the other two species. The reduction in jelly coat size at lower pH suggests that sperm-egg collision rates and fertilisation success will be negatively affected by OA for some species. Egg diameter and jelly coat area differed between females within species suggesting effects of OA on jelly coat size might select against susceptible phenotypes. The results show the importance of considering impacts of stressors on the egg. Variability in egg and jelly coat size of females within and between species and differences in the response of the jelly coat to reduced pH are potential sources of variation. These may contribute to contrasting outcomes of fertilisation in OA experiments using the gametes of free spawning marine invertebrates.

2.2 Introduction

Due to increased atmospheric CO_2, the ocean is expected to decrease in surface ocean pH by approximately 0.3 pH units by the end of the century (IPCC, 2013) Many marine invertebrates release their gametes into the overlying water and so they are directly exposed to water conditions (Pechenik, 1987) Echinoderm gametes and fertilisation are commonly used in ecotoxicology to assess effects of contaminants, including increased CO_2 (Byrne, 2011, 2012). Virtually all CO_2-driven ocean acidification (OA) research on gametes have focussed on the sperm cell, investigating sperm motility, respiration, swimming velocity and intracellular pH (Martin et al., 2011; Schlegel and Havenhand, 2012; Schlegel et al., 2015). Only one study has investigated the effects of OA on the egg cell showing that intracellular pH decreased with exposure to pH 7.6 (Bogner et al., 2014). Low pH seawater (~pH 5) is

long known to remove the jelly coat (Inoue and Yoshioka, 1980; Podolsky, 2002) indicating that it is also important to understand the impacts of OA on eggs.

The jelly coat that surrounds the eggs of many marine invertebrates (Bonnell et al., 1994; Farley and Levitan, 2001; Suzuki, 1989) serves many roles before and during fertilisation (Kanatani and Nagahama, 1983; Podolsky, 2002). Jelly coats provide an economical method to increase egg target size for sperm thereby facilitating fertilisation success by increasing sperm-egg collision frequency (Farley and Levitan, 2001; Podolsky, 2002; Vogel et al., 1982). The jelly coat also contains specific-specific gamete recognition compounds that stimulate sperm metabolism and promote the acrosome reaction (AR) in a species-specific manner (Matsui et al., 1986). In sea urchins, the egg jelly coat contains short peptides, speract and resact, which attract sperm and promote directional swimming and orientation of the sperm, increasing the probability of fertilisation (Islam et al., 2008; Matsumoto et al., 2003; Miller, 1985). These peptides switch on signaling pathways in the sperm, resulting in an increase in intracellular Ca^{2+} resulting in increased sperm respiration and motility (Islam et al., 2008; Matsumoto et al., 2003).

The egg jelly coat of sea urchins is a polysaccharide fibre network embedded in a glycoprotein matrix that hydrates in contact with seawater (Bonnell et al., 1984; Pomin, 2015; Suzuki, 1989). These fibre networks reduce the mechanical stress the egg experiences when passing through the gonopore during spawning because the jelly coat preferentially compresses rather than the egg (Bolton et al., 2000; Thomas and Bolton, 1999). For *Arbacia punctulata* and *Lytechinus variegatus*, eggs with their jelly coats removed are destroyed at levels of shear stress that eggs normally encounter in nature (Thomas and Bolton, 1999). The jelly coat also protects against polyspermy where up to 90% of sperm remain trapped in the jelly coat, even at high sperm to egg ratios (Bohus-Jensen, 1953; Hagstrom, 1953). Complete or partial removal of the jelly coat is required to achieve hybridisation between some sea urchin species and thus the jelly coat acts as a barrier preventing interspecies fertilisation (Raff et al., 1999).

A large component of the egg jelly coat consists of sulphated glycans with varying numbers of glycosidic linkages and attached sulfate groups (Pomin, 2015; SeGall and Lennarz, 1979), where the patterns of glycosidic linkages and sulfation are responsible for the species specific induction of the AR (Vilela-Silva et al., 2008). A "pH-jelly water balance" model proposed by Shu et al., (2015) suggests that the decrease in jelly coat size at lowered pH is due to water loss and depends on the surface charge of the glycans (Menkhorst and Selwood, 2008; Shu et al., 2015).

In developmental biology research, jelly coats are often removed by exposing eggs for several minutes to acidified seawater (pH 5-5.5) through addition of mineral acid (Dale and DeFelice, 2011; Podolsky, 2002; Vacquier, 2011). Low pH water solubilises the jelly coat, an effect that may also occur with CO_2-driven OA exposing molecules that are usually concealed in the gel matrix (Dale and DeFelice, 2011). The effect of removal of the jelly coat on fertilisation is not well understood, with conflicting results (Podolsky, 2002). Studies that reported little or no effects of jelly coat removal involved short experiments with high levels of sperm, where removal of the jelly coat increased fertilisation rate by removal of a barrier (Hagstrom, 1959; Vacquier et al., 1978). In contrast, studies investigating fertilisation in sperm limiting conditions found a decreased fertilisation success (McLaughlin and Humphries, 1978; Styan, 1998). Removal of the jelly coat using acidified seawater is detrimental for some species, as seen in the clumping of dejellied eggs in *Arbacia punctulata* and *Strongylocentrotus franciscanus* (Vacquier, 2011).

Our understanding of the effects of OA on marine invertebrate gametes are based almost entirely on the sperm cell, with contrasting behavioural responses of sperm and outcomes for fertilisation reported (Martin et al., 2011; Schlegel and Havenhand, 2012; Schlegel et al., 2015). Thus far, there is only one OA study on eggs and no studies have considered the effects of OA on the egg jelly coat (Bogner et al., 2014). As both gametes have to be functional for fertilisation, and the function of the egg and its extracellular coat are likely to be affected by OA, this study investigated the effects of OA on the size of the jelly coat of four echinoid species with different egg sizes (three with small eggs, 70-111 μm diameter: *Heliocidaris tuberculata*, *Centrostephanus rodgersii*, *Echinometra mathaei*, and one with large eggs, 390 μm diameter: *Heliocidaris erythrogramma*). We hypothesised that CO_2-driven ocean acidification would impact the size of the jelly coat and that this may differ between species given the key role that the jelly coat plays in fertilisation. Inherent differences in egg jelly coats and their sensitivity to OA may contribute to variation in results between studies on marine invertebrates investigating the impact of OA on fertilisation (Byrne, 2012).

2.3 Methods

2.3.1 Study species, collection sites and spawning procedure

Heliocidaris erythrogramma, *Centrostephanus rodgersii* and *Heliocidaris tuberculata* were collected from Bottle and Glass Point (33.84833° S, 151.26944° E), Sydney, NSW. Animals were transported in ambient seawater in a cool box and transferred promptly to flow through

aquaria. *Echinometra mathaei* were collected from Woolgoolga (30°06′38″S 153°12′02″E) and transferred to large flow through aquaria at the National Marine Science Centre in Coffs Harbour, NSW. All animals were used for experiments within days of collection. Spawning was induced by injection of 2-4 mL of 0.5 M KCl. Eggs were examined for consistency in shape and transferred to a beaker (500mL) of fresh filtered seawater (FSW, 1 μm).

2.3.2 Experimental conditions

Experimental treatments consisted of two pH_T levels (Mean ± SE, control 8.00 ± 0.02 and 7.59 ± 0.01, n= 4) (Table 2.1). Treatments were based on model projections for end of century surface ocean waters in south-east Australia (IPCC, 2013) To achieve experimental treatments, FSW was bubbled with a mixture of air and CO_2 where pH adjustment was tracked using a pH meter (WTW—Wissenschaftilich-TechnischeWerkstatten GmbG P4) and probe (WTW SenTix 41 pH electrode). Probes were calibrated using NIST high precision buffers pH 4.0, 7.0 and 10.0 (ProSciTech). The actual pH on the total scale was determined using the spectrophotometric approach with *m*-cresol purple indicator dye (Acros Organics lot AO321770) and a USB4000 spectrophotometer following the procedures outlined in SOP 6b of Dickson et al., 2007 and the equations of Liu et al., 2011. Experiments were conducted at room temperature (Mean = 20.48˚C, SE = 0.49, n = 4). The mean salinity of treatment water was 34.7 psu, and dissolved oxygen remained >90%.

Samples of the water (250 mL) were collected at the conclusion of each experiment and fixed with 100 μL of saturated HgCl. These were used to determine total alkalinity (TA) by potentiometric titration (Metrohm 888 Titrando) using certified reference standards (Dickson et al., 2007). Experimental pCO_2 (Table 2.1) were determined from TA, temperature, pH_T and salinity data using CO2SYS using the dissociation constants of Mehrbach et al. 1973 as refitted by Dickson and Millero 1987.

2.3.3 Jelly coat experiments

For each of the four species, five females were used. For each female, egg counts were determined in 100 μL aliquots from the egg suspension and transferred to treatments within minutes of spawning. Approximately 200 eggs were placed into containers (100mL glass jars) one for each of the two treatments (control pH: 8.1, pH 7.6) and one for each of the five time points (0, 5, 10, 15 and 30 minutes). Thus, each time point for each pH treatment had an

Table 2.1. Water conditions in jelly coat experiments for each species. Values for pH_T measured per treatment are shown. pCO_2 and the saturation states of calcite (ΩCa) and aragonite (ΩAr) were calculated in CO2SYS using the data on total alkalinity, temperature and salinity.

pH	Measure	*E. mathaei*	*H. tuberculata*	*C. rodgersii*	*H. erythrogramma*
8.1	pH_T	8.04	7.95	8.00	8.01
	pCO_2	289.6	375.7	312.0	317.9
	ΩCa	5.57	4.63	4.69	5.27
	ΩAr	3.63	3.01	3.04	3.43
7.6	pH_T	7.55	7.62	7.59	7.59
	pCO_2	1076.6	902.5	927.9	978.0
	ΩCa	2.17	2.43	2.11	2.34
	ΩAr	1.41	1.58	1.37	1.52
	TA	2290.22	2305.39	2204.92	2300.65
	Temperature	21.2	20.2	19.2	21.3
	Salinity	34.5	34.9	35.0	34.3

independent jar. Jelly coats expand to approximately 80% of their maximum thickness within minutes of contact with seawater and so the time points covered the greatest changes in jelly coat size (Podolsky, 2001). At each time point, a sample of eggs was taken from each pH treatment and suspended in Sumi ink (Holbein) so that the jelly coat could be visualised microscopically (Leica) (Figure 2.1). This method was repeated for the eggs of five females per time point for each of the four species.

Using Image J (U. S. National Institutes of Health), jelly coat size indices were obtained by taking the ratio of the diameter of jelly coat plus egg over the diameter of the egg only. As the spawned eggs were consistently spherical, the diameter was determined by measuring across the centre of the eggs (Figure 2.1). This allowed the size of the jelly coat to be compared across different females, which produce eggs of varying size. To determine effects of decreased pH on the jelly coat, the total area of the egg plus jelly coat minus the egg area only measured in Image J, provided an estimate of jelly coat area. Egg and jelly coat area measurements indicated changes in egg target size (*sensu*, fertilisation models, Podolsky, 2004) in response to decreased pH.

2.3.4 Statistical analyses

To investigate variation in the size of the egg and jelly coat within species, data on the size of the egg and jelly coat at time 0 were analysed using a one-way analysis of variance (ANOVA) in GMAV[42] with female as a random factor. For determining the effect of decreased pH on jelly coat area, females represented separate replicates. The mean jelly coat size per female (determined from five eggs) was used as the datum for analysis. Data were analysed using a three way ANOVA with time and pH as fixed factors, and species as a random factor. The three way ANOVA indicated a significant species x pH interaction thus data for each species was analysed separately using a two way ANOVA with time and pH as fixed factors.[43] The assumptions of homogeneity of variance were confirmed using Cochran's test. Where there were significant effects, Student Newman-Keuls (SNK) tests were used for post hoc analyses.

2.4 Results

2.4.1 Comparison of the egg and jelly coat size within species

For all species except *E. mathaei*, egg diameter differed significantly among females (small egg species ± 9µm; large egg species ± 43µm) (Tables 2.2 & 2.3). For all four species, the
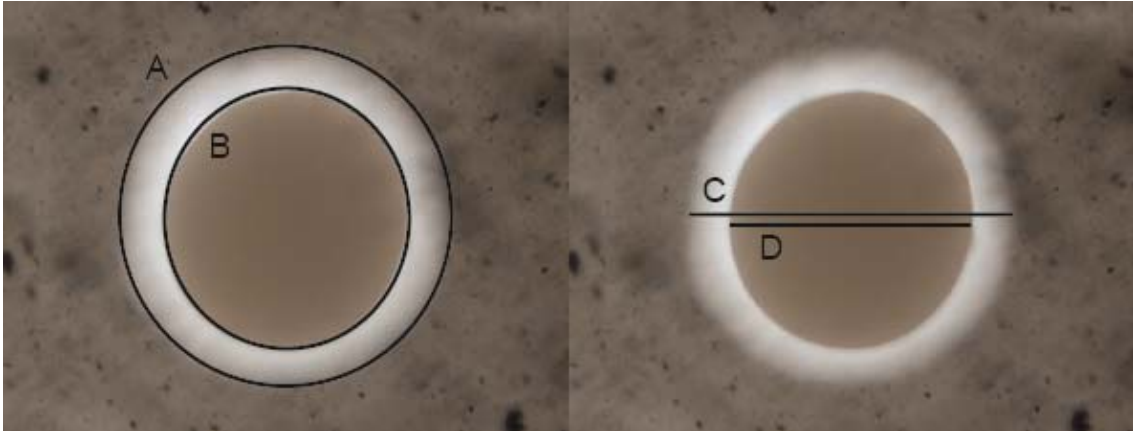
Figure 2.1. Eggs of *Centrostephanus rodgersii* suspended in Sumi Ink allow the extracellular jelly coat to be visualised. The total area of the egg plus jelly coat (A) minus the egg area only (B) determined in Image J gave an estimate of jelly coat area. An index of relative jelly coat size (jelly coat index) was obtained by taking the ratio of (C) diameter of jelly coat plus egg over (D) diameter of the egg only. Scale bar = 100μm.

jelly coat index, i.e. the size of the jelly coat relative to the egg diameter, significantly differed across the five females (Tables 2.2 & 2.4). It appears that as egg diameter increases, the relative size of the jelly coat decreases (Table 2.2).

2.4.2 Effects of ocean acidification on jelly coat size

The three way ANOVA analysis indicated that there was a significant species x pH interaction (Table 2.5). Thus to investigate the effects of pH on each species individually, separate ANOVA analyses for each species were run.[43]

2.4.3 Echinometra mathaei

For *E. mathaei*, egg jelly coat area in controls remained stable for five minutes and then decreased by ~10% at 15 minutes, although this was not significant (Figure 2.2). There were significant effects of decreased pH on the jelly coat area (Table 2.6; Figure 2.2). By five minutes in decreased pH, the jelly coat area was reduced by ~45%. There were no significant effects of time, or time x pH. Thus, after the jelly coat decreased in size in the first five minutes, there was little change thereafter in the low pH treatment.

2.4.4 Heliocidaris tuberculata

For *H. tuberculata*, egg jelly coat area in controls decreased by ~10%, five minutes post spawning, although this was not significant (Figure 2.2). There were no significant effects of decreased pH, time or time x pH. In decreased pH, the egg jelly coat area was reduced but this was not significantly different to the reduction seen for the egg jelly coat area in controls (Table 2.6; Figure 2.2).

2.4.5 Centrostephanus rodgersii

For *C. rodgersii* there were significant effects of time, decreased pH and time x decreased pH on egg jelly coat area (Table 2.6; Figure 2.2). The effects of decreased pH on egg jelly coat area were evident by five minutes, with a ~45% decrease in jelly coat area. The post hoc tests for the time x decreased pH interaction indicated that at 30 minutes, the jelly coat area in controls significantly increased by ~20% 30 minutes post spawning however this increase was not reflected for egg jelly coats in decreased pH (Figure 2.2).

Table 2.2. Egg diameter, egg plus jelly coat diameter and relative size indices of the jelly coat for four species of echinoid. For each female, egg diameter and egg plus jelly coat diameter were measured for five eggs.

Female	Egg diameter (µm±SE)	Range (µm)	Egg + JC diameter (µm±SE)	Range (µm)	Relative JC size index (±SE)
			E. mathaei		
1	70.84 (1.21)	(69-74)	121.91 (5.30)	(109-140)	1.70 (0.06)
2	69.76 (1.44)	(66-74)	119.47 (2.10)	(112-125)	1.71 (0.03)
3	70.06 (1.02)	(68-73)	133.74 (1.30)	(132-139)	1.91 (0.03)
4	67.70 (1.57)	(67-70)	126.58 (3.16)	(115-132)	1.88 (0.08)
5	70.84 (1.62)	(64-73)	116.90 (3.20)	(108-126)	1.65 (0.04)
MEAN	69.84 (1.37)		123.72 (3.01)		1.77 (0.05)
			H. tuberculata		
1	93.24 (1.65)	(88-99)	166.74 (2.42)	(160-175)	1.80 (0.04)
2	90.88 (1.01)	(87-94)	175.50 (5.76)	(162-182)	1.93 (0.06)
3	96.68 (0.79)	(94-99)	155.25 (2.31)	(151-163)	1.60 (0.03)
4	93.49 (0.99)	(93-96)	151.76 (7.42)	(135-171)	1.62 (0.064)
5	97.18 (0.77)	(95-99)	166.48 (5.37)	(152-179)	1.71 (0.05)
MEAN	94.29 (1.04)		163.15 (4.66)		1.73 (0.05)
			C. rodgersii		
1	117.00 (1.66)	(113-121)	180.91 (2.64)	(171-186)	1.55 (0.02)
2	118.33 (2.15)	(112-124)	193.07 (2.75)	(187-203)	1.63 (0.01)
3	104.60 (1.35)	(100-109)	158.04 (2.81)	(150-167)	1.51 (0.02)
4	108.27 (2.28)	(103-116)	185.74 (5.99)	(182-207)	1.72 (0.06)
5	116.52 (1.69)	(114-120)	193.29 (2.40)	(187-201)	1.66 (0.04)
MEAN	112.94 (1.83)		182.21 (3.32)		1.61 (0.03)
			H. erythrogramma		
1	390.46 (5.97)	(375-407)	506.69 (7.94)	(476-521)	1.29 (0.02)
2	394.25 (8.45)	(368-421)	502.59 (15.61)	(494-532)	1.27 (0.02)
3	420.92 (12.17)	(423-441)	550.07 (7.90)	(530-575)	1.27 (0.01)
4	395.05 (8.63)	(368-418)	542.86 (10.07)	(514-569)	1.38 (0.01)
5	377.27 (5.20)	(340-392)	480.60 (14.91)	(448-525)	1.27 (0.03)
MEAN	395.59 (8.08)		516.56 (11.29)		1.29 (0.02)

Table 2.3. Results of ANOVA analysis on differences in egg diameter across females within species. Significant results (p ≤ 0.05) are indicated in bold.

Source	df	MS	F	P
E. mathaei				
female	4	8.3204	0.84	0.5184
residual	20	9.9549		
H. tuberculata				
female	4	35.9369	5.99	**0.0024**
residual	20	5.9984		
C. rodgersii				
female	4	191.0083	10.82	**0.0001**
residual	20	17.6509		
H. erythrogramma				
female	4	2042.4284	9.10	**0.0002**
residual	20	224.3471		

Table 2.4. Results of ANOVA analysis on differences in relative size indices of the jelly coat across females within species. Significant results (p ≤ 0.05) are indicated in bold.

Source	df	MS	F	P
E. mathaei				
female	4	0.0629	4.81	**0.0070**
residual	20	0.0131		
H. tuberculata				
female	4	0.0909	7.32	**0.0008**
residual	20	0.0124		
C. rodgersii				
female	4	0.0347	5.42	**0.0040**
residual	20			
H. erythrogramma				
female	4	0.0095	4.46	**0.0097**
residual	20	0.0021		

Table 2.5. Results of three way ANOVA analysis on the effects of decreased pH on jelly coat area for four echinoid species. Significant results ($p \leq 0.05$) are indicated in bold.

Source	df	MS	F	P
species	3	7303.43	20.71	**0.0000**
time	3	71.38	0.42	0.7400
pH	1	23627.06	2.97	0.1832
species x time	9	168.02	0.48	0.8882
species x pH	3	7952.81	22.55	**0.0000**
time x pH	3	93.53	0.39	0.7655
species x time x pH	9	241.99	0.69	0.7203
residual	128	352.63		

Table 2.6. Results of individual two way ANOVA analyses on the effects of decreased pH on jelly coat area for four echinoid species. Significant results ($p \leq 0.05$) are indicated in bold.

Source	df	MS	F	P
E. mathaei				
time	3	42.21	0.10	0.9583
pH	1	13143.09	31.76	**0.0000**
time x pH	3	223.52	0.54	0.6582
residual	32	413.79		
H. tuberculata				
time	3	167.22	0.42	0.7370
pH	1	174.65	0.44	0.5104
time x pH	3	97.59	0.25	0.86
residual	32	394.22		
C. rodgersii				
time	3	635.7227	6.17	**0.0020**
pH	1	26168.5963	253.82	**0.0000**
time x pH	3	339.9145	3.30	**0.0328**
residual	32	103.1003		
H. erythrogramma				
time	3	162.90	0.67	0.5750
pH	1	23.20	0.10	0.7589
time x pH	3	36.9344	0.15	0.9273
residual	32	242.12		
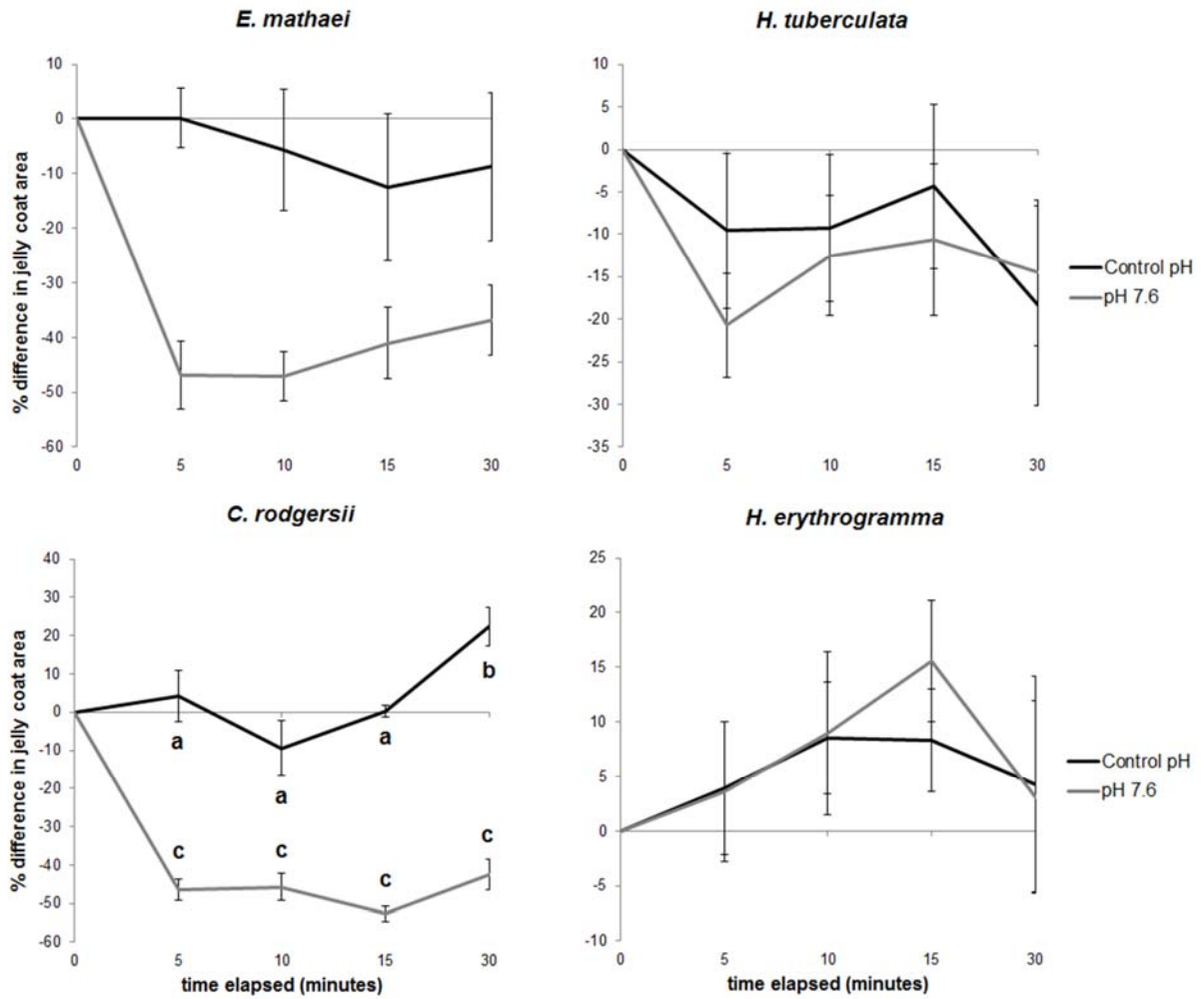
Figure 2.2. The effect of decreased pH on jelly coat area over time for four echinoids.
The percentage difference relative to the starting area (line from 0) is shown for eggs in the different pH treatments. Data represent means for five females. Error bars represent standard error.

2.4.6 Heliocidaris erythrogramma

For *H. erythrogramma*, the egg jelly coat area in controls increased over the first 15 minutes followed by a decrease at 30 minutes, however this change in size was not significant (Figure 2.2). There were no significant effects of decreased pH or time on the jelly coat area with jelly coats in decreased pH showing similar behaviour to that of the control (Table 2.6; Figure 2.2).

2.5 Discussion

The results here with sea urchin eggs have implications for other marine invertebrate species whose eggs are surrounded by a jelly coat, including other echinoderms, molluscs and some cnidarians (Farley and Levitan, 2001; Hofmann, 2013; Plickert, 2013; Rosati, 1995; Schatten and Chakrabarti, 2013; Suzuki, 1989). The eggs of commercially important species such as abalone (Mozingo et al., 2005; Shiroya and Sakai, 1995) and oysters (Loosanoff and Davis, 1950) have jelly coats similar to those of sea urchins (Suphamungmee et al., 2010). Extracellular layers around these eggs play an important role in fertilisation. Abalone sperm exposed to egg jelly water increase in motility indicating that the jelly coat contains chemo-attracting molecules (Suphamungmee et al., 2010). Similar chemo-attractive properties exist for the jelly coat of many asteroids (Miller, 1985). Thus chemo-attractants found in the jelly coats of echinoids, asteroids and abalone create a chemical sphere around the eggs thereby increasing their effective target size for sperm (Jantzen et al., 2001; Miller, 1985). The jelly coat around the eggs of abalone is also sensitive to pH (Lewis et al., 1982) and thus may be vulnerable to OA, as seen here for sea urchins. In cnidarians the jelly may be less important because the jelly coat is extremely thin and transitory (Plickert, 2013; Schatten and Chakrabarti, 2013).

For two of the echinoid species, *C. rodgersii* and *E. mathaei*, we showed that the jelly coat decreased in area in response to CO_2-driven low pH (pH 7.6), similar to the that seen in early studies, albeit at lower pH levels and with addition of mineral acid (pH 5-5.5; e.g. Menkhorst and Selwood, 2008; Podolsky, 2002). In contrast, the jelly coat of the eggs of *H. tuberculata* and *H. erythrogramma* appeared more resilient and was not affected by decreased pH. The sea urchins used in this study are often sympatric in the same reefs, and it is known that the jelly coat is an important structural barrier in preventing cross fertilisation (Raff et al., 1999). The reduction in the size of the jelly coat due to OA could thus have an effect on species-specific gamete recognition, influencing gamete wastage and hybridisation between species.

Because of the important role of jelly coat constituents in stimulating sperm metabolism and species-specific gamete recognition, as well as influencing egg target size, the decrease in jelly coat in near future OA conditions would be expected to lead to a decrease in fertilisation, especially in sperm-limiting conditions (Farley and Levitan, 2001; Podolsky, 2002). For the sea urchin *L. variegatus*, eggs with jelly coats accrue 2.2 more collisions across a range of sperm concentrations compared to those without jelly coats. Eggs stripped of the jelly coat require double the amount of sperm to accrue 50% fertilisation compared to eggs with jelly coats (Farley and Levitan, 2001). Although this was attributed to egg target size, the removal of sperm attractants in stripped eggs is also a likely contributing factor. Furthermore, sperm have been shown to differentially select eggs based on jelly coat chemical cues (Evans et al., 2012) and thus, the reduction of the jelly coat in low pH may impact on mate choice. Thus, the reduction in jelly coat size could encourage the mating of incompatible partners (Evans et al., 2012).

In control pH water, jelly coats across the four echinoids exhibited different behaviour, where for some species the jelly coat appeared to increase in size, while others decreased in size. The behaviour of the jelly coat thus differed between species and is likely due to differences in the chemical constituents of the jelly coat and the dynamics of hydration (Shu et al., 2015; Vilela-Silva, 2008). The significant interaction of time x pH found for *C. rodgersii* indicated that decreased pH affects the normal dynamics of hydration because the increase in jelly coat area in the controls was not observed for jelly coats in decreased pH. Thus, time can have a significant influence on the outcome for jelly coat integrity. This could be a factor influencing the variable responses of fertilisation tests within and between species in OA conditions as studies investigating the effects of low pH on fertilisation typically score fertilisation around five minutes, or at a later stage (Byrne, 2011).

It is not known why the jelly coat area of the two *Heliocidaris* species was not affected by decreased pH. The difference in the jelly coat responses to low pH between this genus and the other two species is likely due to differences in hydration levels and jelly constituents including sialic acid and glycans which are known to differ among species, as shown for sea urchins and frogs (Jondeung and Czihak, 1982; Pomin, 2015; Shu et al., 2015). In frogs, these components are sensitive to pH (Shu et al., 2015). Furthermore, the surface charge of the glycans contributes to the level of hydration in the jelly coat, a feature greatly affected by the pH of the water. Intra-specific variation in glycosylation in egg jelly coats could drive different levels of hydration (Menkhorst and Selwood, 2008; Shu et al., 2015) and hence the

differences in jelly coat size seen within and between species in controls and in the sensitivity to decreased pH.

For three of the four echinoids examined, there was significant variation in the diameter of the eggs between females of the same species. The relative size of the jelly coat also significantly differed between females of the same species. The degree to which energy is invested in egg jelly coat production varies among echinoids where a greater volume of jelly coat correlates with more energy invested in this extracellular structure (Bolton et al., 2002). Our study and these previous results show that not all eggs are created equal, and that the jelly coat around the eggs of some females might be better able to endure effects of low pH contributing to a more resilient fertilisation response in OA conditions. If this is a heritable trait, OA might select against the more susceptible phenotypes (Foo et al., 2012, 2014; Schlegel and Havenhand, 2012). Environmental stressors such as OA, which have strong effects on jelly coat size may induce strong selection on marine species that have egg coats as ocean pH changes (Shu et al., 2015).

For the frog *Rana arvalis*, egg coats displayed extensive intra-specific and inter-population variation in sensitivity to decreased pH. Low pH water caused severe water loss in the jelly and subsequently reduced the hatching success of the embryos. However the eggs of populations adapted to low pH conditions retained a greater amount of water in their jelly coat (Shu et al., 2015). For this frog species, jelly coats mediated the effects of low pH on developing embryos. Although this example is for a vertebrate with eggs that are laid in freshwater, it is possible that a similar situation has occurred for the sea urchins examined in this study. The *Heliocidaris* species may be already adapted to low pH conditions which in turn reduces the sensitivity of their jelly coats to low pH.

Inherent variability in the size of eggs and jelly coats of females within and between echinoid species and differences in the response of the jelly coat to reduced pH as seen here, along with other factors such as differences in gamete compatibility (e.g. bindin-egg bindin receptor system, Evans and Sherman, 2013) provide insights into potential sources of variation in the contrasting outcomes of OA fertilisation experiments even with the same species (Byrne, 2011 review). Another important factor revealed here is the handling of eggs in OA experiments, as the time that the eggs are in treatment prior to introduction of sperm may influence how much of the jelly coat remains and therefore attractiveness of eggs to sperm. This would be especially important in low sperm concentrations.

2.5.1 Conclusions

The jelly coat performs a number of important functions before and during fertilisation including an increase in effective egg target size and chemo-attraction of sperm. The decrease in jelly coat area observed for species with small eggs could affect all these functions, contributing to a reduction in fertilisation in OA conditions. The higher sensitivity of the small eggs to low pH may be due to different chemical compositions in the jelly coat and different surface charges of the glycans, which help to control the level of hydration in the coat. However, as eggs and jelly coats show significant variation between females for all species, less susceptible individuals could be selected for in an acidifying ocean. The results of these experiments suggest that some species are less vulnerable to OA, at least with respect to integrity of the egg jelly coat. This may help protect against the effects of OA from the outset of spawning.

CHAPTER THREE: CONTRIBUTIONS OF GENETIC AND ENVIRONMENTAL VARIANCE IN EARLY DEVELOPMENT OF THE ANTARCTIC SEA URCHIN *STERECHINUS NEUMAYERI* IN RESPONSE TO INCREASED OCEAN TEMPERATURE AND ACIDIFICATION[1]

3.1 Abstract

Breeding designs such as the North Carolina II can be used to identify the sources of genetic and environmental variances in embryo performance. Here this approach is used for the Antarctic sea urchin *Sterechinus neumayeri* to explore how the contribution of sire and dam can influence the performance of cleavage stage embryos and blastulae, and how these contributions differ when exposed to stress from increased temperature and acidification. The interrelationship of sire-dam effects was also compared across developmental stages. The effects of warming (+3°C) and acidification (-0.3 and -0.5 pH$_T$ units) on 24 sire-dam crosses was investigated. These stressors decreased cleavage success and the percentage of normal blastulae, with a negative interactive effect between stressors. The response to these factors differed among the sire-dam pairs indicating the influence of gamete compatibility. A positive genetic correlation indicated that genotypes that performed well as blastulae in low pH also performed well at increased temperatures. Performance at cleavage was a good predictor of performance at the later blastula stage. Significant dam by temperature interactions indicated differential performance among maternal half-siblings in response to increased temperature. Adaptation depends on additive genetic variance for stress tolerance being present in populations, however there were no sire by stressor interactions found. This indicates that *S. neumayeri* will need to rely on phenotypic plasticity to persist through an ocean decreasing in pH and warming, at least with respect to early development.

3.2 Introduction

Offspring fitness is determined by the intrinsic quality of maternal and paternal haplotypes and their interaction (Evans et al., 2008). High latitude marine ecosystems are among the most vulnerable to global change and so understanding the contributions of genetic and environmental sources of variance in development will provide insights into their vulnerability in a changing ocean (Peck, 2005; Turley et al., 2010). The Southern Ocean has the greatest increase in anthropogenic CO_2 and is predicted to reach a CO_2 concentration of 1000 µatm by 2100, equivalent to a drop in 0.4 pH units (IPCC 2013). In parallel with ocean acidification, sea surface temperatures are expected to increase by a further 2.6˚C by 2100 (IPCC, 2013). Faced with such rapid change, polar regions are considered a bellwether for climate induced changes in other oceans (Fabry et al., 2008).

The sea urchin *Sterechinus neumayeri* is the most abundant echinoid in coastal Antarctica and is vital for ecosystem functioning due to its role as a grazer and predator (Bosch et al., 1987). In studies of the impacts of warming and/or acidification on fertilisation and development in *S. neumayeri,* where multiple parents were used to generate progeny, fertilisation and early development (cleavage) were resilient to near-future acidification and/or warming (pH 7.6/+3˚C) (Ericson et al., 2010; Ho et al., 2013). With single sire-dam crosses however, effects varied with pairs with deleterious effects evident at pH 7.8 in most crosses, with two pairs exhibiting enhanced performance (Sewell et al., 2014). By the blastula stage, deleterious effects are evident in embryos reared from fertilisation at 3˚C at all pH levels tested (pH 8, 7.7 and 7.5) (Ericson et al., 2012). It is clear that the multiple parent spawning approach provides the group mean response but cannot allow assessment of effects on individual genotypes (Schlegel and Havenhand, 2012; Sewell et al., 2014). The spawner versus individual genotype experimental approaches could influence differences in results, thus an understanding of genetic and environmental variances in performance to stressors will help to more accurately identify vulnerabilities in a changing ocean.

Experimental breeding designs where gametes of sires and dams are crossed in all combinations allow the contribution of genetic and environmental effects to be estimated through the generation of paternal and maternal half siblings, and full siblings (Lynch and Walsh, 1998). As DNA is the only contribution from the father to offspring, estimation of paternal variance can be used to determine additive genetic variance and species' adaptive potential, as paternal effects

are largely genetic (Lynch and Walsh, 1998). The ability to adapt to a changing environment depends on the existence of additive genetic variance present within populations; the proportion of genetic variation that responds to natural selection (Billington and Pelham, 1991; Kelly et al., 2013). There is increasing evidence, however, that the sire component does not exclusively represent additive genetic variance (Crean and Bonduriansky, 2014; Jensen et al., 2014; Marshall, 2015) and thus estimating genetic variance from paternal lines may not be accurate (Crean and Bonduriansky, 2014). It can still offer important insights into adaptive potential by providing evidence of whether different genotypes are present in the population.

Maternal variance represents both genetic and environmental effects, where components cannot be separated in quantitative genetic experiments (Räsänen and Kruuk, 2007). Maternal effects are also important in population evolutionary dynamics because they influence the rate and direction of genetic change under selection (Falconer, 1989; Hoffmann and Sgro, 2011). Significant dam and temperature/pH interactions would indicate effects of both maternal provisioning (e.g. environmental) and/or additive genetic variation. Production of offspring of higher fitness is also influenced by the genetic compatibility of a sire and dam haplotype (sire x dam variance) and represents non-additive genetic effects (Falconer, 1989).

As the negative effects of changing ocean conditions on marine biota are of major concern, it is important to understand the capacity of species to acclimatise and adapt to change (Munday et al., 2013; Sunday et al., 2014). For polar species, persistence in changing environments may be facilitated and highly depend on phenotypic plasticity to acclimatise to changing conditions as Antarctic marine species are assumed to have narrow adaptive capacity due to environmental thermal stability over evolutionary timescales (Enzor et al., 2013; Peck, 2015).

In this study, the contributions of genetic and environmental variance in early development of *S. neumayeri* to concurrent ocean warming and ocean acidification was assessed using the North Carolina II quantitative genetic design, which involves mating a set of N_s sires with N_d dams in all combinations (Neff and Pitcher, 2005). Genetic correlations for *S. neumayeri* early embryos were determined to quantify the relationship of the performance of genotypes across multiple warming/ acidification environments (Sgro and Blows 2004; Astles et al., 2006; Bell, 2013). To determine whether performance at one stage can predict performance later on in development, pair performance was contrasted across both stages.

3.3 Materials and Methods

3.3.1 Study species and collection sites

Sterechinus neumayeri were collected (20-25 m depth) by SCUBA diving from Winter Quarters Bay, Ross Island, Antarctica (77˚ 50' S, 165˚ 0' E) in November 2013 during their peak spawning period (Brockington et al., 2007). Animals were transported in ambient seawater in a cool box and transferred to flow through aquaria (100 L; -1.1˚ C) shortly after collection. Individuals were spawned for experiments within two days of collection. The animals were collected under an Antarctic Marine Living Resources Act 1981 Permit (Permit No: AMLR13/R03/Lamare/K068). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

3.3.2 Fertilisation and the North Carolina II design

Spawning of *S. neumayeri* was induced by injection of ~1 ml of 0.5 M KCl and the eggs from each dam were placed in separate beakers of fresh, filtered seawater (FSW; 1 µm) following routine procedures for sea urchins (Foltz et al., 2004). Sperm from each sire was stored dry in an Eppendorf tube at -1.1˚C until use (< 1 hour). Egg density was determined in counts of 100 µl aliquots from the egg suspension. Haemocytometer counts of sperm samples diluted with FSW were used to determine the amount of sperm solution required to achieve a final sperm concentration of 1×10^4 sperm ml^{-1} and sperm:egg ratio of ~740:1 . Based on the approach in Foo et al., (2012, 2014), sire-dam crosses were made in three consecutive experimental runs (blocks) with each block using gametes from 2 dams and 4 sires crossed in all combinations. Each block thus resulted in 8 full-sib families, resulting in a total of 24 families for the experiment. For fertilisation, approximately 11,000 eggs from each dam were placed in containers (800 ml glass beakers) containing FSW. Eggs were fertilised and after 10 minutes, the water in each container was changed to remove excess sperm and prevent polyspermy. Immediately after rinsing, embryos were transferred into rearing containers; 50mL falcon tubes (approx. 600 embryos per tube) with mesh covered windows; and exposed to six flow-through experimental temperature/pH treatments. Falcon tubes were fully immersed in the six treatment tanks.

Each family was exposed to each of 6 combinations of pH and temperature treatments with three replicates for each family by treatment combination. Thus each block used a total of

144 falcon tubes (2 dams x 4 sires x 3 pH levels x 2 temperature x 3 replicates). At 24 h and 72 h, a haphazardly selected subsample of approximately 50 embryos was pipetted from each replicate, placed into 1.5 mL eppendorf tubes and fixed with formalin in FSW to a final concentration of 2%. The first 30–50 embryos from each tube were examined microscopically and scored for successful development. At 24 h, the percentage of cleavage stage embryos was determined. Given the slow development of this species, embryos were exposed to treatments for 24 h to assess the effects of treatments on cleavage. At 72 h, the percentage of blastulae was calculated from counts of normal/abnormal and arrested embryos as illustrated in Ericson et al., (2010). The number of embryos arrested at fertilisation (e.g., fertilisation envelope only) was low in controls (<1%) indicating that polyspermy was minimal.

3.3.3 Experimental conditions – temperature and pH treatments

Experimental treatments consisted of two temperatures (Mean ± SE, control -0.94 ± 0.001˚C and 2.02 ± 0.003˚C) and three pH_T levels (Mean ± SE, control 7.97 ± 0.02, 7.66 ± 0.04, and 7.50 ± 0.02, n = 24) in all combinations (Table 3.1). Treatments are within model projections for near future (2100) conditions for the Southern Ocean (IPCC 2013). Filtered experimental FSW was supplied from a flow through system (ambient pH_T 8.00 - 0.94˚C) at Scott Base, Ross Island, Antarctica. Water temperature was controlled by aquarium heaters (300W, Jager) and mixers supplying 80 L tanks. The experimental pH was regulated by injection of pure CO_2 into two of these tanks using an automatic CO_2 injection system with feedback to solenoids through two pH_{NIST} calibrated controllers (TUNZE pH/CO_2 controllers 7074/2, TUNZE AQUARIENTECHNIK GMBH, Penzberg, Germany) set at ppm equivalent to pH 7.6 and pH 7.8. Unmanipulated FSW served as the ambient control. The actual pH on the total scale that was delivered to treatments and tanks was monitored twice daily (n = 24 per treatment across blocks) using the spectrophotometric approach with *m*-cresol purple indicator dye (Sigma Aldrich batch 2303-01-7) and a Hitachi U-1100 spectrophotometer (Dickson et al., 2007). Temperature was constantly monitored with HOBO loggers at five minute intervals. The salinity of treatment water was 35 psu, and dissolved oxygen remained > 90% (n = 24 per treatment across blocks).

Water samples (1L) were collected at the beginning of each block, filtered through a 0.45 mm syringe filter, and fixed with 100 µl of saturated HgCl. These were used to determine total

Table 3.1. Experimental conditions in experiments with *Sterechinus neumayeri* embryos. Average values ($\pm$ SE) of pH_T, pCO_2) were calculated from CO2SYS using data on total alkalinity (TA = 2349.3 ± 0.6 µmol/kg, n = 12), salinity (35 ± 0.00, n = 24) and temperature for each treatment.

	-1.0			2		
	pH 8.1	**pH 7.75**	**pH 7.6**	**pH 8.1**	**pH 7.75**	**pH 7.6**
Temp (˚C)	-1.01 (0.002)	-0.94 (0.002)	-0.87 (0.003)	2.07 (0.007)	1.95 (0.005)	2.06 (0.003)
pH_T	8.00 (0.02)	7.67 (0.06)	7.51 (0.03)	7.93 (0.01)	7.65 (0.02)	7.49 (0.01)
pCO_2	442.1 (22.59)	1009.7 (147.45)	1443.8 (106.02)	527.6 (7.45)	1065.3 (48.59)	1532.5 (36.72)

alkalinity (TA) by potentiometric titration. Experimental pCO_2 and pH_T (Table 3.1) was determined from TA, temperature, and salinity data using CO2SYS (Pierrot et al., 2006) applying the dissociation constants of Mehrbach et al., (1973) as refitted by Dickson and Millero, (1987).

3.3.4 Statistical Analyses

Percentage of cleavage stage embryos and blastulae data were analysed using permutational analysis of variance (PERMANOVA; Anderson, 2001) with temperature and pH as fixed factors, experimental block as a random factor, and sire and dam as random factors nested within blocks. Since some significance tests involved quasi-F ratios (in which significance tests derived from the F distribution are unreliable (Quinn and Keough, 2002)), significance of the F statistics was calculated using 9999 permutations of the raw data for all factors in the PERMANOVA routine of Primer V6 (Anderson et al., 2008). To check that treatments were not different among blocks, a three-way ANOVA was run with temperature, pH and block as fixed factors.

Reaction norms (interaction plots, see Quinn and Keough, 2002) were plotted to visualize the interactions between sire genotypes across a range of environments (Lynch and Walsh, 1998). The genetic correlation of embryo performance (% of normal embryos) across temperature and pH environments were used to quantify the genotype x environment interaction using variance components derived from restricted error maximum likelihood (REML) estimates calculated in the R package lme4 (available at http://cran.r-project.org/web/packages/lme4/index.html). Variance components for the random factors were calculated in a single analysis with all factors (Temperature, pH, Block, Sires, dams). Genetic correlations were calculated using the causal variance components associated with the sire effects (additive genetic (V_A)) and the interaction effects between sires and each of the environmental factors of temperature (V_{AT}), pH ($V_{A\ pH}$) and both temperature and pH ($V_{AT\ pH}$). Genetic correlations for the same trait averaged over both types of environments (r^*_G), the genetic correlation for the same trait within one environmental class (i.e. temperature; $r^*_{G(T)}$) and the genetic correlation within the other environmental class (i.e. pH; $r^*_{G(pH)}$) were calculated using equations from Eisen and Saxton, (1983):

$$r^*_G = V_A/(V_A + V_{AT} + V_{A\ pH} + V_{AT\ pH})$$

$$r*_{G(T)} = (V_A + V_{AT})/(V_A + V_{AT} + V_{A\,pH} + V_{AT\,pH})$$

$$r*_{G(pH)} = (V_A + V_{A\,pH})/(V_A + V_{AT} + V_{A\,pH} + V_{AT\,pH})$$

Linear regression analyses were performed in Microsoft Excel (2013) to assess the relationship between performance across the two different life history stages; cleavage and blastula using percentage performance data for each pair over both stages over the six treatments.

3.4 Results

3.4.1 Cleavage stage embryos

For embryos collected 24 h post-fertilisation, decreased pH had a significant effect on cleavage success (= percentage of cleavage stage embryos) with a significant interactive effect with increased temperature (PERMANOVA, Figure 3.1; Table 3.2). As displayed in the box and whisker plots, the percentage of cleavage stage embryos in control conditions was 88% and this was reduced to 84% and 83% in pH 7.8 and 7.6 respectively. At 3 ˚C, the percentage of cleavage stage embryos was 82%. When coupled with low pH, the percentage of cleavage stage embryos was unchanged at pH 7.8 (82%) but further reduced at pH 7.6 (79%) showing the negative synergistic effect of both stressors and exemplifying the temperature x pH interaction (Figure 3.1; Table 3.2).

There were significant sire x dam (pair), and sire x dam x temp x pH interactions showing the influence of parental combinations, resulting in variable responses among families to the same treatment as displayed in the scatter plot (Table 3.2; Figure 3.2). For example, pair 24 showed only a 4% reduction in performance in pH 7.6/+3 ˚C compared to 23% reduction for pair 14. Dam identity also influenced cleavage success and contributed the second highest percentage (22%) of variance at this developmental stage. The block x temperature interaction found may be due to the different performances of sire and dam to temperature within each block as temperature treatments were consistent across blocks (see Table 3.3). Genetic correlations were not calculated at this stage as there were no significant sire effects.

3.4.2 Blastulae

At ambient temperatures, decreased pH significantly reduced the percentage of normal blastulae from 86% to 79% (ANOVA, Table 3.4, Figure 3.3). The box and whisker plots show that when

low pH was coupled with +3˚C, the percentage of blastulae was further reduced to 80% and 77% in pH 7.8 and 7.6 respectively. The block x temperature x pH interaction found is likely to be due to the different interactive effects of stressors on performances of sire and dam within each block as treatments were consistent across blocks (see Tables 3.3 and 3.5).

The effects of sire and dam on the percentage of normal blastulae were significant. Dam contributed the highest percentage (38%) of variance. This maternal effect indicates the presence of both additive genetic and environmental effects (Table 3.4). Furthermore, the interaction between dam and temperature was significant where different slopes in the reaction norm show that progeny of some dams performed worse in high temperature, while some benefitted in the same treatment (Figure 3.4). Sire accounted for 1.4% of the total variance however no sire x stressor interactions were found.

The genetic correlation ($r*G$) in the blastula trait across the temperature/pH environments based on paternal half siblings was 0.34. This indicates that the half siblings that were less sensitive to increased temperature performed the same in decreased pH. There were also positive genetic correlations across the two temperature levels ($r*G(t) = 0.34$) and across the three pH levels ($r*G(pH) = 0.81$). Thus, half siblings that performed well at control temperatures also performed the best in high temperatures and likewise for pH.

3.4.3 How does performance at the blastula stage compare with performance at cleavage?
To address the notion that progeny of pairs perform consistently across developmental stages, scatter plots of the relationship between fertilisation success and percentage of normal blastula were plotted. Pairs were shown to perform consistently across all environments with significant positive correlations shown for all environments (Figure 3.5; Table 3.6). R^2 values for scatter plots ranged from 0.17 to 0.32 across treatments (Figure 3.5). Thus, genotypes that performed well at cleavage were good genotypes as blastulae across all temperature and pH environments tested.
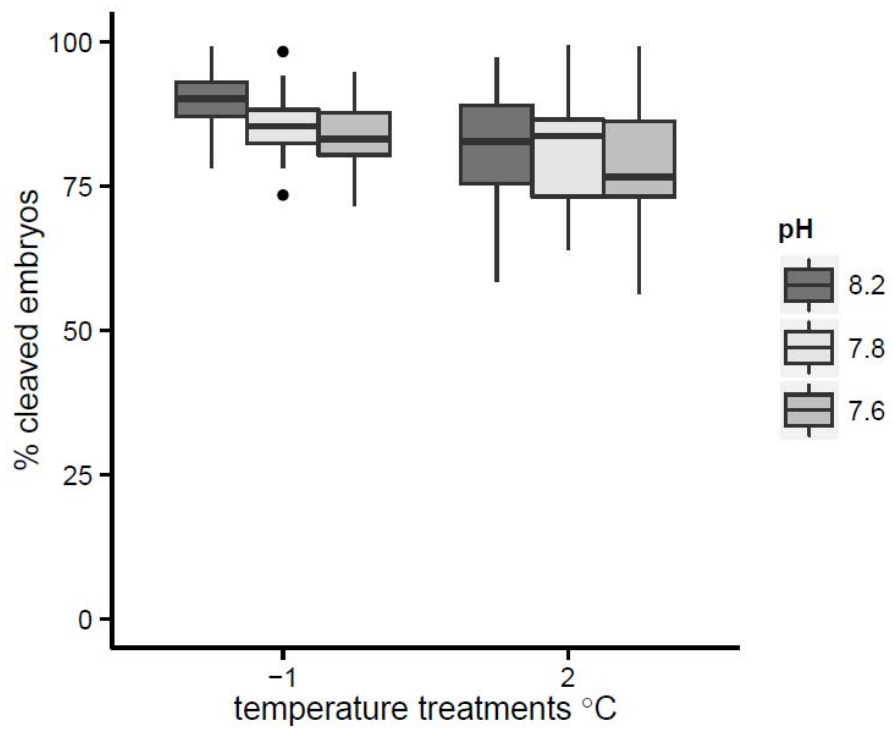
Figure 3.1. Percentage of *Sterechinus neumayeri* cleavage stage embryos across 24 pairs in the six experimental treatments. The solid bar represents the median, with the box representing the 25th and 75th percentiles, the whiskers the 1.5 interquartile range, and outliers indicated by dots.
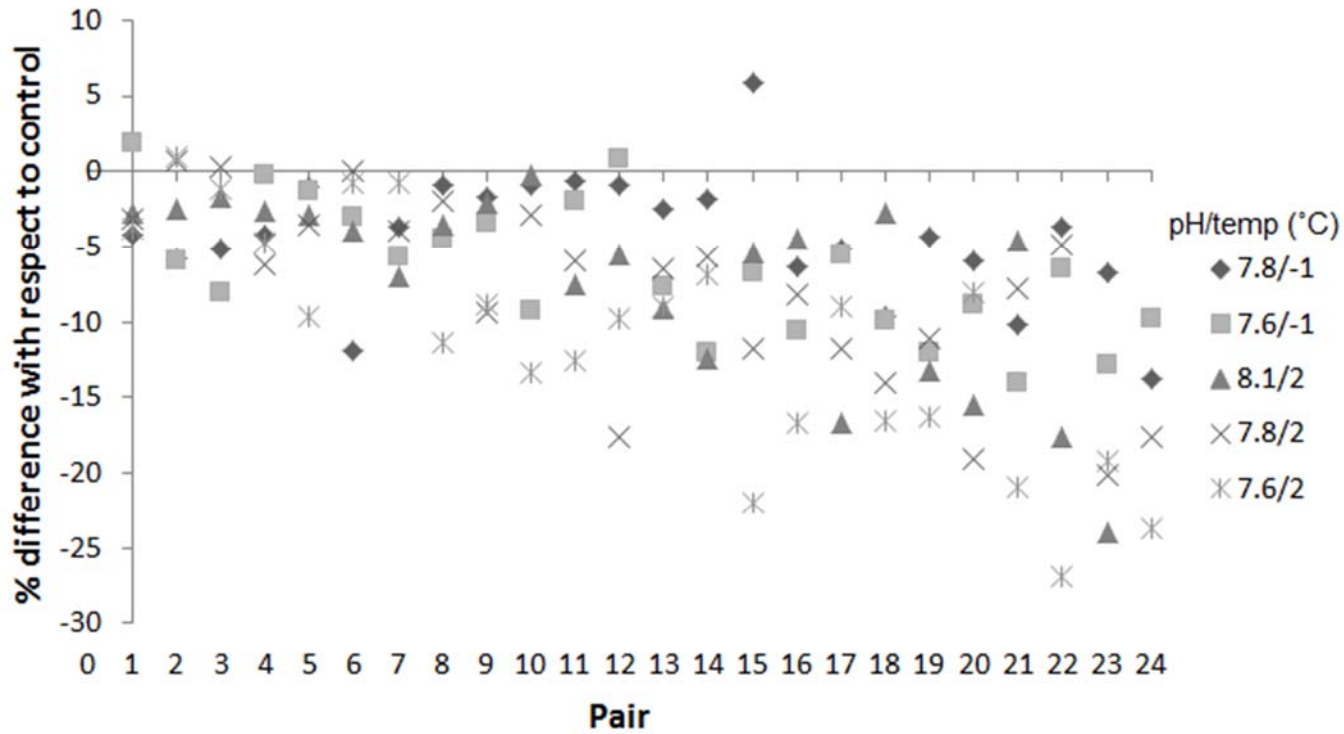
Figure 3.2. Scatter plot showing the difference in percentage of cleavage with respect to the control treatment in 24 different *Sterechinus neumayeri* sire-dam pairs across five experimental temperature-pH treatments. Negative effects are indicated by data below the x axis with positive effects of treatment above the x axis from left to right. Pairs are illustrated from the best to the worst performing.

Table 3.2. Permutational ANOVA of percentage cleavage stage embryos of *Sterechinus neumayeri* in single dam-sire crosses across various temperature (Te) and pH conditions. Temperature and pH were fixed factors, with experimental block (Bl) as a random factor, and sire (Ma) and dam (Fe) identity as random factors nested within block. The percentage of total variance from REML estimates of variance components are shown for random factors (nt = not tested). Significant effects are shown in bold.

Source	df	MS	F	P	%
Fixed effects					
Te	1	2943.3	3.3071	0.2261	nt
pH	2	846.52	22.111	**0.0106**	nt
TexpH	2	226.42	8.978	**0.0328**	nt
Random effects					
Bl	2	5907.3	3.0081	0.0599	24.58
Ma(Bl)	9	78.409	1.0202	0.4919	<0.01
Fe(Bl)	3	1916.3	25.076	**0.0002**	21.92
BlxTe	2	892.34	6.5072	**0.0054**	12.37
BlxpH	4	38.29	0.53971	0.875	<0.01
Ma(Bl)xFe(Bl)	9	76.857	2.0711	**0.0336**	1.95
Ma(Bl)xTe	9	24.33	0.69293	0.7068	<0.01
Ma(Bl)xpH	18	46.03	1.0749	0.434	<0.01
Fe(Bl)xTe	3	118.66	3.3772	0.0678	1.63
Fe(Bl)xpH	6	103.92	2.4313	0.0658	1.20
BlxTexpH	4	24.984	1.7628	0.1303	<0.01
Ma(Bl)xFe(Bl)xTe	9	35.112	0.9462	0.4964	<0.01
Ma(Bl)xFe(Bl)xpH	18	42.822	1.154	0.2923	0.76
Ma(Bl)xTexpH	18	33.878	0.45448	0.9473	<0.01
Fe(Bl)xTexpH	6	22.349	0.29981	0.9246	<0.01
Ma(Bl)xFe(Bl)xTexpH	17	74.543	2.0088	**0.0122**	1.93
Res	286	37.109			33.66

Table 3.3. ANOVA analysis of pH treatments across blocks for *Sterechinus neumayeri*.
Two-way ANOVA testing for significant differences in $pH_{(T)}$ among the three $pH_{(T)}$ treatment levels (8.0, 7.7, 7.5), across the three experimental periods (blocks), and the interaction of block with pH (pH level x block). Seawater pH readings are not transformed, with variances homogeneous among pH levels (Levene's test, $p = 0.372$) and blocks (Levene's test, $p = 0.947$). Measurements were normally distributed.

Source	df	SS	F	P
pH level	2	2.947	970.27	<0.001
Block	2	0.0059	1.948	0.149
pH level x Block	4	0.0021	0.348	0.844
Error	86			
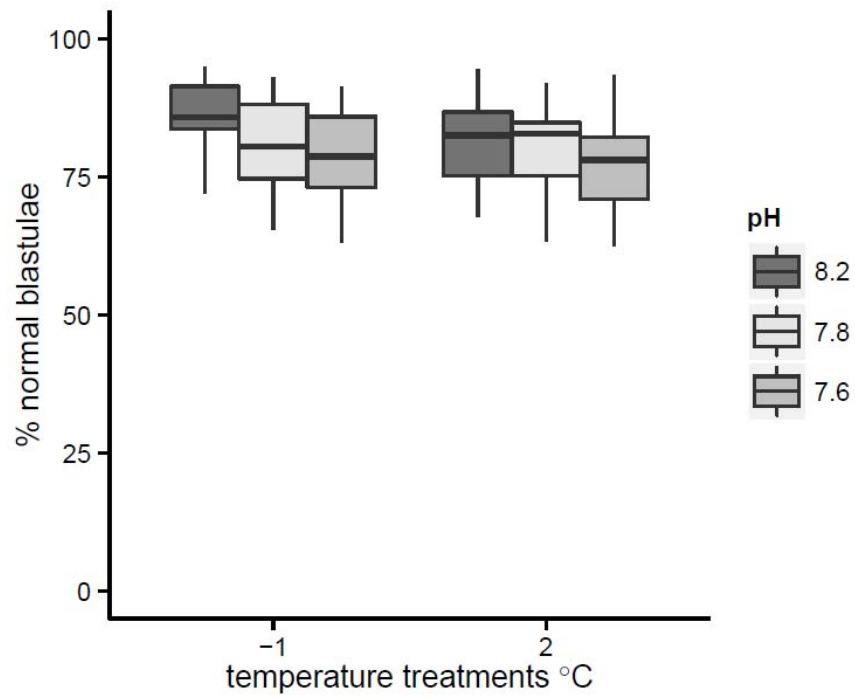
Figure 3.3. Percentage of normal *Sterechinus neumayeri* blastulae across 24 pairs in the six experimental treatments. The solid bar represents the median, with the box representing the 25th and 75th percentiles and the whiskers the 1.5 interquartile range.
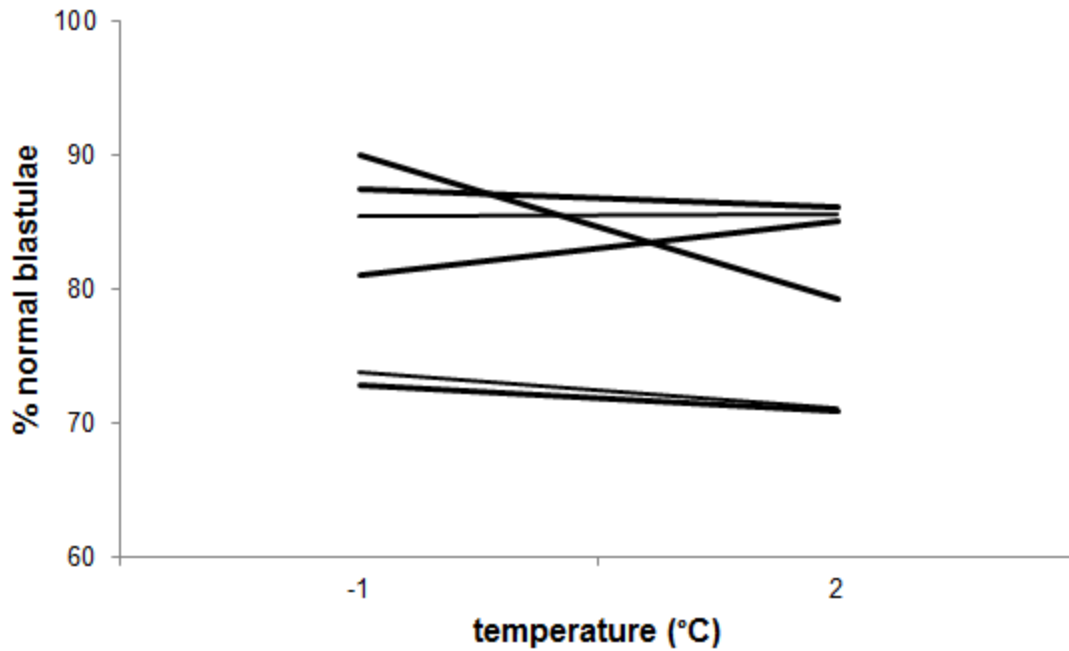
Figure 3.4. Reaction norm showing the responses of *Sterechinus neumayeri* progeny of six dam genotypes to increased temperature. The reaction norm shows the percentage of normal blastulae in experimental temperatures pooled for pH. Lines represent the mean percentage of normal blastulae for maternal half siblings (n = 6).

Table 3.4. Permutational ANOVA of percentage blastulation data of *Sterechinus.*
neumayeri **in single dam-sire crosses across various temperature (Te) and pH conditions.**
Temperature and pH were fixed factors, with experimental block (Bl) as a random factor, and
sire (Ma) and dam (Fe) identity as random factors nested within block. The percentage of total
variance from REML estimates of variance components are shown for random factors (nt = not
tested). Significant effects are shown in bold.

| Source	df	MS	F	P	%
Fixed effects					
Te	1	351.74	0.69633	0.563	nt
pH	2	1424.7	108.61	**0.0029**	nt
TexpH	2	177.02	8684	0.5582	nt
Random effects					
Bl	2	2549.2	0.70681	0.6691	<0.01
Ma(Bl)	9	134.71	5.4419	**0.0101**	1.37
Fe(Bl)	3	3517	140.78	**0.0001**	37.59
BlxTe	2	506.4	1.1974	0.3843	<0.01
BlxpH	4	12.963	0.46125	0.935	<0.01
Ma(Bl)xFe(Bl)	9	24.754	0.54423	0.8405	<0.01
Ma(Bl)xTe	9	58.284	0.7784	0.6434	<0.01
Ma(Bl)xpH	18	94.11	1.4677	0.2108	1.86
Fe(Bl)xTe	3	428.05	5.7415	**0.0166**	9.02
Fe(Bl)xpH	6	72.298	1.1322	0.3833	0.18
BlxTexpH	4	258.69	3.4892	**0.0065**	3.03
Ma(Bl)xFe(Bl)xTe	9	74.876	1.6462	0.099	<0.01
Ma(Bl)xFe(Bl)xpH	18	64.121	1.4097	0.1254	<0.01
Ma(Bl)xTexpH	18	61.417	1.1011	0.4229	0.74
Fe(Bl)xTexpH	6	29.588	0.53049	0.7816	<0.01
Ma(Bl)xFe(Bl)xTexpH	17	55.776	1.2262	0.2383	2.65
Res	286	45.485			43.56

Table 3.5. ANOVA analysis of temperature treatments across blocks for *Sterechinus neumayeri*. Two-way ANOVA testing for significant differences in temperature between the two treatment levels (-1, 2 ˚C), across the three experimental periods (blocks), and the interaction of block with temperature (temp x block). Seawater temperatures readings are not transformed, with variances homogeneous among pH level (Levene's test, p = 0.717) and among blocks (Levene's test, p = 0.390). Measurements were normally distributed.

Source	df	SS	F	P
Temp	2	0.149	0.038	0.967
Block	2	0.389	0.088	0.9152
Temp x Block	4	0.225	0.026	0.998
Error	14			

3.5 Discussion

For *S. neumayeri*, decreased pH significantly reduced cleavage success, the magnitude of which depended on the individual pair indicating the importance of sire-dam compatibility, a feature characteristic of sea urchin fertilisation. For later development, the percentage of normal blastulae was reduced in ocean acidification scenarios. Significant dam by temperature interactions indicated differential performance among maternal half-siblings in response to increased temperature. On the other hand, no significant sire by stressor interactions were found. As adaptation depends on additive genetic variance for stress tolerance being present in populations, this may mean that at least with respect to early development, *S. neumayeri* will need to rely on maternal effects to persist through an ocean decreasing in pH and warming.

3.5.1 Contributions of genetic and environmental variance across early development
Previous studies on *S. neumayeri* using embryos generated from multiple parents, found that fertilisation and early development were resilient to future changes (Ericson et al., 2010; Ho et al., 2013). In contrast, with single sire-dam crosses, results indicate significant effects of decreased pH on the percentage of cleavage stage embryos, with significant influences of dam and combination of sire and dam. This is likely due to not only differences in experimental design, but an effect of differences in gamete compatibility typical of sea urchin fertilisation (Palumbi, 1999, Schlegel and Havenhand, 2012; Sewell et al., 2014). The magnitude of the response to increased temperature and acidification differed among sire-dam pairs, with three pairs exhibiting slightly positive responses to decreased pH scenarios, similar to that found by Sewell et al., (2014) for fertilisation in *S. neumayeri*. The significant contribution of dam to embryo performance may be due to the presence of maternal protective factors (e.g. stress proteins) loaded into sea urchin eggs during oogenesis (Hamdoun and Epel, 2007). For *Sterechinus neumayeri*, maternal protective factors are important in reducing oxidative damage to the lipids of embryos where adult sea urchins exposed to oxidative stress produce eggs with greater levels of antioxidants (Lister et al., 2015).
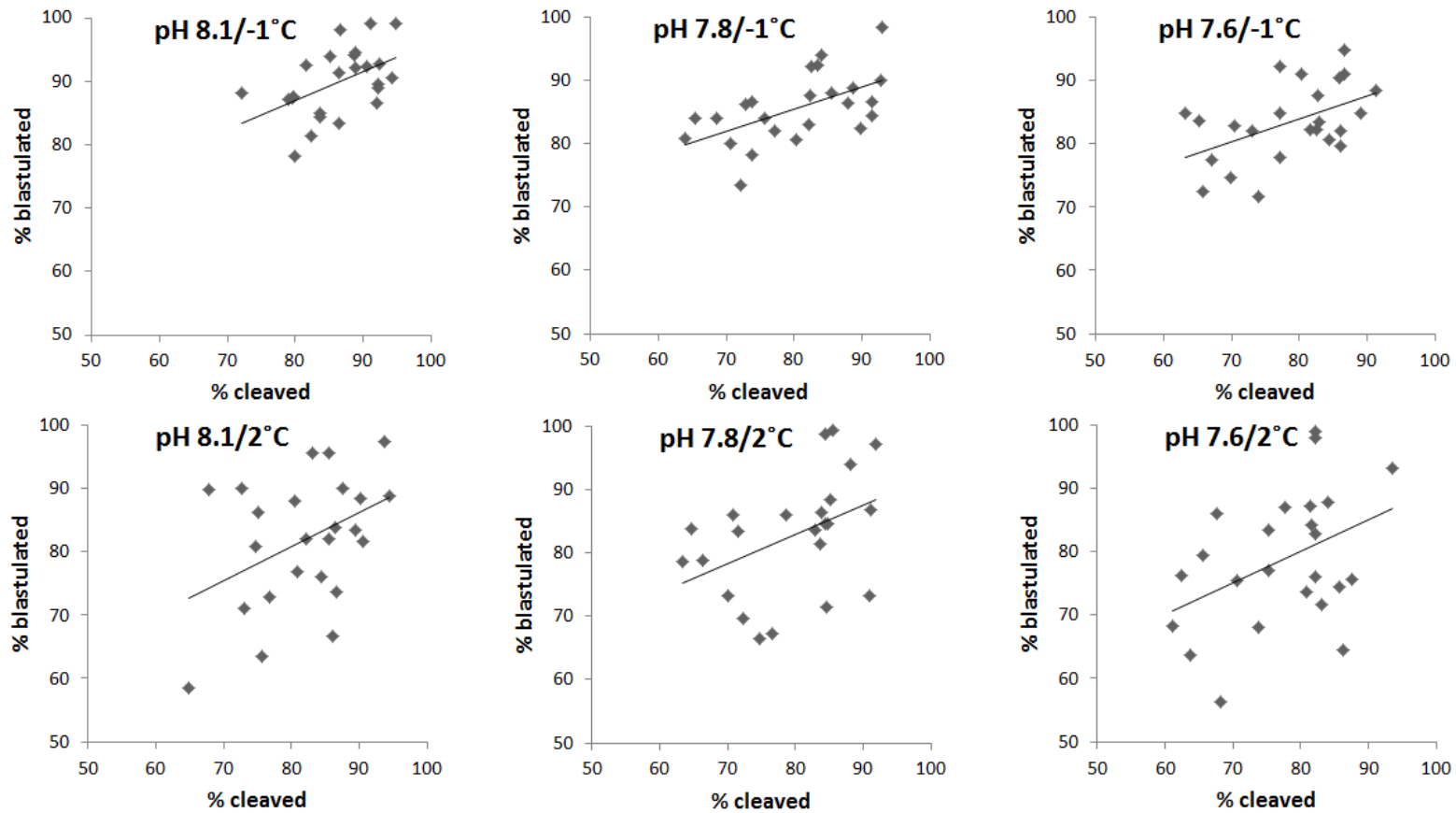
Figure 3.5. Scatter plots of the relationship between pair performance at cleavage (y-axis) and at blastulation (x-axis) for *Sterechinus neumayeri*. Each point represents the mean performance of an individual pair in each treatment across both stages. Positive relationships were evident for all treatments: pH 8.1/-1°C ($R^2 = 0.24$, p = 0.01), pH 8.1/+3°C ($R^2 = 0.17$, p = 0.04), pH 7.8/-1°C ($R^2 = 0.32$, p = 0.004), pH 7.8/+3°C ($R^2 = 0.18$, p = 0.04), pH 7.6/-1°C treatments ($R^2 = 0.24$, p = 0.01) and pH 7.6/+3°C treatments ($R^2 = 0.17$, p = 0.04).

Table 3.6: Linear regression analyses of the relationship between pair performance at cleavage and at blastulation across treatments for *Sterechinus neumayeri*. Significant effects are shown in bold.

Treatment	df	SS	F	P
pH 8.1/-1 ˚C	1	188.40	7.089	**0.014**
pH 7.8/-1 ˚C	1	574.59	10.19	**0.004**
pH 7.6/-1 ˚C	1	383.71	7.03	**0.015**
pH 8.1/2 ˚C	1	248.87	4.61	**0.043**
pH 7.8/2 ˚C	1	305.35	4.66	**0.042**
pH 7.6/2 ˚C	1	300.08	4.47	**0.046**

Blastulae were sensitive to increased acidification but not increased temperature. There was a significant interaction between dam and temperature where eggs of some dams were more sensitive to increased temperature than others. This could be due to both genetic and environmental effects. Therefore, dams may buffer embryos through phenotypic plasticity and impact the rate of selection from ocean change stressors (Räsänen and Kruuk, 2007).

The response of different dam-sire pairs to experimental treatments differed in the magnitude of the effect on percentage development. These effects of parental combination could help to buffer the effects of ocean change. The identity of sire significantly contributed to the development of progeny indicating the presence of additive genetic variance in blastulation of *S. neumayeri* in control scenarios. However there were no sire by stressor interactions found which may indicate limited selection of genotypes in response to warming and acidification. In response to the effects of warming and acidification, animals have shown mixed responses in the amount of additive genetic variance present. The lack of additive genetic variance in response to stressors found for *S. neumayeri* could be due to evolution in a stenothermal environment where isolation in cold waters over evolutionary timescales is also associated with other losses from the genetic tool kit (Harrison and Gerstein, 2002; Hoffmann and Willi, 2008).

The temperate sea urchin *Centrostephanus rodgersii* and the tropical sea urchin *Pseudoboletia indiana* showed great additive genetic variation in the responses of each genotype to pH, temperature and pH x temperature (Foo et al., 2012, 2014). In single stressor studies, the sea urchins *Strongylocentrotus franciscanus* and *S. purpuratus* exhibited high genetic variation in larval growth in response to low pH conditions (Sunday et al., 2011; Kelly et al., 2013). These three species inhabit regions which experience greater natural fluctuations in pH and temperature (Hofmann et al., 2013) than *S. neumayeri*, potentially influencing the genetic variance seen in response to the stressors (Etterson and Shaw, 2001).

On the other hand, family lines of the copepod *Tigriopus californicus* exposed to thermal gradients showed no additive genetic variation in their response to increased temperature (Kelly et al., 2011). Similarly, clones of the bryozoan, *Celleporaria nodulosa* showed no additive genetic variation in tolerance to temperature and pH (Durrant et al., 2013). The absence of genotype x environment interactions found for *S. neumayeri* shows that the performance of family lines does not differ among the pH/temperature scenarios and could be due to the narrow geographical ranges that this sea urchin inhabits. As our design consists of 6 dams and 12 sires,

resulting in 24 genotypes, this may not have been sufficient enough to detect additive genetic effects. A larger design, especially one using many more sires would be more powerful (Conner and Hartl, 2004), but would be difficult to achieve.

As the blastula stage was negatively affected by decreased pH for all *S. neumayeri* genotypes, this developmental stage may represent a bottleneck of sensitivity with negative flow on effects for subsequent stages in development. The magnitude of the effects of pH and increased temperature was relatively small, with a reduction of 10% in the most extreme treatment as compared with the control for both stages. Development to the 3-day old blastula stage was investigated, while *S. neumayeri* has a total pelagic larval duration of up to 115 days (Bosch et al., 1987). During this long planktonic phase, seawater conditions directly affect development and so with a decrease in normal development noted by day 3, this could have flow on effects further down the track (Lamare and Barker, 1999). Larvae of *S. neumayeri* reared in various ocean acidification/warming scenarios have been shown to have significantly shorter arms and abnormal changes in body allometry, with consequential effects for feeding and swimming (Clark et al., 2009, Byrne et al., 2013; Yu et al., 2013).

Although the early post-metamorphic settlement stage may be a mortality bottleneck as the case for many benthic invertebrates (Gosselin and Qian, 1997), small subadult *S. neumayeri* have been shown to very tolerant of high temperature limits (Peck et al., 2013). Kapsenberg et al., (2014) found that ~80% of *S. neumayeri* embryos that reached the blastulae stage at pH 7.9 and 7.7 at 2.6˚C survived thermal shock of up to 15˚C. As these temperatures are much higher than they ever will experience, this indicates that survivors may be relatively robust to environmental stress albeit in acute scenarios. After acclimation of adult *S. neumayeri* to increased temperature (+2˚C) and deceased pH (-03–0.5 units) for 6-8 months, there were no detectable effects of stressors on growth, feeding or behaviour (Suckling et al., 2015).

There was a genetic correlation of 0.34 for the blastulation trait between increased temperature and decreased pH treatments. Positive correlations indicate that selection on one trait is not constrained by selection on the other trait, which is the case for negative correlations. This means that overall, the progeny of parents that performed the best in the lower pH environment also performed the best in the warmer environment. This may be due to an overlap in the gene sets that contribute to genetic variation in performance in response to these two stressors (Via and Lande, 1985; Sgro and Blows, 2004). There were also positive genetic

correlations among the three levels of pH indicating that genotypes that performed the best in the control conditions also performed the best in the stressed pH environment and similarly for the different temperature environments.

3.5.2 Linking performance across pre and post-zygotic developmental stages

As found for *S. neumayeri*, it is often assumed that certain genotypes that perform the best during early development will continue to have superior performance across all developmental stages (Marshall and Keough, 2008). In contrast, for the tropical sea urchin *Pseudoboletia indiana*, performance across stages became unpredictable in stressful environments. For *P. indiana*, performance of pairs at fertilisation predicted performance at the later stage of gastrulation however this relationship was lost in decreased pH scenarios (Foo et al., 2014).

The positive genetic correlations for *S. neumayeri* across all six treatments show that best performing genotypes may have already been selected for in this species. This may be due to evolution in stable environments where long generation times and slow growth of Antarctic invertebrate species have created populations with low genetic diversity adapted to a specific environment (Peck, 2005, 2015; Pörtner, 2007). Antarctic organisms have undergone various genetic changes and adaptations essential for life in the cold, and these often underlie losses of other traits not required under stable thermal conditions found in the Southern Ocean (Pörtner et al., 2007; Somero, 2010). As pairs were shown to perform differently across treatments, where some even showed enhanced performance in stressful environments, these could be the genotypes selected for in the future.

3.5.3 Conclusions

The contributions of genetic and environmental variance in response to ocean stressors was tested here using a North Carolina II design of 24 sire-dam crosses exposed to conditions predicted for 2100 (IPCC, 2013). Although decreased pH and increased temperature impacted cleavage success and the percentage of normal blastulae, the response to these factors differed among the sire-dam pairs. Furthermore, significant dam by temperature interactions indicated differential performance among maternal half-siblings in response to increased temperature due to both genetic and environmental effects. A positive genetic correlation indicated that genotypes that performed well as blastulae in low pH also performed well at high temperatures.

Performance at cleavage was a good predictor of performance at the later blastula stage. On the other hand, there were no sire by environment interactions found indicating limited adaptive potential at these stages of development. Thus, at least with respect to early development, *S. neumayeri* will likely need to rely on phenotypic plasticity to persist through an ocean decreasing in pH and warming at least in the short term. Dams may buffer embryos through phenotypic plasticity and influence selection under ocean change stressors.

CHAPTER FOUR: INCREASED TEMPERATURE, BUT NOT ACIDIFICATION, ENHANCES FERTILISATION AND DEVELOPMENT IN A TROPICAL URCHIN: POTENTIAL FOR ADAPTATION TO A TROPICALIZED EASTERN AUSTRALIA[1]

4.1 Abstract

To predict effects of global change on marine populations, it is important to measure the effects of climate stressors on performance and potential for adaptation. Adaptation depends on heritable genetic variance for stress tolerance being present in populations. We determined effects of near-future ocean conditions on fertilisation success of the sea urchin *Pseudoboletia indiana*. In 16 multiple dam-sire crosses, we quantified genetic variation in tolerance of warming (+3°C) and acidification (-0.3-0.5 pH units) at the gastrulation stage. Ocean acidification decreased fertilisation across all dam-sire combinations with effects of pH significantly differing among the pairings. Decreased pH reduced the percentage of normal gastrulae with negative effects alleviated by increased temperature. Significant sire by environment interactions indicated the presence of heritable variation in tolerance of stressors at gastrulation and thus the potential for selection of resistant genotypes, which may enhance population persistence. A low genetic correlation indicated that genotypes that performed well at gastrulation in low pH did not necessarily perform well at higher temperatures. Furthermore, performance at fertilisation was not necessarily a good predictor of performance at the later stage of gastrulation. Southern range edge populations of *Pseudoboletia indiana* may benefit from future warming with potential for extension of their distribution in south east Australia.

4.2 Introduction

Anthropogenic CO_2 emissions are causing the climate in the ocean to change at an unprecedented rate (Doney et al., 2012). Many studies show the deleterious effects of concurrent ocean warming and acidification on early development of marine organisms (reviews: Byrne 2011; Byrne and Przewalski 2013). Populations can respond to climate

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[1] Chapter is published:
Foo, S.A., Dworjanyn, S.A., Khatkar, M. S. Poore, A.G.B., Byrne, M., 2014. Increased temperature, but not acidification, enhances fertilisation and development in a tropical urchin: potential for adaptation to a tropicalized eastern Australia. Evol. Appl. 7, 1226–1237. See Appendix 1 for statement of author contributions.

change through shifts in distribution, phenotypic plasticity and/or genetic adaptation or otherwise risk extinction. Predicting the prospects for long-term persistence in marine populations requires a better understanding of the capacity for phenotypic adjustment, a plastic response to environmental changes, and adaptation, a genetic response (Yeh and Price 2004; Gienapp et al., 2008; Hoffman and Sgro 2011; Hansen et al., 2012).

Shifts in distribution allow species to track favourable environmental conditions. In response to ocean warming, poleward range shifts have been observed in marine species including fish, plankton, sea urchins and macroalgae (Perry et al., 2005; Parmesan 2006; Ling et al., 2009; Wernberg et al., 2011; Poloczanska et al., 2013). Some species however, have a limited ability to shift their distributions in the time frames needed due to constraints such as limited dispersal potential, long generation times and lack of suitable habitat to migrate to (Hansen et al., 2012). For these species, the ability to produce multiple phenotypes under different conditions (phenotypic plasticity) can facilitate persistence in changing environments (Scheiner 1993; Via et al., 1995). Phenotypic plasticity may thus convey short term tolerance to climate change stressors providing a temporal window for adaptive genetic change to occur (Thompson 1991; Chevin et al., 2010).Long term persistence of populations will likely depend on genetic adaptation in the face of ocean change.

To predict whether marine populations will persist, it is important to determine the effects of ocean change stressors on performance, and the potential for adaptation which is dependent on the levels of heritable variation for stress tolerance. Taxa like sea urchins where male and female gametes can be isolated for experimental matings, provide a tractable and controllable model system for quantifying the contribution of heritable genetic variation to the overall phenotypic variation. Sires are considered to only contribute genetic effects to offspring performance (but see Crean et al., 2013) and so sire x environment interactions can be used to determine the presence of additive genetic variation in the response of offspring to environmental change (Lynch and Walsh 1998). On the other hand, dam effects include both additive genetic effects and environmental effects such as variation in egg provisioning (Mousseau and Fox 1998).

Despite the clear need to understand genetic variation in stress tolerance present in marine populations (Munday et al., 2013; Sunday et al., 2013), relatively few studies have used the tools of quantitative genetics in global change studies with marine organisms. Results to date indicate mixed outcomes for populations with respect to the presence of additive genetic variation in response to stressful environments. The copepod *Tigriopus californicus* showed little adaptive potential in response to a selection regime of increased

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temperature (Kelly et al., 2012). Similarly, the bryozoan, *Celleporaria nodulosa* showed no variation in tolerance to temperature and pH among clones (Durrant et al., 2013). On the other hand, studies on sea urchins, mussels, bryozoans and macroalgae have found significant levels of variation among genotypes, providing the potential for adaptation to ocean warming and acidification (Pistevos 2011; Sunday et al., 2012; Foo et al., 2012; Kelly et al., 2013; Clark et al., 2013). These studies have largely investigated adaptation to a single stressor (temperature: Meyer et al., 2009, Kelly et al., 2012; Clark et al., 2013; acidification: Sunday et al., 2012; Kelly et al., 2013; Pespeni et al., 2013; Sunday et al., 2013) with three studies investigating the response to both stressors concurrently (Foo et al., 2012; Pistevos et al., 2011; Durrant et al., 2013). Thus, we have limited understanding of possible interactions between genotypes and multiple stressors.

In this study, the potential for adaptation to increased temperature and acidification in the tropical sea urchin *Pseudoboletia indiana* was investigated with a quantitative genetics approach using male-female crosses in all combinations of parents under warming-acidification regimes. This species has a broad Indo Pacific distribution, from Madagascar to Hawaii and Easter Island and from Japan to Australia (Turner and Graham 2003). With its recent poleward extension into the Tasman Sea (Pope 1964; Australian Museum records), *P. indiana* is also found in the warm temperate waters of Sydney Harbour representing its southern range end. In east Australia, poleward range extension of tropical species is occurring due to increased southerly flow of the East Australian Current and as temperature continues to rise, *P. indiana* and other tropical species have the potential to migrate poleward in this region and elsewhere (Johnson et al., 2011; Sunday et al., 2013). The impact of increased temperature on echinoderm development is well understood, especially for temperate species (Review: Byrne 2010). For several Australian temperate sea urchin species, a 3 ˚C increase in temperature is deleterious to early development (Foo et al., 2012; Byrne 2012). Therefore, it is of interest to assess the effects of regional ocean warming on populations of a tropical sea urchin species at its warm temperate edge.

The responses of echinoderm fertilisation to increased acidification and temperature have been mixed with outcomes depending on the species and whether the experimental design incorporated multiple male and female parents (spawner population approach) or individual pair responses (Byrne 2011,12; Schlegal et al., 2012). While experimental designs that pool multiple males and females find that fertilisation is fairly robust todecreased pH, results withsingle male-female crosses aremore variable (Foo 2012; Schlegal et al., 2012; Sewell et al., 2014). In sea urchins, fertilisation is mediated by the protein bindin which

controls sperm attachment to the egg (Vacquier and Moy 1977). Eggs show strong discrimination depending on male bindin genotype mating most successfully with sperm having a similar bindin genotype to the egg (Palumbi 1999; Zigler 2008; Evans and Sherman, 2013). Intense sperm competition at fertilisation and differences in compatibility between parental haplotypes are non-additive genetic differences and can explain the differences in experimental outcomes using multiple males/females vs individual pairs.

Here, we use the North Carolina II quantitative genetic design (Lynch and Walsh 1998), where sires and dams are mated in all combinations to determine if additive genetic variance underlies the tolerance of *P. indiana* embryos to ocean change scenarios predicted for this region for 2060 and beyond (Hobday and Lough 2011; IPCC 2014). Maternal influence wanes as the zygotic genome takes over in sea urchin development soon after fertilisation (Tadros and Lipschitz 2009; Hamdoun and Epel 2007). Thus while fertilisation was undertaken in experimental conditions to better reflect future scenarios, genetic performance was assessed at the embryo stage with respect to the contributions of sire and dam to determine if *P. indiana* has the additive genetic variance required to adapt changing ocean conditions. Furthermore, investigating the performance of genotypes across multiple environments, as in this study, allows calculation of genetic correlations among traits, the proportion of variance that two genetic traits share (Sgro and Blows, 2004). We addressed the following questions (1) Is fertilisation robust across future ocean change scenarios? (2) Does the performance of pairs differ across the different treatments? (3) Will the percentage of normal gastrulae be reduced in ocean warming and ocean acidification scenarios? (4) How does performance of genotypes among treatments compare across two stages (fertilisation and gastrulation) and (5) Will significant additive genetic variation (significant interactions of sire with warming and acidification treatments) facilitate persistence of *P. indiana*?

4.3 Materials and methods

4.3.1 Study species and collection sites

Pseudoboletia indiana was collected from 4-6 m depth at Camp Cove, Sydney Harbour, New South Wales (33˚ 50' 21.32 S, 151˚ 16'42.2 E) in April 2013 during their peak spawning period (Zigler et al., 2008). Animals were transported in ambient seawater in a cool box and transferred to large flow through aquaria (80 L; 22˚ C) shortly after collection. They were used for experiments within days of collection. The temperature during the collection period, as indicated by sea surface temperature (SST) recordings during the spawning season, ranged

between 21.5 to 22.5˚ C (http://www.metoc.gov.au/products/data/aussst.php). The animals were collected under permit (NSW DPI: P00/0015-6.0).

4.3.2 Fertilisation and the North Carolina II design

Spawning of *P. indiana* was induced by injection of 2 – 4 ml of 0.5 M KCl. Following routine procedure, eggs from each female were placed in separate beakers of fresh, filtered seawater (FSW; 1 µm). Sperm from each male was stored dry at 4˚C until use. Egg density was determined in counts of 100 µl aliquots from the egg suspension. Approximately 200 eggs were placed in rearing containers (100 ml glass jars) containing experimental seawater 20 minutes prior to fertilisation. Thus eggs were fertilised in experimental temperature/pH conditions (see below). Haemocytometer counts of semen samples diluted with experimental FSW were used to determine the amount of sperm solution required to achieve a final sperm to egg ratio of 500:1; 1×10^3 sperm/ml. Eggs were fertilised with the sperm solutions and after 10 minutes, the water in each jar was changed to remove excess sperm and prevent polyspermy.

Single sire-dam crosses were done in two experimental runs (blocks) with each block using gametes from 2 dams and 4 sires crossed in all combinations. Each block thus resulted in 8 full-sib families (total of 16 families) and were run concurrently. Each family was exposed to each of the 6 combinations of pH and temperature treatments with three replicates for each family by treatment combination. Thus each block had a total of 144 jars (2 females x 4 males x 3 pH levels x 2 temperature x 3 replicates). At 1 h and 24 h, a haphazardly selected sample of approximately 50 embryos was pipetted from the containers, placed into tubes and fixed with 2% glutaraldehyde in FSW. The first 30–50 embryos haphazardly selected from each tube were examined microscopically (Leica) and scored for successful development. At 1 hour, the percentage of successfully fertilised embryos was determined based on the presence of a fertilisation envelope and/or cleavage. At 24 hours, the percentage of gastrulae was calculated from counts of normal/abnormal and arrested embryos (see Gilbert 2000). The number of embryos arrested at fertilisation (e.g., fertilisation envelope only) was low (<1%) indicating that polyspermy was minimal.

4.3.3 Experimental conditions

Experimental treatments consisted of two temperatures (Mean ± SE, control 22.08 ± 0.06 ˚C and 25.04 ± 0.04 ˚C) and three pH_{NIST} levels (Mean ± SE, control 8.12 ± 0.004, 7.85 ± 0.031, and 7.69 ± 0.006) in all combinations (Table 4.1). Treatments were based on model

projections for near future (2060) surface ocean waters in the southeast Australia global change hot spot where SST have been warming appreciably for decades (Hobday and Lough 2011; IPCC 2014).

Filtered (1 µm) experimental FSW was supplied from a flow through system (ambient pH_{NIST} 8.12, 22.1 ˚C) at the Sydney Institute of Marine Science. Water temperature was controlled by thermal mixers supplying 80 L header tanks. Experimental pH was controlled via a mixed CO_2 supply where a pH controller (Parker) regulated the amount of CO_2 gas supplied into the airline. The required amount of air and CO_2 was bubbled through ceramic diffusers into the 80L header tanks controlled by an automatic CO_2 injection and pCO_2 feedback system (BioSys custom system), set at ppm equivalent to pH 7.6 and pH 7.8. The controls were FSW at ambient temperature and pH. .

Temperature, pH and salinity were measured in all treatments (n = 9 per treatment across both blocks) using a pH meter (WTW —Wissenschaftilich-TechnischeWerkstätten GmbG P4) and probe (WTW SenTix® 41 pH electrode; precision ± 0.01 pH units). These parameters were measured at the beginning of the experiment with the water used to fill jars and measured in 8 randomly selected jars from each treatment at the end of the experiment (24 hours). The salinity of treatment water was 34 psu, and dissolved oxygen remained > 90%. Probes were calibrated using NIST high precision buffers pH 4.0, 7.0 and 10.0 (ProScitec).

Water samples (250 ml) were collected at the beginning and conclusion of the experiment, filtered through a 0.45 mm syringe filter, and fixed with 100 µl of saturated HgCl. There were used to determine total alkalinity (TA) by potentiometric titration (Metrohm 888 Titrando) using certified reference standards (Dickson et al., 2007) and total dissolved carbon (TCO_2) using the Apollo SciTech DIC Analyzer AS-C3 (http://www.apolloscitech.com/DIC.htm). Experimental pCO_2 and pH_T (Table 4.1) was determined from TA, TCO_2, temperature, pH_{NIST} and salinity data using CO2SYS (Pierrot et al., 2006) using the dissociation constants of Mehrbach et al., 1973 as refitted by Dickson and Millero 1987.

Table 4.1. Experimental conditions in experiments with *Pseudoboletia indiana*. Mean values (±SE, n = 9) for pH$_{NIST}$ measured daily per treatment is presented with pH$_T$ (determined in CO2SYS using data for dissolved inorganic carbon (DIC) and TA) for comparison. pH$_T$, pCO$_2$ and the saturation states of calcite (Ω_{ca}) and aragonite (Ω_{ar}) were calculated in CO2SYS using data on DIC and total alkalinity (TA = 2258.1 ± 15.6 µmol/kg, n = 12), salinity (34.1 ± 0.04, n = 12) and temperature for each treatment.

	22˚C			25˚C		
	pH 8.1	pH 7.8	pH 7.6	pH 8.1	pH 7.8	pH 7.6
Temp	21.81 (0.02)	21.91 (0.01)	22.5 (0.02)	24.77 (0.05)	25.20 (0.00)	25.14 (0.02)
pH$_T$	8.00 (0.02)	7.86 (0.02)	7.56 (0.03)	7.95 (0.04)	7.69 (0.02)	7.64 (0.01)
pH$_{NIST}$	8.08 (0.00)	7.88 (0.00)	7.71 (0.01)	8.11 (0.00)	7.82 (0.00)	7.66 (0.01)
pCO$_2$	347.75 (2.51)	616.93 (5.59)	923.72 (21.49)	319.00 (3.65)	706.15 (6.18)	1070.67 (14.86)
ΩCa	4.84 (0.02)	3.44 (0.02)	2.42 (0.05)	5.76 (0.04)	3.35 (0.02)	2.46 (0.03)
ΩAr	3.16 (0.02)	2.25 (0.02)	1.58 (0.03)	3.79 (0.02)	2.21 (0.01)	1.62 (0.02)

4.3.4 Statistical analyses

Percentage fertilisation and percentage of normal gastrulae data were analysed using analysis of variance (ANOVA) with temperature and pH as fixed factors, experimental block as a random factor, and sire and dam as random factors nested within blocks. Since some significance tests involved quasi F ratios (in which significance tests derived from the F distribution are unreliable (Quinn and Keough 2002), we calculated significance of the F statistics using 9999 permutations of the raw data for all factors in the PERMANOVA routine of Primer V6 (Anderson et al., 2008). The assumptions of normality and homogeneity of variance were checked by frequency histograms of the residuals and scatter plots of residuals versus estimates. The distribution of the residuals was normal and no transformation was necessary.

For the gastrulation trait, the stage by which the zygotic genome is fully operational (Tadros and Lipschitz 20009), reaction norms were plotted to visualize the interactions between male genotypes across a range of environments (Lynch and Walsh 1998). The genetic correlation of embryo performance (% of normal gastrulae) across temperature and pH environments were used to quantify the genotype x environment interaction using variance components derived from restricted error maximum likelihood (REML) estimates calculated in the R package lme4 (available at http://cran.rproject.org/web/packages/lme4/index.html). Variance components for the random factors were calculated in a single analysis with all factors (Temperature, pH, Block, Males, Females). Genetic correlations were calculated using the causal variance components associated with the sire effects (additive genetic (V_A)) and the interaction effects between sires and each of the environmental factors of temperature (V_{AT}), pH ($V_{A\,pH}$) and both temperature and pH ($V_{AT\,pH}$). Genetic correlations for the same trait averaged over both types of environments ($r*_G$), the genetic correlation for the same trait within one environmental class (i.e. temperature; $r*_{G(T)}$) and the genetic correlation within the other environmental class (i.e. pH; $r*_{G(pH)}$) were calculated using equations from Eisen and Saxton (1983):

$$r*_G = V_A/(V_A + V_{AT} + V_{A\,pH} + V_{AT\,pH})$$
$$r*_{G(T)} = (V_A + V_{AT})/(V_A + V_{AT} + V_{A\,pH} + V_{AT\,pH})$$
$$r*_{G(pH)} = (V_A + V_{A\,pH})/(V_A + V_{AT} + V_{A\,pH} + V_{AT\,pH})$$

Linear regression analyses were performed to assess the relationship between performance across the two different life history stages; fertilisation and gastrulation using percentage performance data for each pair over both stages over the six treatments.

Heritability was estimated using animal, sire and dam models for fertilisation and gastrulation data across all treatments (Knott et al., 1995; Lynch and Walsh,1998). The animal model considers all relationships in the pedigree and computes additive genetic variance based on the additive genetic relationship matrix. A detailed description and application of the animal model are given in Kruuk (2004). Multiple observations on the same genotype were included in the models as random effects and were used to compute repeatability (variation between replicates). Temperature and pH were fixed effects and block a random effect. The models were fitting using ASReml (Gilmour et al., 2009). Heritability estimates were also calculated for each treatment combination.

4.4 Results

4.4.1 Effects of increased temperature and decreased pH on fertilisation and the importance of pair compatibility

Fertilisation success in the 16 sire/dam crosses for *Pseudoboletia indiana* ranged between 29% and 93% in the control conditions (mean of 63.5% ± SE 4.6). Decreased pH had a significant effect in reducing fertilisation success. Both factors were strongly influenced by male/female pairings as indicated by significant sire x dam x temp and sire x dam x pH interactions (Table 4.2; Fig. 4.1). The sire x dam x temp x pH interaction indicates the effect of increased temperature in reducing the negative effect of decreased pH on percentage fertilisation (Table 4.2; Fig. 4.1). This is also evident in the scatter plot as seen in the comparison of fertilisation in the 22 ˚C /pH 7.6 and 25˚C/7.6 treatments (Fig. 4.1). The most extreme pH treatment lowered fertilisation success across all but two pairs showing the influence of gamete compatibility resulting in different responses to the same treatment (Fig. 4.1).

Sire and dam identity also influenced fertilisation success with significant interactions between sire and temperature and between dam, temperature and pH (Table 4.2). The sire x temperature interaction indicates that the effect of +3˚C varied among paternal half-siblings. Similarly, the effects of pH and temperature varied among maternal half-siblings with an increase in fertilisation success at +3˚C and a decrease with lowered pH (Table 4.2).
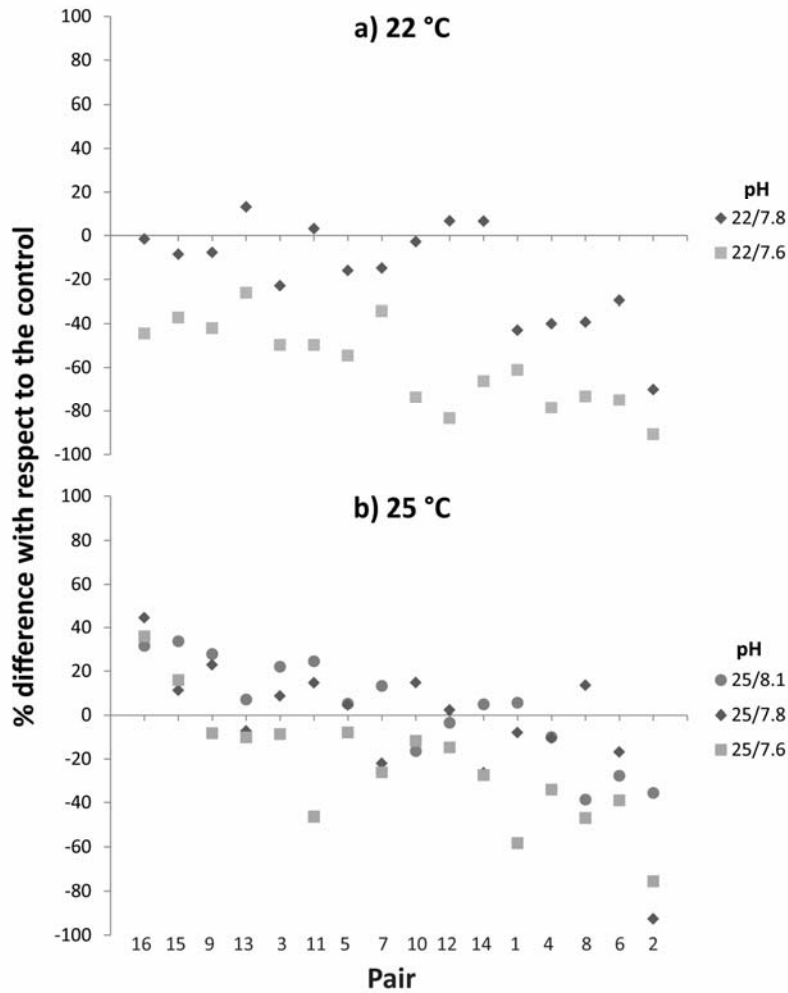
Figure 4.1. The difference in fertilisation success with respect to the control treatment (22 ºC, pH 8.1) in 16 different male-female pairs across five experimental treatments for *Pseudoboletia indiana*. Mean fertilisation success per genotype is displayed for the different pH levels across the control temperature (a) and increased temperature (b). Symbols above the line display higher fertilisation success than the control, while fertilisation success was lower than the control for those symbols below the line. Pairs are ranked from the best to the worst performing from left to right.

Table 4.2. ANOVA of percentage fertilisation data of *Pseudoboletia indiana*. ANOVA of fertilisation data of single dam-sire crosses across various temperature (Te) and pH conditions. These were fixed factors, with experimental block (Bl) as a random factor, and male (Ma) and female (Fe) identity as random factors nested within block. Significant effects are shown in bold ($p < 0.05$).

Source	df	MS	F	P
Bl	1	94.712	3.35E-02	0.9998
Te	1	21958	13.714	0.1692
pH	2	45612	20.5	**0.047**
Ma(Bl)	6	1986.6	7.0254	**0.0193**
Fe(Bl)	2	9266.1	32.769	**0.0012**
BlxTe	1	1601.1	0.76159	0.5789
BlxpH	2	2225	2.3551	0.0837
TexpH	2	6921.6	3.7581	0.2195
Ma(Bl)xFe(Bl)	6	282.77	1.607	0.1482
Ma(Bl)xTe	6	1934.1	4.63	**0.0438**
Ma(Bl)xpH	12	893.52	1.6226	0.2046
Fe(Bl)xTe	2	716.66	1.7156	0.2522
Fe(Bl)xpH	4	285.05	0.51764	0.7227
BlxTexpH	2	1841.8	0.99945	0.4698
Ma(Bl)xFe(Bl)xTe	6	417.73	2.374	**0.0341**
Ma(Bl)xFe(Bl)xpH	12	550.68	3.1295	**0.0004**
Ma(Bl)xTexpH	12	261.83	0.52167	0.8594
Fe(Bl)xTexpH	4	2083.1	4.1505	**0.0257**
Ma(Bl)xFe(Bl)xTexpH	12	501.9	2.8523	**0.0013**
Res	192	175.96		

4.4.2 Effects of increased temperature and decreased pH on gastrulation

Increased temperature significantly increased the percentage of normal gastrulae, with temperature increasing performance at the two decreased pH treatments but not at the control pH (Table 4.3). The effects of sire and dam on the percentage of normal gastrulae were significant as were the interactions between sire and temperature, and dam and temperature. The sire by environment interactions are illustrated in reaction norms showing the response of paternal half-siblings to pH and temperature treatments (Fig. 4.2). The significant sire x temperature interaction indicates that the effect of the $+ 3$ ˚C treatment varied among paternal half-sib families, as shown by the different slopes in the reaction norms. Male and female compatibility was also important with significant sire x dam, sire x dam x temp, sire x dam x pH, and sire x dam x temp x pH interactions (Table 4.3).

There was a low genetic correlation ($r*_G$) of 0.1 in the gastrulation trait across all environments indicating that genotypes that performed well in a particular combination of temperatures and pH might not necessarily perform similarly in other environmental combinations. However, there were stronger positive genetic correlations across the three temperature levels ($r*_{G(t)} = 0.47$) and across the three pH levels ($r*_{G(pH)} = 0.63$). Thus, genotypes that performed well at control temperatures also performed the best in high temperatures and similarly for pH.

Table 4.3. ANOVA of percentage of normal gastrulae of *Pseudoboletia indiana.* ANOVA of gastrulation data of single dam-sire crosses across temperature (Te) and pH treatments. Temperature and pH are fixed factors, experimental block (Bl) a random factor, and male (Ma) and female (Fe) identity random factors nested within block. Significant effects are shown in bold ($p < 0.05$).

Source	df	MS	F	P
Bl	1	590.18	0.31628	0.8814
Te	1	23030	249.9	**0.0377**
pH	2	65919	15.575	0.058
Ma(Bl)	6	1422.8	8.056	0.0116
Fe(Bl)	2	1001.7	5.6715	**0.043**
BlxTe	1	92.157	6.59E-02	0.9977
BlxpH	2	4232.5	2.8429	**0.0464**
TexpH	2	27451	919.88	**0.0019**
Ma(Bl)xFe(Bl)	6	176.61	2.2257	**0.041**
Ma(Bl)xTe	6	1178.3	6.251	**0.0216**
Ma(Bl)xpH	12	1248	2.4223	0.0677
Fe(Bl)xTe	2	3079.1	16.336	**0.0037**
Fe(Bl)xpH	4	422	0.81907	0.53
BlxTexpH	2	29.841	0.65838	0.7135
Ma(Bl)xFe(Bl)xTe	6	188.49	2.3754	**0.0296**
Ma(Bl)xFe(Bl)xpH	12	515.22	6.493	**0.0001**
Ma(Bl)xTexpH	12	280.2	0.39025	0.9441
Fe(Bl)xTexpH	4	855.69	1.1918	0.3676
Ma(Bl)xFe(Bl)xTexpH	12	718	9.0486	**0.0001**
Res	192	79.35		
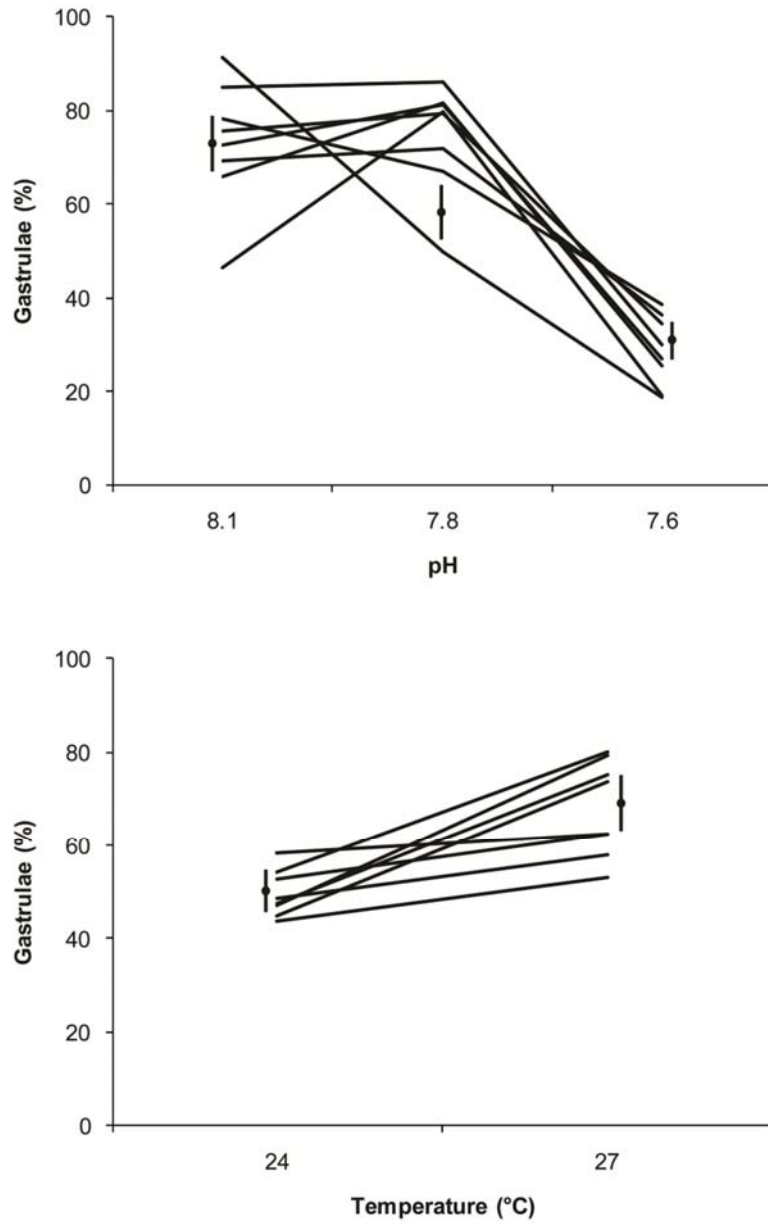
Figure 4.2. Reaction norms showing the responses of the progeny of eight male genotypes to increased temperature and reduced pH for *Pseudoboletia indiana*. The reaction norms show the percentage of normal gastrulae in experimental temperatures pooled for pH (A) and in experimental pH levels pooled for temperature (B). Lines represent the mean percentage of paternal half siblings with standard errors indicated (n = 8).

4.4.3 Does performance at fertilisation predict gastrulation success?

The relationships between fertilisation success and percentage of normal gastrulae show that the pairs did not perform consistently across all environments (Fig. 4.3). A positive relationship was evident for the control pH/22.08 ˚C, pH 7.9/+2.99˚C and pH 7.7/+2.99˚C environments. Here, genotypes that had a high percentage of fertilisation also had the highest percentage of normal gastrulae. However, this does not hold true when decreased pH is considered in isolation. Thus, genotypes that perform well at fertilisation were good genotypes at gastrulation, but only under certain pH/temperature conditions.

4.4.4 Repeatability and heritability estimates for fertilisation and gastrulation

The heritability estimate from the animal model at fertilisation was moderate (0.222 ± 0.1), contributed mainly by the dam component as the heritability estimate from the sire component was zero (Table 4.4). Estimates of repeatability (0.0356 ± 0.0323) and heritability (0.062 ± 0.047) from the animal model for gastrulation were quite low, with the heritability based on sire component lower when compared to fertilisation (0.094 ± 0.075) albeit with equally large standard error. Heritability estimates were also calculated for each of the six treatments. However as estimates were quite variable across different models and between different treatments these have comparatively large standard errors (Table 4.5).

4.5 Discussion

This study examined the performance of replicate male-female pairs of *Pseudoboletia indiana* at fertilisation and the potential to adapt to climate change stressors at gastrulation. Decreased pH significantly reduced fertilisation success, the magnitude of which depended on the individual pair, indicating the importance of pre-zygotic effects and the sperm bindin-egg bindin receptor system characteristic of sea urchin fertilisation (Palumbi 1999). The percentage of normal gastrulae was reduced in ocean acidification scenarios but increased in warming scenarios. There was a slight positive genetic correlation between performance in increased temperature and decreased pH. Most importantly, the significant interaction between male and each stressor indicated the presence of additive genetic variance in the response of progeny to increased temperature and acidification. Interestingly, performance at fertilisation did not necessarily predict performance at gastrulation.
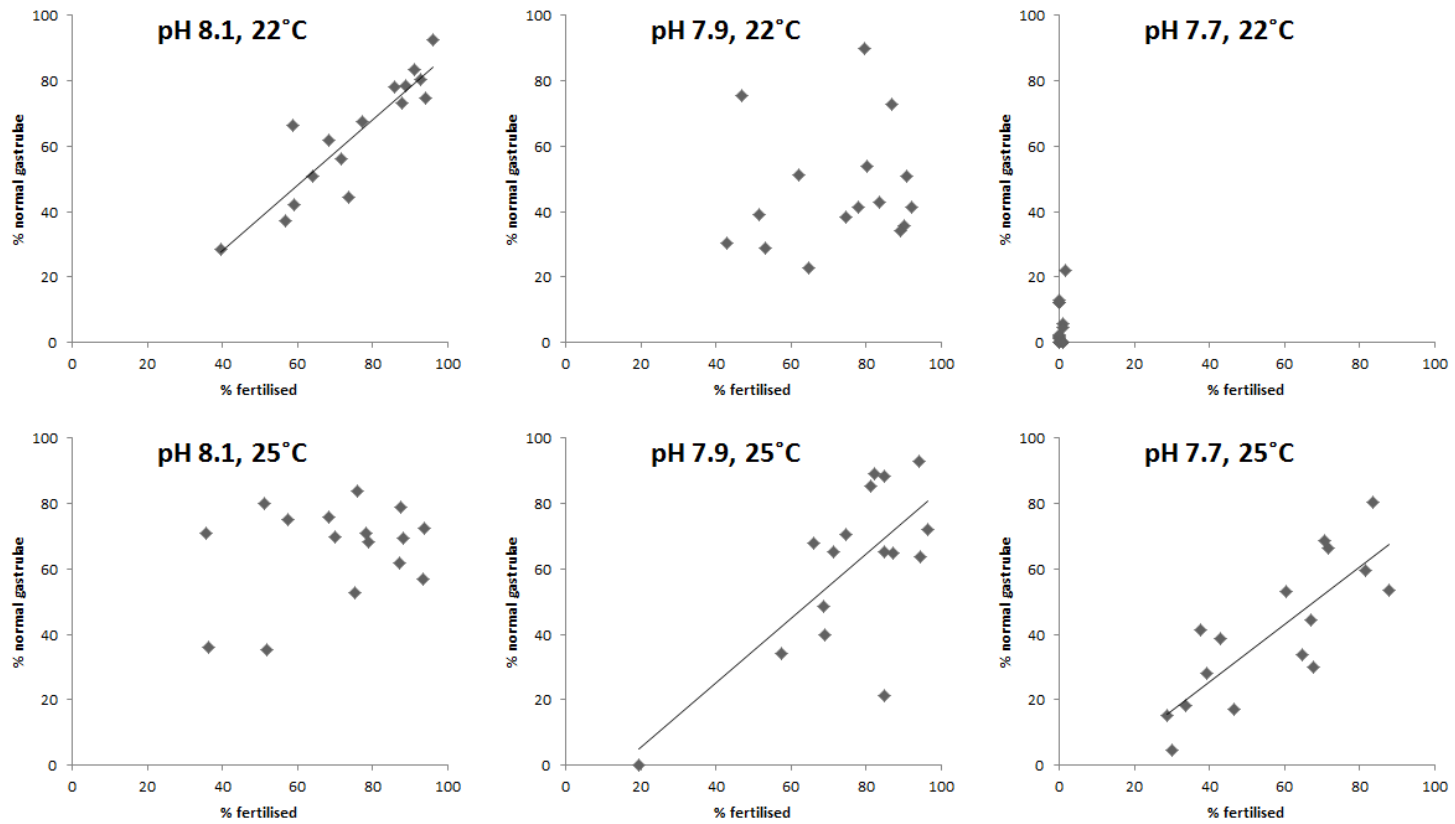
Figure 4.3. Scatter plots of the relationship between pair performance at fertilisation (y-axis) and at gastrulation (x-axis) for *Pseudoboletia indiana*. Each point represents the mean performance of an individual pair in each treatment across both stages. A positive relationship was evident for the control pH/control temp ($R^2 = 0.81$, p = 0.000002), pH 7.8/+3˚C ($R^2 = 0.51$, p = 0.002) and pH 7.6/+3˚C treatments ($R^2 = 0.66$, p = 0.0001).

Table 4.4. Heritability estimates at fertilisation and gastrulation for *Pseudoboletia indiana.* Animal, sire and dam models were used to estimate heritability for fertilisation and gastrulation across all treatments. Multiple observations on the same genotype were included in the model as random effects and were used to compute repeatability. Temperature and pH were fixed effects and experimental block a random effect. The models were fitted using ASReml.

Trait	Model	Parameter	Estimate	SE
Fertilisation	IntraFamily	Repeatability	0.1932	0.0721
Fertilisation	Animal	Heritability	0.2217	0.0951
Fertilisation	Sire	Heritability	0.1582	0.1079
Fertilisation	Dam	Heritability	0.3251	0.2461
Gastrula	IntraFamily	Repeatability	0.0356	0.0323
Gastrula	Animal	Heritability	0.0621	0.0472
Gastrula	Sire	Heritability	0.0904	0.0754
Gastrula	Dam	Heritability	0.0206	0.0423

Table 4.5. Heritability estimates at fertilisation and gastrulation for *Pseudoboletia indiana* separated across the six experimental treatments. Animal, sire and dam models were used to estimate heritability for fertilisation and gastrulation across each treatment. Multiple observations on the same genotype were included in the model as random effects and were used to compute repeatability. Temperature and pH were fixed effects and experimental block a random effect. The models were fitted using ASReml.

Cleavage stage embryos				
Treatment	**Model**	**Parameter**	**Estimate**	**SE**
22/8.1	IntraFamily	Repeatability	0.3481	0.2137
	Animal	Heritability	0.4581	0.3077
	Sire	Heritability	0.2324	0.2277
	Dam	Heritability	0.9236	0.8808
22/7.8	IntraFamily	Repeatability	0.454	0.2189
	Animal	Heritability	0.6504	0.1356
	Sire	Heritability	0.5087	0.3415
	Dam	Heritability	0.83	0.4861
22/7.6	IntraFamily	Repeatability	0.3454	0.2402
	Animal	Heritability	0.3493	0.2912
	Sire	Heritability	0	0
	Dam	Heritability	1.0973	1.0709
25/8.1	IntraFamily	Repeatability	0.2956	0.1653
	Animal	Heritability	0.3082	0.202
	Sire	Heritability	0.2286	0.2898
	Dam	Heritability	0.3915	0.3678
25/7.8	IntraFamily	Repeatability	0.7922	0.0792
	Animal	Heritability	0	0
	Sire	Heritability	0.509	0.6304
	Dam	Heritability	0	0
25/7.6	IntraFamily	Repeatability	0.6547	0.1176
	Animal	Heritability	0.6328	0.1623
	Sire	Heritability	0.5942	0.3773
	Dam	Heritability	0.6789	0.4653
Gastrulae				
22/8.1	IntraFamily	Repeatability	0.3648	0.2432

	Animal	Heritability	0.5092	0.3646
	Sire	Heritability	0.3431	0.3043
	Dam	Heritability	0.8666	0.873
22/7.8	IntraFamily	Repeatability	0.7349	0.0966
	Animal	Heritability	0.2946	0.3511
	Sire	Heritability	0.4095	0.5361
	Dam	Heritability	0.2108	0.4083
22/7.6	IntraFamily	Repeatability	0	0
	Animal	Heritability	0	0
	Sire	Heritability	0	0
	Dam	Heritability	0	0
25/8.1	IntraFamily	Repeatability	0.5989	0.2681
	Animal	Heritability	0.1926	0.4094
	Sire	Heritability	0.3911	0.5657
	Dam	Heritability	0.0272	0.3078
25/7.8	IntraFamily	Repeatability	0.8684	0.2079
	Animal	Heritability	0	0
	Sire	Heritability	0.5594	0.7079
	Dam	Heritability	0	0
25/7.6	IntraFamily	Repeatability	0.7118	0.103
	Animal	Heritability	0.3491	0.3280
	Sire	Heritability	0.4223	0.5004
	Dam	Heritability	0.2892	0.4205

4.5.1 Fertilisation success: Prezygotic effects

For *P. indiana*, fertilisation success decreased at low pH with significant influences of sire, dam and combination of sire and dam. For other sea urchin species using the multiple parent (population) approach (e.g. *Heliocidaris erythrogramma*, *H. tuberculata*), fertilisation is fairly robust to future ocean change scenarios (Byrne 2012; Byrne and Przeslawski 2013). For *P. indiana*, this is not the case for single male-female crosses as also found for other sea urchin species using a similar approach (Schelgal et al., 2012; Sewell et al., 2014) The multiple parent spawning approach does not allow detection of intra-specific variation. To allow a full assessment of offspring response to treatments, the genotype of the parents was taken into account, as in the design of our experiment.. For *P. indiana*, none of the males consistently performed the best across all treatments indicating there is no universally good male. Reduced pH can decrease the number of motile sperm (i.e. moving) in some sea urchins (Schlegal et al., 2012) and this may have influenced the results with *P. indiana*. Swimming speed of sea urchin sperm is either not affected (Schelgal et al., 2012) or actually enhanced (Caldwell et al., 2011) in ocean acidification scenarios. It should be noted that within-ejaculate variability of individual sperm, as shown to be important by Crean et al., (2012) where the offspring of the tunicate *Styela plicata* sired by longer-lived sperm performed better in control conditions, remains unexplored for *P. indiana*.

Although decreased pH had an overall negative effect on percentage fertilisation, the scatter plot revealed that two pairs actually showed a positive response in decreased pH conditions with an increase of 36% and 16% compared to the control, also seen for *Sterechinus neumeyeri* and *Centrostephanus rodgersii* in response to acidification scenarios (Sewell et al., 2014; Foo et al., 2012). As non-additive genetic differences (i.e. non heritable) due to parental haplotype compatibility (e.g. sperm bindin – egg bindin receptor system) dominates the fertilisation biology of sea urchins (Palumbi 1999; Zigler 2008; Levitan and Ferrell 2006) this difference was expected.

Certain individuals may disproportionately contribute to the success of future generations (Schlegal et al., 2012), although following through to gastrulation as in this study for *P. indiana* indicated that this is not straight forward. It is clearly important to follow through with zygotic (post-fertilisation) development to better understand adaptive potential (e.g Sunday et al., 2012). Furthermore, this is supported by heritability estimates at fertilisation where it can be seen that the dam contributes most to heritability at this stage compared to the sire component.

4.5.2 Gastrulation: Additive genetic variance, genetic correlations and adaptive potential
At gastrulation (a post-zygotic trait), normal development decreased at low pH for all genotypes of *P. indiana* with warming significantly increasing the percentage of normal gastrulae. The interaction between pH and temperature indicated that warming somewhat alleviated the effects of decreased pH. If this was a single stressor study, one may have concluded that normal development could not occur in a decreased pH environment, but here we show that coupled with increased temperature, normal development can occur. For the biota of eastern Australia, temperature is the more important and contemporary stressor (Hobday and Lough 2011) with negative effects of present day and near future warming reported for several echinoderm species (Nguyen et al., 2012). Thus it is interesting that decreased pH is the more important negative stressor for *P. indiana*.

The significant contribution of sire to progeny performance, and the interaction between sire and temperature and with pH at gastrulation indicates the presence of additive genetic variation in tolerance to ocean warming and acidification conditions. It can be seen that the offspring of some males were more strongly affected by acidification than others but only at increased temperatures. Selection mediated by increased temperature and acidification would be expected to favour the more tolerant *P. indiana* genotypes allowing species persistence in future ocean change conditions. Most importantly, the significant sire x pH x temperature interaction, and non-significant sire x pH interaction indicate that adaptation to ocean acidification would not occur in this species in isolation from ocean warming.

For many echinoderms, maternal effects result in variable egg quality likely due to phenotypic effects associated with the maternal nutritive history, egg nutrients and maternal environmental history (Byrne et al., 2008, 2011). Normal development in sea urchins is influenced by maternal transcripts which may be influenced by maternal stress history (Hamdoun and Epel 2007). Thus, the significant interaction between dam and temperature where eggs of some females were more susceptible to stressors than others could be due to both genotypic and environmental/phenotypic effects.

Development in *P. indiana* was enhanced in the $+3°C$ treatments. If spawning time in this species is influenced by temperature, as appears the case for many sea urchins (Byrne 1999), there is potential that adult *P. Indiana* may be able to track favourable temperature conditions for offspring, to spawn at times to facilitate developmental success, a phenotypic adjustment of reproduction. Phenological change in biological events such as spawning is a major response to marine global change as seen for diatoms, copepods and fish larvae

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(Edwards and Richardson 2004). Developmental plasticity may provide short term tolerance to climate change allowing the time for adaptive evolution to occur (Sultan 2007). Furthermore, epigenetic (non-genetic inheritance) can also affect progeny's response to environmental change (Bonduriansky et al., 2011). The environmental conditions the parent experiences can influence parts of its phenotype that can affect the development of its progeny (Visser 2008, Bonduriansky and Day 2009).

There was a genetic correlation of close to zero for the gastrulation trait among all temperature and pH treatments. This means that the progeny of parents that performed the best in the lower pH environment did not necessarily mean they performed the best in the warmer environment. This may indicate that there is little overlap in the gene sets that contribute to genetic variation in performance in response to these two stressors. Thus, evolution is not constrained in adapting to both stressors simultaneously. A previous study on a temperate sea urchin species found a high positive genetic correlation indicating similar gene sets influence performance in both ocean acidification and warming environments, which could enhance the speed at which natural selection can occur (Foo et al., 2012). For *P. indiana* there were positive genetic correlations among the three levels of pH indicating that genotypes that performed the best in the control conditions also performed the best in the stressed pH environment and similarly for the different temperature environments.

The presence of standing genetic variation as indicated by significant sire x stressor interactions, and absence of a trade-off between tolerance to both pH and temperature, contributes to the potential of *P. indiana* to adapt to concurrent ocean acidification and warming and adds to the resilience of this important species in a changing ocean. When comparing heritability estimates across both stages it can be seen that the dam contribution is much larger at fertilisation, suggesting that the pre-zygotic stage is dominated by maternal effects. Sire effects remained similar throughout both developmental stages. However estimates, especially at gastrulation, were quite low with proportionately large standard errors. Moreover, there were large differences in the estimates from animal, sire and dam models. As estimates of heritability should be lower as compared to their respective repeatability estimates, these results suggest that more data incorporating a greater number of genotypes are required to obtain more precise estimates of heritability and to better understand the genetic architecture of these traits (Lynch and Walsh, 1998).

4.5.3 Linking performance across different life history stages

When comparing the performance of pairs across fertilisation and gastrulation, there was a positive relationship for the control environment, and the two treatments with combined pH and increased temperature. Pairs that had the highest fertilisation success in these environments also had the highest percentage of normal gastrulae. However, when decreased pH is considered in isolation, this does not hold true for pairs with performance being unpredictable.

It is often assumed that certain genotypes that perform the best will continue to have superior performance across all developmental stages, e.g. larger larvae in various marine taxa typically show higher performance as juveniles and adults (Marshall and Keough 2008). Here we show however that pairs that perform best in pre-zygotic stages did not necessarily predict their performance in post-zygotic stages, or in stressed conditions. This indicates the lack of connection between pair performance at both stages and shows that looking only at prezygotic effects (e.g. fertilisation) cannot be used to predict performance. Here we only considered the gastrulation trait and the disconnection between fertilisation success and gastrulation success for some pairs highlights the importance of considering all developmental stages.

4.5.4 Implications for Pseudoboletia indiana and the tropicalisation of eastern Australia

The gastrulae stage of many echinoderms is sensitive to warming with an increased temperature of 4˚C above ambient being deleterious to many species (Byrne 2011, Byrne et al., 2010). In this study however, *P. indiana* exhibited a higher percentage of normal gastrulation at 3˚C above ambient, with this level of warming being beneficial to early development. Temperature is likely to have been an important factor in establishing populations of this tropical species at its southern range edge in Sydney. Elsewhere in its range, *P. indiana* experiences temperatures 28˚C and above (Clark and Rowe 1971). In Sydney, this species spawns in Summer and Autumn at temperatures ranging from 22 to 23 ˚C (Zigler et al., 2012). Although the cool temperature tolerance of development is not known, it seems likely that the population in Sydney may be living where temperatures are just warm enough for successful development. This is similar for newly established *Centrostephanus rodgersii* populations in Tasmania where they live at their lower limit of developmental tolerance (12 ˚C) (Hardy et al., 2014; Ling et al., 2009).

Ocean warming may facilitate the success of *P. indiana* in the temperate waters of Eastern Australia and may provide an opportunity for poleward range extension as seen with

other tropical echinoids and asteroids (Hardy et al., 2014, Pecorino et al., 2013). Tropical echinoids tend to have a broader developmental thermal tolerance compared with temperate species facilitating poleward range extension to cooler climes (Hardy et al., 2014; Sunday et al., 2011). Southerly expansion of *P. indiana* is possible as there appears to be suitable habitat for this rocky reef species. As the latitudinal distribution of many marine species is related to the thermal tolerance of planktonic stages, understanding species' potential for poleward range extension with respect to the thermal tolerance of development is key to understanding how the seascape will change with further warming and acidification as not all species are affected equally (Sunday et al., 2012).

In conclusion, early development in *P. indiana* was sensitive to acidification at pH 7.6 while warming (+3 ˚C) alleviated the negative effects of acidification at gastrulation. Male/female compatibility significantly affected performance however the pairs that performed the best in control conditions did not necessarily perform the best in stressed environments. Furthermore it was clear that performance of pairs across different developmental stages was particularly unpredictable in ocean acidification conditions. Our analyses revealed the presence of significant additive genetic variation underlying success at gastrulation in response to ocean acidification and warming scenarios. Furthermore, due to *P. indiana*'s increased performance in warmer conditions, this species has potential to expand its population in Sydney at its range edge and beyond. The presence of tolerant genotypes indicates that Sydney Harbour populations of *P. indiana* are resilient to +3 ˚C and combined with the lack of a negative correlation between tolerance to both decreased pH and warming will contribute to the potential of early development in *P. indiana* to adapt to a changing ocean. In eastern Australia, the Sydney region approximates the southern range edge of many tropical sea urchin species (e.g *Diadema* spp.) (Miskelly 2002), that like *P. indiana* would be expected to have enhanced performance as the ocean warms with potential for population expansion locally and extension of their distribution poleward.

CHAPTER FIVE: ADAPTIVE CAPACITY OF THE SEA URCHIN *HELIOCIDARIS ERYTHROGRAMMA* TO OCEAN CHANGE: RESPONSES FROM FERTILISATION TO THE JUVENILE

5.1 Abstract

To accurately predict impacts of ocean acidification and warming on the responses of marine populations, it is important to determine an organism's capacity for phenotypic plasticity and the potential for genetic adaptation. We determined the effects of near-future acidification and warming across the life cycle of *Heliocidaris erythrogramma* from fertilisation to metamorphosis in the progeny of 16 sire-dam crosses. Sources of variation in tolerance to warming (+3 °C) and acidification (-0.3-0.5 pH units) were investigated for fertilisation, larval success and juvenile metamorphosis. Across all life stages, maternal legacy was important, with dam identity significantly interacting with stressors. Across the genotypes tested, fertilisation was negatively affected by increased temperature, but not pH. Larval development was compromised in low pH, but not temperature. By the settled juvenile stage, no impact of warming or acidification was evident and this was likely due to selective mortality of sensitive individuals. Across all environments tested, the juveniles exhibited a similar ability to calcify. The impact of warming and acidification on development after fertilisation was influenced by parental identity, with the offspring of some dam-sire pairs more sensitive than others. That the progeny of some sire-dam pairs showed high stress tolerance indicates the potential for selection of resistant genotypes and adaptation that could facilitate the persistence of *H. erythrogramma* populations. Performance of progeny at one stage could not predict the performance later in development and shows the importance of assessing impacts of ocean change across the life cycle of marine invertebrates.

5.2 Introduction

The world's oceans are changing due to anthropogenic gas emissions, creating an imperative to assess the potential impacts of climate change on marine populations (Byrne, 2012; Bernhardt and Leslie, 2013). In response to environmental change, animals can respond by shifting their distribution, adjusting their phenotype or genetically adapting (Gienapp et al. 2008; Hoffmann & Sgro 2011). While shifts in distribution as species track favourable environmental conditions are now a global phenomenon (Burrows et al., 2014; Garcia Molinos et al., 2015; Sunday et al., 2015), this phenotypic response is not an option for all

species, such as those that have restricted dispersal, physical barriers and habitat fragmentation (Hansen et al. 2012; Kinlan & Gaines, 2003). For these species, acclimatisation to changing conditions and genetic adaptation will be essential for survival (Gienapp et al. 2008; Visser, 2008). Acclimatisation allows species to adjust to a new or changing environment in their lifetime with potential for these effects to be passed on to offspring and across generations (Ghalambor et al., 2007; Whitman and Agrawal, 2009; Chown et al., 2010).

The ability to genetically adapt to a changing marine environment depends on the existence of additive genetic variance within the population (Billington and Pelham, 1991). Standing genetic variation can provide a reservoir of resilience to stressors (Anttila et al., 2013; Kelly et al., 2013; Pespeni et al., 2013) especially if the variation is present for the trait of interest. Within a population, the presence of genetic variance influences the response of life history traits, such as fertilisation and larval success, to increased temperature and acidification (e.g. sea urchins, mussels, Sunday et al., 2011; Foo et al., 2012, 2014).

The environment the species experiences can influence the presence and pattern of genetic variation across the range that the species inhabits. Variable conditions experienced among populations across the species range have been shown to result in the presence of locally adapted genotypes (Sanford & Kelly, 2010). Thus species with a broad latitudinal distribution across a range of thermal or pH environments are likely to have populations with an in-built capacity to persist in changing oceans (Bradshaw and Holzapfel, 2001).

For free spawning marine invertebrates, the gametes of the male and female parent can be isolated for experimental matings, making it possible to compare the performance of offspring genotypes in different environments. The North Carolina II quantitative breeding design where sires and dams are crossed in all combinations, allows variance in traits within a population to be partitioned into additive, maternal, interactive and environmental components, with the potential for genetic adaptation strongly related to the levels of additive genetic variation (Lynch and Walsh, 1998). The opportunity to control matings provides a model system to investigate selection in different environments from fertilisation in offspring generated in experimental conditions (Foo et al., 2012, 2014).

Studies have used this breeding design to investigate the responses of the offspring of sea urchins, mussels and macroalgae in response to climate change stressors and have found significant levels of variation in stress tolerance among genotypes, indicating the potential for adaptation to those stressors (Sunday et al., 2011; Foo et al., 2012; Clark et al., 2013; Kelly et al., 2013; Lymbery and Evans, 2013; Foo et al., 2014). These studies largely involve a single

stressor (temperature: Chirgwin et al., 2015; Clark et al., 2013; Lymbery and Evans, 2013; acidification: Kelly et al., 2013; Sunday et al., 2011) with two studies investigating the response to both stressors in combination (Foo et al., 2012, 2014).

We used the short development time of the sea urchin *Heliocidaris erythrogramma,* with access to the juvenile in 3–5 days, as a model system to assess the response of genotypes to both increased temperature and acidification across the life cycle using the North Carolina II design. *Heliocidaris erythrogramma* is a widely distributed and ecologically important sea urchin endemic to southern Australia (Keesing, 2013). Previous studies have investigated the outcome of sire-dam crosses in this species using a single stressor or considering performance at one life history stage only. There is previous evidence for significant additive genetic variance in embryos at metamorphosis (Evans et al., 2007) and for embryos fertilised in control conditions and then transferred to increased temperature treatments (+3°C), significant sire x temperature interactions and pair x temperature effects were evident at hatching (Lymbery and Evans, 2013). In sire-dam crosses, fertilisation success was reduced (Havenhand et al., 2008) or variable with some pairs performing better than others (Schlegel et al., 2012) in low pH (-0.4 pH units) conditions. Studies using gametes from multiple parents found that fertilisation in *H. erythrogramma* is resilient to increased acidification and temperature (-0.3-0.5 pH units, +4˚C) (Byrne et al., 2009, 2010a) with larvae and juveniles sensitive to increases in temperature (+4-6˚C) but tolerant to 2˚C warming (Byrne et al., 2009; Byrne et al. 2011). In multistressor studies, juveniles survived temperature increases of +4˚C coupled with decreases in pH up to 0.7 units however the number of abnormal juveniles increased in response to combined effects of increased temperature (+2-4˚C) and (-0.3–0.5 pH units) (Wolfe et al., 2013).

In this study, the performance of the progeny of 16 male-female crosses of *H. erythrogramma* were followed from fertilisation to identify the sources of variation in tolerance to warming (+3°C) and acidification (-0.3-0.5 pH units) for fertilisation, larval success and juvenile metamorphosis. As calcification in *H. erythrogramma* (Wolfe et al., 2013) and other marine calcifiers is negatively affected by ocean acidification (Kroeker et al., 2013; Przeslawski et al., 2015), we also tested for variation in calcification among genotypes. We took the approach of initiating the stressor treatments with gametes prior to fertilisation, as these cells are known to be highly sensitive to stressors (e.g. Reuter et al., 2011; Schlegel et al., 2012). In contrast to fertilising in control conditions and subsequent transfer of zygotes to stress treatments, this approach provides a more realistic assessment of stressor responses for a free spawning marine invertebrates in the carry over effects from the parental (sperm,

eggs) to the zygotic genotype. For *H. erythrogramma*, it was predicted that adaptive capacity is likely to stem from standing genetic variation maintained through balancing selection across a large spatial environmental mosaic along the coast of Australia (Keesing, 2013) as shown for *Strongylocentrotus purpuratus* (Pespeni et al., 2013).

5.3 Materials and methods

5.3.1 Study species, spawning and fertilisation

Heliocidaris erythrogramma was collected (3-5 m depth) near Coffs Harbour, New South Wales (30˚ 15' S, 153˚ 08' E) in April and transferred to large flow through aquaria (3500 L) shortly after collection. Spawning was induced by injection of 2 ml of 0.5 M KCl. Eggs from each female were placed in separate beakers of fresh, filtered seawater (FSW; 1 μm) and sperm from each male was stored dry at 4 ˚C until use. Egg density was determined in counts of 100 μl aliquots from the egg suspension. Approximately 1000 eggs were placed in rearing containers; 100 ml plastic jars, with mesh sides to allow water to flow through. Positioning of the window ensured at least 40 ml of water remained in each container at any time as it was constantly renewed. The eggs were supplied with flowing experimental FSW, with randomly assigned temperature/pH conditions for approximately 20 minutes before sperm were introduced. Haemocytometer counts of semen samples were used to determine the amount of sperm required to achieve a consistent sperm concentration. Just prior to fertilisation, 1 μl of the semen sample was activated in 1 ml of experimental FSW. The amount of diluted sperm to add into each rearing container to achieve a sperm to egg ratio of 200:1 was determined from the original sperm count. Before addition of sperm, the flow through system was turned off to allow fertilisation and turned back on after 10 minutes to remove excess sperm.

5.3.2 Manipulation of temperature and pH

The experiments were conducted in a flow through water system with a flow rate 7.8 mL/min, ambient pH of 8.18, temperature of 23.7˚C, salinity of 33.6 psu, and dissolved oxygen >90%. Experimental treatments were based on model projections for near future (2100) surface ocean waters in the southeast Australia region (IPCC 2013; CSIRO and BOM, 2015). The treatments consisted of two temperatures (control 23.7 ˚C, 26.52 ˚C) and three pH_{NIST} levels (control 8.11, 7.86, 7.7 pH units) in all combinations (Table 5.1). The experiments were conducted in UV sterilised and filtered (1 mm) FSW that was supplied to three 60 L header tanks. The experimental pH was regulated by injection of pure CO_2 into two of these tanks using an automatic CO_2 injection system with two pH controllers (Tunze),

set at pH 7.6 and pH 7.8. The CO_2 was mixed in these tanks using a vortex mixer (Red Sea). A third header tank was allowed to track ambient pH. All header tanks (control and experimental treatment water) were continuously bubbled with air to promote mixing and to maintain dissolved oxygen >90%. A constant volume was maintained in the header tanks using a float valve. Water from the header tanks was fed into sub-header tanks (20 L) where it was warmed to the required temperature (+2.8˚C) using an aquarium heater (200 W, Jager) or unmanipulated for the ambient control. Temperature was automatically regulated using temperature sensors in the rearing containers and a temperature controller (Tunze) connected to the heaters. Water from each sub-header tank was continually circulated using 20 watt pumps to maintain even temperatures within each treatment. Water was delivered individually into individual rearing containers using irrigation dripper valves.

Temperature, pH and salinity were measured daily in all treatments across random containers (n =33 per treatment) using a Hach Hqd Portable Multiprobe. The probe was calibrated frequently using NIST buffers pH 4.0, 7.0 and 10.0 (Oakton) with pH on the total scale determined through calibration with TRIS buffers [mean ± SE pH_T in the three treatments was 8.10 ± 0.01 (control), 7.80 ± 0.00, and 7.63 ± 0.00]. Temperature was also monitored with this meter [mean ± SE temperatures were 23.66 ± 0.08 ˚C (control) and 26.48 ± 0.08 ˚C, see Table 5.1]. Water samples (250 ml) were collected at the beginning and end of the experiment for each treatment, filtered through a 0.45 mm syringe filter, and fixed with 150 µl of saturated HgCl. These water samples (n=12) were then used to determine total alkalinity by potentiometric titration using an automatic titrator (Metrohm 888 Titrando) and calibrated against certified reference standards (Dickson et al., 2007). Experimental pCO_2 (Table 5.1) was determined from TA, temperature, pH_{NIST} and salinity data using CO2SYS (Pierrot et al., 2006) using the dissociation constants of Mehrbach et al., 1973) as refitted by Dickson and Millero (1987).

5.3.3 The North Carolina II breeding design
Single sire-dam crosses were completed in two experimental runs (blocks) with each block using gametes from 2 dams and 4 sires crossed in all combinations. Each block thus resulted in 8 full-sib families (total of 16 families). Each family was exposed to each of 6 treatments. Thus each block had a total of 144 containers (2 females x 4 males x 3 pH levels x 2 temperature x 3 replicates).

Table 5.1. Experimental conditions in experiments with *Heliocidaris erythrogramma*.
Mean values (±SE, n = 33) for pH_{NIST} measured daily per treatment is presented with pH_T (determined using tris buffers) for comparison. pH_T, pCO_2 and the saturation states of calcite (Ω_{ca}) and aragonite (Ω_{ar}) were calculated in CO2SYS using data on DIC and total alkalinity (TA = 2205.2 ± 9.3 µmol/kg, n = 12), salinity (33.6 ± 0.06, n = 14) and temperature for each treatment.

	24 ˚C			27 ˚C		
	pH 8.1	**pH 7.8**	**pH 7.6**	**pH 8.1**	**pH 7.8**	**pH 7.6**
Temp	23.57 (0.08)	23.60 (0.09)	23.82 (0.08)	26.53 (0.06)	26.21 (0.06)	26.70 (0.13)
pH_T	8.10 (0.01)	7.80 (0.00)	7.63 (0.00)	8.12 (0.00)	7.80 (0.00)	7.63 (0.00)
pH_{NIST}	8.18 (0.01)	7.88 (0.01)	7.69 (0.00)	8.18 (0.01)	7.83 (0.00)	7.70 (0.00)
pCO_2	386.8	864.4	1401.1	391.8	1002.6	1400.1
ΩCa	5.21	2.97	2.08	5.78	3.18	2.24
ΩAr	3.41	1.95	1.36	3.82	2.10	1.48

At the control temperature (24 °C), *H. erythrogramma* develops to the juvenile stage within 4 days. At 2 hours post fertilisation (hpf), 24 hpf and 96 hpf, a random sample of approximately 50 embryos was pipetted from the containers, placed into tubes and fixed with 2% formalin in FSW. The first 30–50 embryos randomly selected from each tube were examined microscopically (Leica) and scored for successful development. At 2 hpf, the percentage of fertilised embryos was determined through counts of unfertilised, fertilised and cleavage stage embryos. At 24 hpf, the percentage of larvae was calculated from counts of normal/abnormal and arrested embryos. At 72 hpf, coralline algae (*Amphiroa anceps*) were added to each container to induce the larvae to settle. At 96 hpf, the percentage of metamorphosed larvae was calculated from counts of normal/abnormal juveniles and arrested embryos. The number of embryos arrested at fertilisation (e.g., fertilisation envelope only) was low (<1%) indicating that polyspermy was minimal.

Photographs of juveniles from each replicate across all genotypes and treatments were taken and the number of spines counted as a proxy for calcification for at least 10 individuals per replicate.

5.3.4 Statistical analyses

Data on development for each time point and spines were analysed using analysis of variance (ANOVA) conducted in the PERMANOVA routine of Primer V6 with temperature and pH as fixed factors, experimental block as a random factor, and sire and dam as random factors nested within blocks. Since some significance tests involved quasi F ratios (in which significance tests derived from the F distribution are unreliable (Quinn and Keough, 2002)), we calculated significance of the F statistics using 9999 permutations of the raw data for all factors (Anderson et al., 2008).

Coefficients of variation (CV) were calculated for each treatment across all developmental stages to determine whether stressful treatments can increase the variability in the response of embryos and juveniles. The CV, (defined as the standard deviation divided by the mean and multiplied by 100 expressed as a percentage using the formula: $\frac{\delta}{\mu}$ x 100 where δ = standard deviation, and μ = mean) expresses the relative variability of a measurement and is less likely to increase as an artefact of increases in the mean (Quinn and Keough, 2002).

Linear regression analyses were performed in Microsoft Excel (2013) to assess the relationship between performance of individual male-female pairs across different life history stages; fertilisation and larvae, and larvae and juveniles. Male-female pairs were used for

these analyses due to significant interactions among males, females and stressors (see Results).

5.4 Results

5.4.1 Fertilisation

Increased temperature significantly reduced the percentage of fertilisation with no significant effect of decreased pH (Table 5.2). There was a significant effect of sire identity on the percentage of fertilisation across all environments and a significant interaction between dam identity and temperature (Table 5.2). Reaction norms of maternal half-siblings show that the effect of increased temperature on the percentage of fertilisation differed between the female parents (Figure 5.1).

5.4.2 Larvae

Decreased pH, but not increased temperature, significantly reduced the percentage of normal larvae (Table 5.2). There was a significant dam x temperature interaction indicating that dam identity was an important source of variation in determining the percentage of normal larvae in increased temperature as shown in the reaction norms (Figure 5.1). In addition, the significant interactions between sire x dam and sire x dam x pH in the percentage of normal larvae indicates the importance of parental pair to the developmental success of their progeny. The different responses of the offspring of the 16 pairs to decreased pH are shown in the reaction norms (Table 5.2; Figure 5.2).

5.4.3 The percentage of metamorphosed larvae

On day 4 when the larvae had settled and metamorphosed, there were no significant effects of increased temperature or decreased pH on the survivors that settled (Table 5.2). There was a significant interaction between sire x dam x pH indicating that success to the settled juvenile stage was significantly affected by sire-dam pair as shown in the reaction norms (Figure 5.2). There was also a significant interaction between dam x pH x temperature where increased temperature greatly reduced the percentage metamorphosed with responses dependent on pH level and maternal identity (Figure 5.3).

Table 5.2. ANOVA of percentage of fertilisation, normal larvae and metamorphosed larvae of *Heliocidaris erythrogramma*. ANOVA of data of single dam-sire crosses across temperature (Te) and pH treatments. Temperature and pH are fixed factors, experimental block (Bl) a random factor, and male (Ma) and female (Fe) identity random factors nested within block. Significant effects are shown in bold (P < 0.05).

Source	df	Fertilisation			Larvae			Metamorphosed		
		MS	F	P(perm)	MS	F	P(perm)	MS	F	P(perm)
Bl	1	672.62	0.23	0.93	8122.60	11.77	**0.01**	354.84	0.25	0.92
pH	2	1509.20	6.43	0.22	4213.00	43.21	**0.02**	2648.30	6.44	0.27
Te	1	11666.00	197.66	**0.05**	1.81E+05	15.81	0.25	44312.00	15.52	0.25
Ma(Bl)	6	2725.40	9.93	**0.00**	718.18	0.40	0.89	1206.70	3.55	0.08
Fe(Bl)	2	1324.90	4.83	0.06	125.98	6.97E-02	0.93	1565.90	4.61	0.07
BlxpH	2	234.87	0.65	0.72	97.51	0.28	0.97	411.17	0.48	0.85
BlxTe	1	59.02	0.13	0.98	11419.00	2.26	0.16	2854.90	1.77	0.23
pHxTe	2	809.25	1.86	0.35	3624.40	14.75	0.07	8162.20	5.15	0.17
Ma(Bl)xFe(Bl)	6	274.57	1.02	0.42	1808.60	4.34	**0.00**	339.81	1.00	0.43
Ma(Bl)xpH	12	406.41	1.51	0.25	1239.80	1.45	0.26	1599.00	1.75	0.17
Ma(Bl)xTe	6	430.71	3.39	0.08	1357.60	2.60	0.14	1372.40	3.36	0.08
Fe(Bl)xpH	4	368.38	1.37	0.30	2153.60	2.52	0.10	1155.60	1.27	0.33
Fe(Bl)xTe	2	1008.30	7.94	**0.02**	3931.90	7.54	**0.02**	467.97	1.15	0.38
BlxpHxTe	2	435.40	1.32	0.31	245.81	0.34	0.94	1585.30	0.71	0.68
Ma(Bl)xFe(Bl)xpH	12	269.95	1.00	0.45	853.17	2.05	**0.02**	913.08	2.69	**0.00**
Ma(Bl)xFe(Bl)xTe	6	126.92	0.47	0.83	521.61	1.25	0.28	408.58	1.20	0.31
Ma(Bl)xpHxTe	12	418.05	1.68	0.19	624.95	1.38	0.30	1053.70	1.87	0.15
Fe(Bl)xpHxTe	4	102.50	0.41	0.80	1420.20	3.13	0.06	1986.40	3.53	**0.04**
Ma(Bl)xFe(Bl)xpHxTe	12	249.09	0.92	0.53	453.52	1.09	0.37	562.25	1.66	0.08
Res	192	270.15			416.36			339.44		
Total	287									
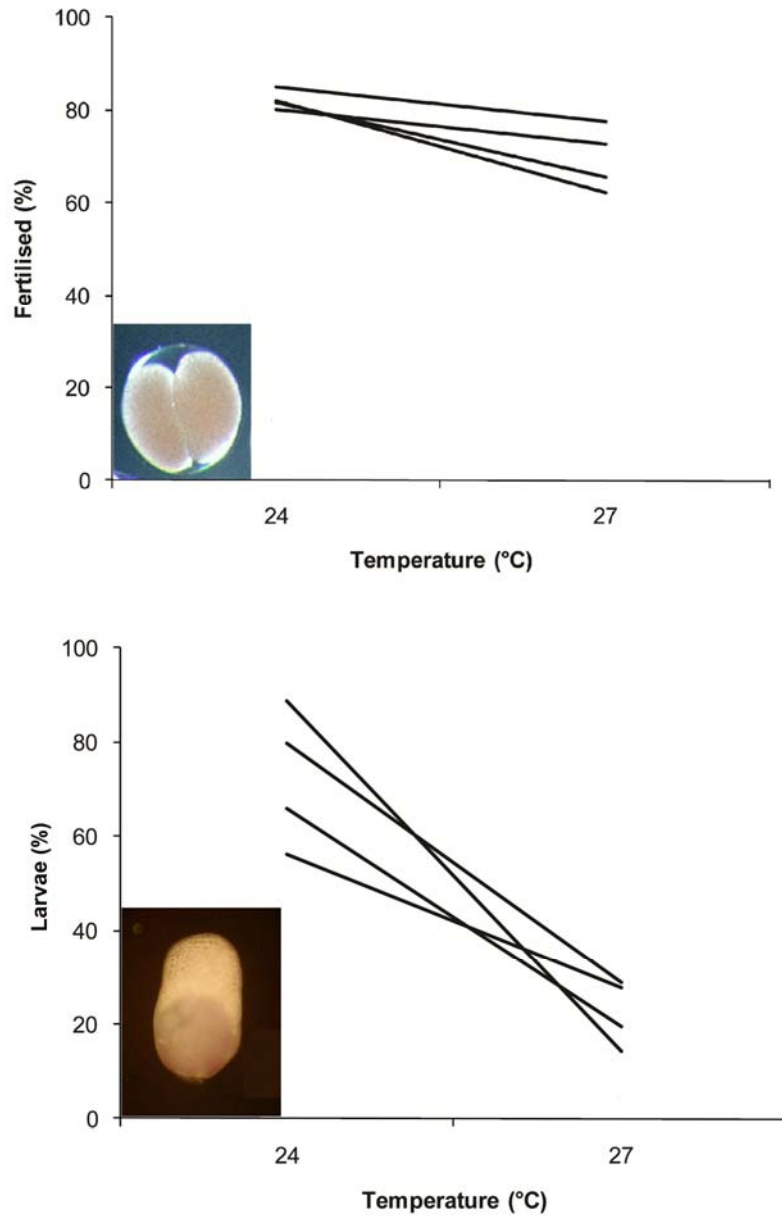
Figure 5.1. Reaction norms showing the percentage of fertilised embryos (top panel) and normal larvae (24 hpf) (bottom panel) of *Heliocidaris erythrogramma* maternal half siblings in response to increased temperature pooled for pH. Lines represent the mean percentage for maternal half siblings (n = 4).
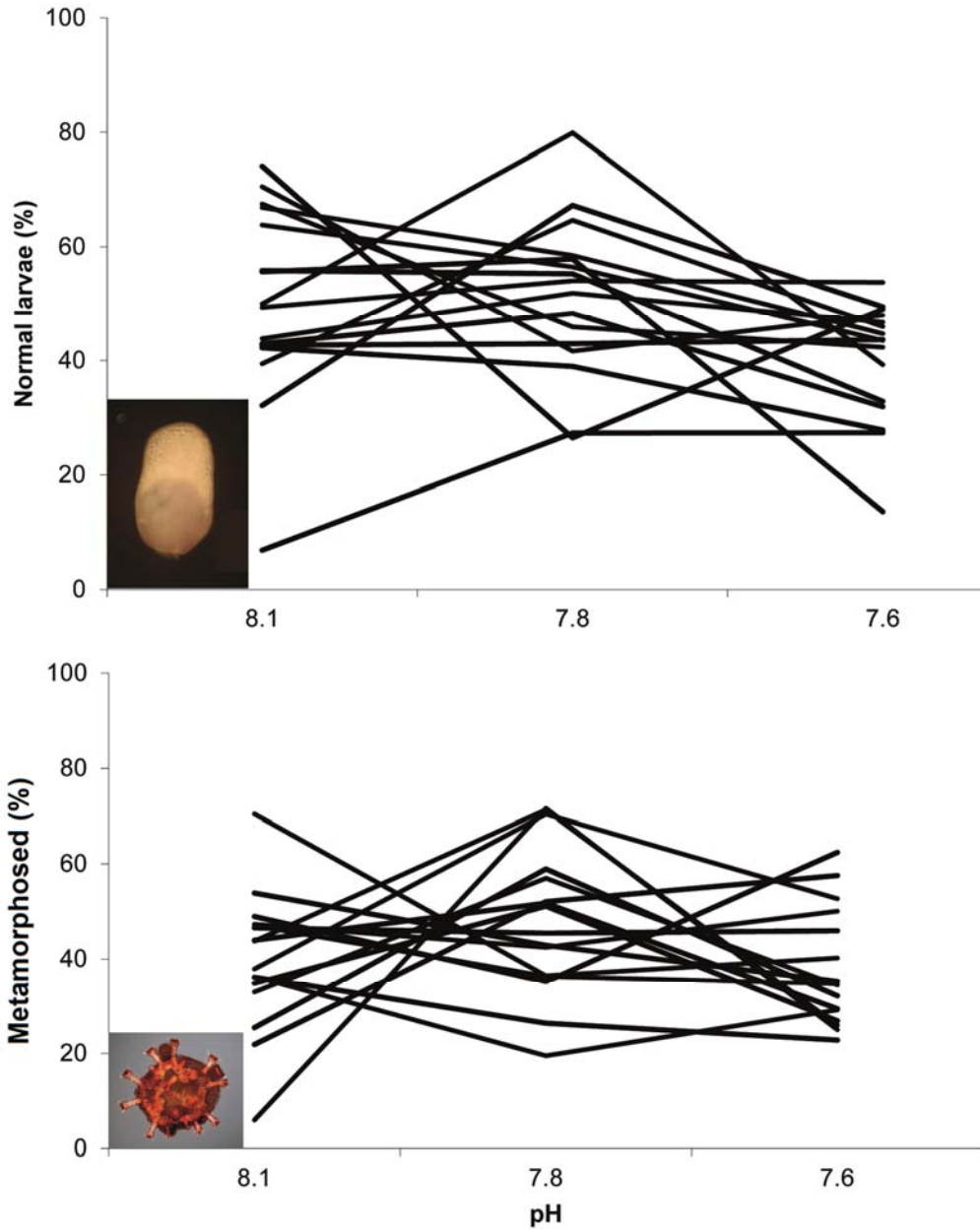
Figure 5.2. Reaction norms show the percentage of normal larvae (24 hpf) (top panel) and metamorphosed juveniles (96 hpf) (bottom panel) across *Heliocidaris erythrogramma* offspring of the 16 sire-dam pairs in response to experimental pH levels pooled for temperature. Lines represent the mean percentage for full siblings (n = 16).
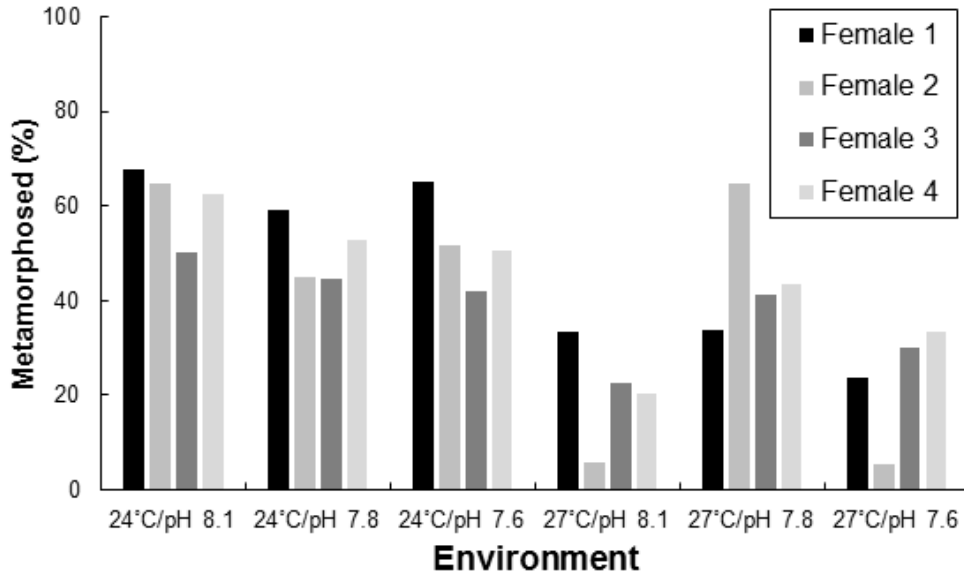
Figure 5.3. Histogram displaying the percentage of metamorphosed juveniles for each *Heliocidaris erythrogramma* female across the six treatments. The significant interaction between female x pH x temperature shows that settlement was influenced by increased temperature and decreased pH however this varied with maternal identity.
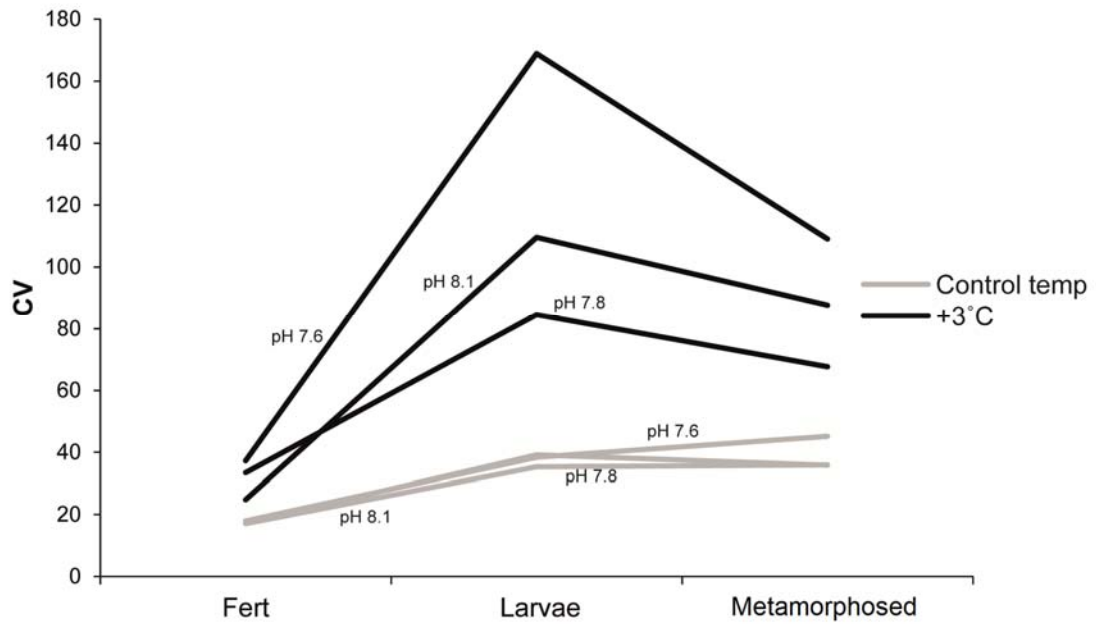
5.4.4 Coefficients of variation

In control temperature, there was only a slight increase in variation from fertilisation to the juvenile stage across all pH levels. Increased temperature greatly increased the variation across all developmental stages. Furthermore, at increased temperature there appears to be a synergistic effect with decreased pH where pH 7.6 increased the variation seen across developmental stages in comparison the control pH of 8.1 (Figure 5.4).

5.4.5 Calcification

There was a significant effect of maternal identity on the number of spines produced per juvenile. There were also significant effects of sire x dam, sire x dam x temp, sire x dam x pH and sire x dam x pH x temp. This indicates the strong influence of parental pair on spine number in response to stressors as shown in the reaction norms (Figure 5.5; Table 5.3).

5.4.6 Performance across life history stages

In none of the six combinations of temperature and pH was there significant relationships between fertilisation success and the percentage of normal larvae (Figure 5.6). Genotypes that had a high fertilisation success did not subsequently have the highest percentage of normal larvae. However, the relationships between percentage of normal larvae and subsequent metamorphosis did show that pairs in certain environments performed consistently (Figure 5.7). Genotypes that had a high percentage of normal larvae in the control pH/27°C and pH 7.6/27°C environments also had the highest percentage of metamorphosed larvae. Thus, performance of specific genotypes at fertilisation did not predict performance of that genotype at the larval stage, however performance at the larval stage did predict metamorphosis in two environments. There was no correlation found for the pH 7.8/27°C environment. With removal of two outliers, a positive correlation became evident but this was not done.

Figure 5.4. Effects of increased temperature and decreased pH on the coefficients of variation of developmental success across fertilisation, larvae and juveniles of *Heliocidaris erythrogramma*.
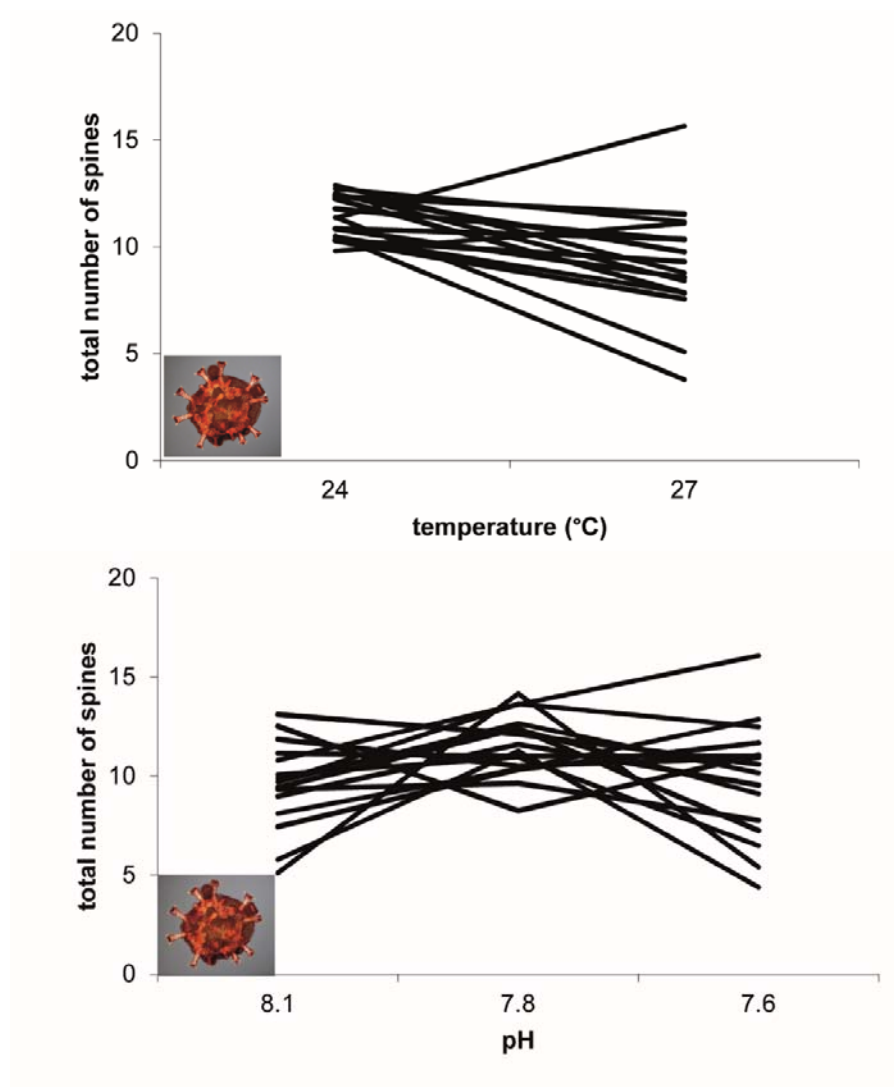
Figure 5.5. Reaction norms showing total number of spines present on *Heliocidaris erythrogramma* juveniles (96 hpf) across offspring of the 16 sire-dam pairs in response to experimental temperatures pooled for pH (top panel) and in experimental pH levels pooled for temperature (bottom panel). Lines represent the mean number of spines for full siblings (n = 16).

Table 5.3. ANOVA of number of spines present on metamorphosed larvae of
Heliocidaris erythrogramma. ANOVA of data of single dam-sire crosses across temperature
(Te) and pH treatments. Temperature and pH are fixed factors, experimental
block (Bl) a random factor, and male (Ma) and female (Fe) identity random factors nested
within block. Significant effects are shown in
bold (P < 0.05).

| Source	df	MS	F	P(perm)
		Spines		
Bl	1	2.38	0.13	0.98
pH	2	113.70	1.56	0.39
Te	1	357.59	79.72	0.07
Ma(Bl)	6	25.94	1.13	0.45
Fe(Bl)	2	170.43	7.43	**0.02**
BlxpH	2	72.87	2.08	0.11
BlxTe	1	4.49	0.51	0.75
pHxTe	2	143.45	3.75	0.21
Ma(Bl)xFe(Bl)	6	22.95	5.53	**0.00**
Ma(Bl)xpH	12	19.92	0.71	0.72
Ma(Bl)xTe	6	34.93	1.02	0.49
Fe(Bl)xpH	4	28.69	1.02	0.43
Fe(Bl)xTe	2	41.44	1.21	0.36
BlxpHxTe	2	38.25	2.32	0.09
Ma(Bl)xFe(Bl)xpH	12	28.10	6.77	**0.00**
Ma(Bl)xFe(Bl)xTe	6	34.29	8.27	**0.00**
Ma(Bl)xpHxTe	12	9.63	0.26	0.99
Fe(Bl)xpHxTe	4	23.23	0.61	0.66
Ma(Bl)xFe(Bl)xpHxTe	12	37.81	9.12	**0.00**
Res	192	4.15		
Total	287			
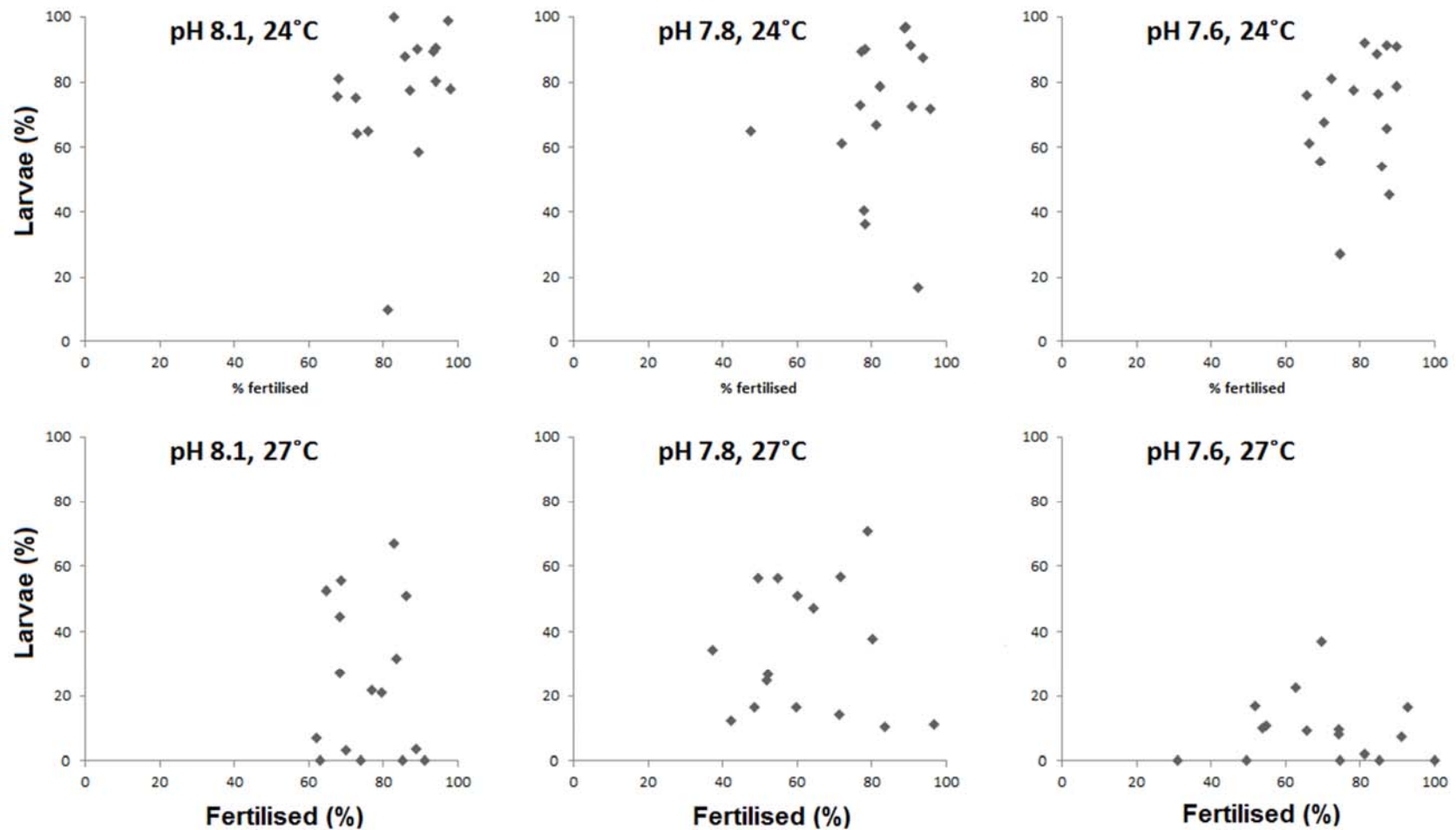
Figure 5.6. Scatter plots of the relationship between pair performance at fertilisation (y-axis) and at the larvae stage (x-axis) of *Heliocidaris erythrogramma*. Each point represents the mean performance of an individual pair in each treatment across both stages. No relationships were evident for any of the six treatments.
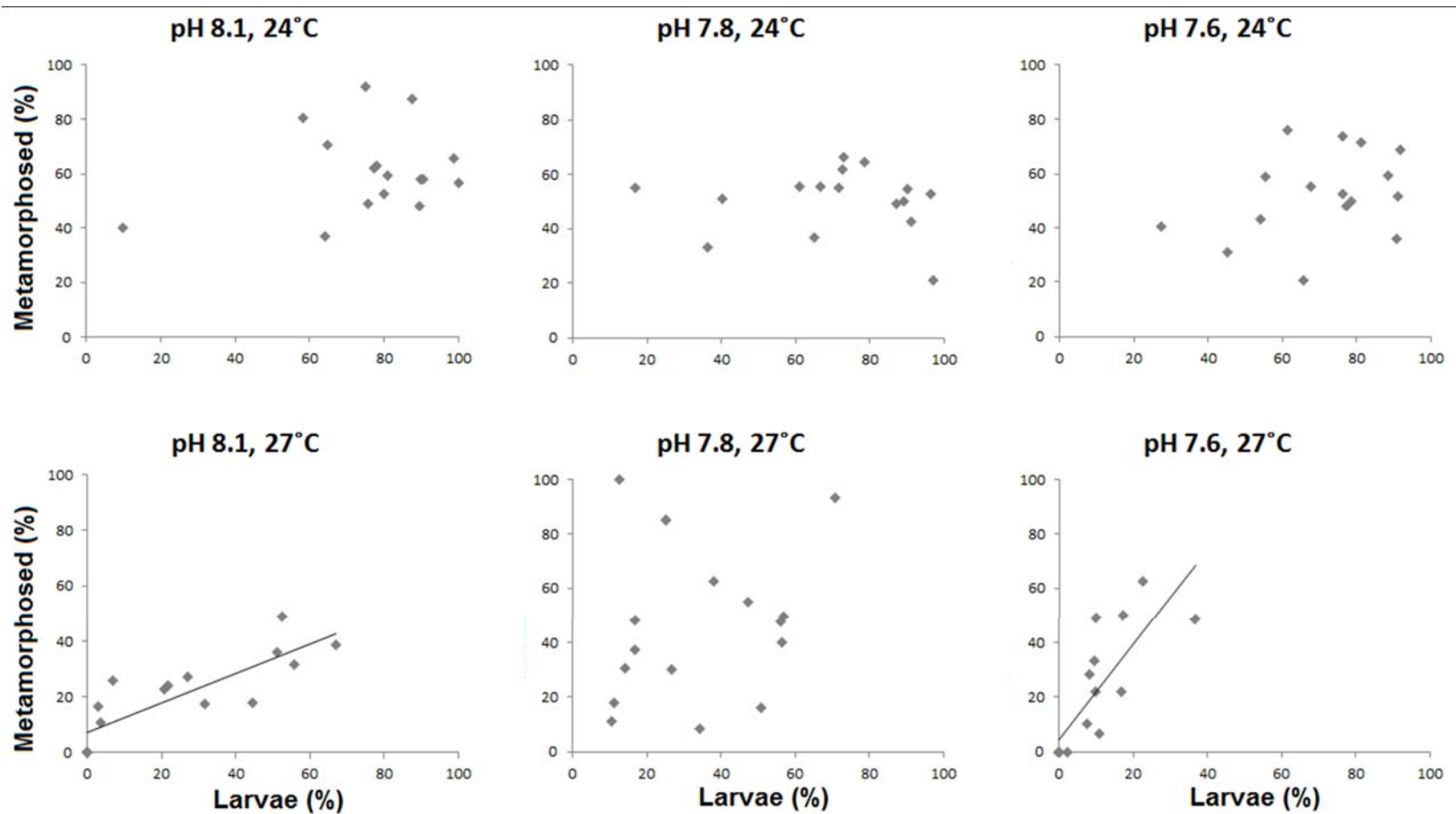
Figure 5.7. Scatter plots of the relationship between pair performance at the larval stage (y-axis) and as metamorphosed larvae (x-axis) of *Heliocidaris erythrogramma*. Each point represents the mean performance of an individual pair in each treatment across both stages. A positive relationship was evident for the pH 8.1/27°C ($R^2 = 0.69$, P = 0.000) and pH 7.6/27°C treatments ($R^2 = 0.64$, P = 0.000).

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5.5 Discussion

Across the genotypes tested, fertilisation was negatively affected by increased temperature, but not by decreased pH. Larval development was compromised in decreased pH, but not by increased temperature. By the juvenile stage, no impact of warming or acidification was evident, likely due to selective mortality of sensitive individuals and resilience of the survivors. Across all environments tested, the juveniles exhibited a similar ability to calcify. Maternal identity and parental pair exerted significant influences on how developmental success in *H. erythrogramma* was affected by environmental stress. That the progeny of some sire-dam pairs showed high stress tolerance indicates that the survival of resistant genotypes could facilitate the persistence of *H. erythrogramma* populations under stressful conditions.

At fertilisation, the significant contribution of sire effects were expected because fertilisation success in *H. erythrogramma* and other sea urchins is significantly influenced by sperm traits such as motility, velocity and viability (Gage et al., 2004; Evans & Marshall, 2005; Garcia-Gonzalez & Simmons, 2005; Evans et al., 2007). The significant dam x temperature interaction at fertilisation, indicates that eggs of different females were differently affected by warming, where eggs of some females were less affected by +3°C with respect to fertilisation success.

The differences in the effects of each stressor varied among females and this remained throughout development of *H. erythrogramma*. Strong maternal effects at fertilisation were anticipated due to known variability in egg size, quality and maturity, attributes which have been shown to be important sources of variation in *H. erythrogramma* and other sea urchins (Styan, 1998; Marshall et al., 2004; Levitan, 2006). The maternal legacy that continues onto larvae and metamorphosis may be due to the presence of maternal protective factors (e.g. stress proteins) loaded into sea urchin eggs during oogenesis (Hamdoun & Epel, 2007). In addition*, H. erythrogramma* produces a large egg that provides all the nutrition needed to support development to metamorphosis and eggs are also supplied with maternal transcripts to facilitate rapid development (Raff & Byrne, 2006). Over evolutionary history, lecithotrophic larvae have been shown to be more resilient to extinction driven by climate change (Uthicke et al., 2009). Significant maternal provisioning, as in *H. erythrogramma*, may provide a strong buffer against stressors (Hamdoun & Epel 2007; Byrne, 2011).

The interaction of sire and dam with stressors becomes apparent after fertilisation and are influenced by gamete compatibility. At the larval and juvenile stages, the progeny of certain pairs were sensitive and others less sensitive to warming and acidification. Pairs that perform better are likely to be selected for in changing ocean conditions (Hoffmann &Parsons, 1991; Gassmann et al., 2009; Ghalambor et al., 2015).

The effects of stressors on calcification were influenced by sire-dam combination. The lack of an overall effect of pH or temperature on calcification across all genotypes tested is similar to that found in other studies of *H. erythrogramma* (Byrne et al., 2009; Byrne et al. 2011; Wolfe et al., 2013). The resilience of the juvenile stage is likely due to selective mortality of sensitive individuals at the larval stage. Due to the flow through conditions, dead offspring would have been washed from the system. To discern how differential mortality could have affected the outcome, we would have had to track a known population of individual *H. erythrogramma* as in Byrne et al., (2010b) where increased temperature caused ~70% mortality in the larvae. That a subset of resilient progeny became juveniles and were able to calcify as normal shows the potential for persistence of this species under stressful conditions. However, survivorship data are needed to more fully understand the influence of sensitive and resistant genotypes on overall adaptive capacity.

The ranking of pair performance across life history stages did not show consistent performance between the fertilisation and larval stages. However, when comparing pairs from larvae to metamorphosis, the pairs that performed the best at the larval stage performed the best at metamorphosis in two of the high temperature treatments. This may suggest that increased temperature can impose selection on specific genotypes, possibly revealing pairs that are likely to be selected for under future ocean warming (Ghalambor et al., 2015).

A marked increase in variation among genotypes occurs with an increase in temperature. The coefficients of variation for each trait also showed a slight increase in variation with development, well known for development in *H. erythrogramma* and other marine invertebrates (Pechenik, 1987). When decreased pH is considered at control temperatures, the variability in the progeny's response is not changed from the control response. However at increased temperature, decreased pH of 7.6 causes an even greater increase in variation. This indicates that at pH 7.6 only, the synergistic effects of increased temperature and decreased pH may make the probability

of success more unpredictable as development progresses, resulting in a larger selection pressure on genotypes (Hoffmann & Merilä, 1999).

Although early development is impacted by decreased pH and increased temperature, by metamorphosis, individuals in the extreme treatments appear similar to those in the control in both development and number of spines. The resilience of *H. erythrogramma* to ocean stressors tested here may be due to the presence of genetic variation across the metapopulation of this species (Sanford & Kelly, 2010). The broad distribution and abundance of *H. erythrogramma* in a variety of intertidal and subtidal environments indicates that this species has a wide environmental tolerance, and if there is local adaption to areas of differing conditions, then gene flow among populations is likely to contribute to the levels of genetic variation in stress tolerance within a given population (Byrne et al., 2010b).

Our results indicate that the effects of environmental stressors and contributions of sire and dam change throughout the life cycle of a sea urchin. For *H. erythrogramma*, maternal and parental pair effects have the strongest influence on the outcome of fertilisation and development. In the face of a warming and acidifying ocean, maternal buffering and sire-dam pairs with high tolerance to stressors will allow for adaptation in this species. These are important mechanisms in persisting through an ocean decreasing in pH and warming for species like *H. erythrogramma* that have a significant maternal investment in production of large eggs.

CHAPTER SIX: GENERAL DISCUSSION

Anthropogenic emissions of carbon dioxide (CO_2) have enhanced the greenhouse effect causing an increase in both atmospheric and sea surface temperature (IPCC, 2013). Oceans have acted as a sink for excess CO_2 with absorption of over 40% of these emissions (Zeebe et al., 2008; IPCC, 2013). This is causing oceans to simultaneously increase in temperature, decrease in pH, increase in partial pressure of CO_2 (hypercapnia) and decrease in $CaCO_3$ saturation (Kerr, 2010; Howes et al. 2015). The rate of ocean warming and acidification differs among regions and is influenced by ocean circulation, coastal processes and ocean chemistry (Poloczanska et al., 2007). Marine animals along the east Australian coast are particularly vulnerable as this region is a climate change hot spot due to ocean warming faster than the global average (Wu et al., 2012; Poloczanska et al., 2013). Additionally, the Southern Ocean is the world's fastest acidifying marine system and is predicted to reach a CO_2 concentration of 1000 ppm by 2100, equivalent to a drop in 0.4 pH units (IPCC, 2013).

Gametes and early embryogenesis form the foundation developmental stage for population persistence (Pechenik, 1987; Byrne, 2010). As marine invertebrate gametes and embryos are vulnerable to changes in pH and temperature of surrounding waters, this thesis used free spawning echinoids as model species to identify effects of ocean change stressors on the egg and investigate the genetic basis of resistance to climate change stressors through application of an animal breeding design.

6.1 Impacts of ocean acidification on the extracellular jelly coat of the egg

The jelly coat of the egg exhibited variable vulnerability to ocean acidification across species, and within species. Eggs are not created equal with respect to egg size and jelly coat sensitivity. The jelly coat around the eggs of some females might be better able to endure effects of low pH contributing to a more resilient fertilisation response in ocean acidification conditions. If this is a heritable trait, an acidifying ocean might select against the more susceptible phenotypes (Foo et al., 2012; Schlegel and Havenhand, 2012, Foo et al., 2014). Thus environmental stressors such as decreased pH, which have strong effects on jelly coat size, may induce strong selection on marine species that have egg coats as ocean pH changes (Shu et al., 2015).

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The different responses of the jelly coat to decreased pH across species indicates that egg incubation time in experimental treatments may be a previously unappreciated source of variance in ocean acidification fertilisation studies. The differences in egg and jelly coat size between females of the same species may contribute to the strong maternal effects seen in the fertilisation response to ocean change scenarios seen here and in other studies (Evans and Marshall, 2005). The strong sire, dam and sire-dam pair effects found across fertilisation for species tested indicates that both individual effects of stressors on gametes (e.g. jelly coat) and gamete compatibility is important in determining fertilisation success. Thus for experiments investigating acclimatisation and adaptive potential of marine invertebrates to ocean change stressors, it is essential that gametes are fertilised in experimental treatments. This thesis presents studies that are the first quantitative genetics studies to fertilise animals in experimental seawater levels (excluding *Sterechinus neumayeri* experiments). This is a novel feature, currently not done in any other marine climate change studies that use the North Carolina II breeding design. It is clear that to reflect real world scenarios, quantitative genetic selection tests need to incorporate the gametes and gamete compatibility traits, allowing selection to occur from the outset of development.

6.2 Investigating the adaptive potential of sea urchins across latitudes, the responses of polar, tropical and temperate species

The levels of phenotypic plasticity and genetic variation in natural marine populations for traits critical for survival and reproduction in future ocean climates remains largely unknown (Munday et al., 2013; Sunday et al., 2014). In response to climate change, marine species can tolerate change through their existing genotype, adapt genetically, migrate or become extinct (Sultan, 2007; Przelawski et al., 2008).

Plastic responses in the parents can alter offspring development, a form of developmental plasticity through the influence of environment on gamete quality (Ghalambor et al., 2007). This "gamete imprinting," is particularly important with respect to the egg, as exemplified by loading of protective factors e.g. hsps in the egg by the mother (Hamdoun and Epel, 2007) and can allow a population to persist in a changing environment (Merilä, 2012).

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Variable conditions experienced by species across their range can result in the presence of locally adapted genotypes (Sanford & Kelly, 2010). This can provide considerable genetic variation across the species metapopulation. Species with a broad latitudinal distribution across a range of thermal or pH environments are likely to have an in-built capacity to persist in changing oceans (Bradshaw and Holzapfel, 2001).

6.2.2 The Antarctic sea urchin Sterechinus neumayeri

For the polar sea urchin *S. neumayeri*, maternal effects and sire-dam effects observed may buffer early development in an ocean decreasing in pH and increasing in temperature. Positive correlations were found across life history stages across all six treatments which shows that performance of pairs at one developmental stage was a good predictor for later development. This supports the long standing hypothesis that that best performing genotypes may have already been selected for in this species (Marshall and Keough, 2008). This may be due to evolution in stable environments where long generation times and slow growth of Antarctic invertebrate species have created populations with low genetic diversity (Peck 2005; Pörtner 2007).

Antarctic marine species are assumed to have narrow adaptive capacity due to environmental thermal stability over evolutionary timescales (Somero, 2009; Enzor et al., 2013). This could explain why no significant sire x stressor interactions influenced the response of *S. neumayeri* to warming and acidification. Species deficient of flexibility in their response to stressors have increased vulnerability to becoming extinct in future ocean change conditions. Maternal effects will need to be significant in buffering *S. neumayeri* embryos through phenotypic plasticity. A caveat for the inferences made here is that development only to the blastula stage was investigated (first 72 hours) but *S. neumayeri* has a long pelagic larval duration of up to 115 days (Bosch et al., 1987). Thus it is not known if genetic variation could be present in the response of later development to stressors.

6.2.3 The tropical sea urchin Pseudoboletia indiana

For the tropical sea urchin *P. indiana*, inherent genetic variation as well the positive effect of increased temperature countering the effects of low pH will facilitate persistence of the species in its current range and potentially expansion of populations

in NSW. This strengthens the notion that multistressor studies are required to better represent real life scenarios (Byrne 2012; Przeslawski et al. 2015) as a single stressor ocean acidification study may have concluded high vulnerability of *P. indiana* to future ocean change.

When comparing the performance of pairs across fertilisation and gastrulation, there was a positive relationship for the control environment, and the two treatments with combined pH and increased temperature. Pairs that had the highest fertilisation success in these environments also had the highest percentage of normal gastrulae. However, when decreased pH was considered in isolation, this did not hold true for pairs with performance being unpredictable. Here, we found that decreased pH had the potential to make compatible pairs incompatible. Thus these results run counter to the hypothesis that performance at one stage can predict performance later further on in development (Marshall and Keough, 2008), especially when ocean change stressors are considered.

Pseudoboletia indiana displays the largest latitudinal distribution of the species examined and showed heritable genetic variation in response to stressors. Species with large ranges may be winners in a changing ocean as seen for other marine invertebrates with large distributions (Pespeni et al., 2013; Garcia Molinos et al., 2015). However as our experiments were conducted in NSW, a region in which *P. indiana* has recently expanded (Pope, 1964), these experiments should be conducted in the natural, tropical range of *P. indiana* to identify whether local adaptation has occurred in NSW.

6.2.4 The temperate sea urchin Heliocidaris erythrogramma

For the temperate sea urchin *H. erythrogramma*, inherent resilience likely due to preadaptation to a habitat which highly fluctuates in temperature and pH levels will facilitate survival. This species also shows a resilient response of the egg jelly coat to decreased pH as well as survival of resilient juveniles in ocean stressor scenarios.

The ranking of pair performance across life history stages did not show consistent performance between fertilisation and larvae. However, when comparing pairs from larvae to metamorphosis, pairs that performed the best at the larval stage, performed the best at metamorphosis, but only in two of the high temperature treatments. Therefore increased temperature may impose greater selection on specific

genotypes in comparison to low pH (Ghalambor et al., 2015). These results are similar to that of *P. indiana* where in increased temperature scenarios, the more resilient phenotypes performed best across both stages.

Thus, the assessment of ocean change stressors across a range of life-history stages is essential in identifying where the vulnerabilities might lie in adaptation to ocean change. One can not necessarily predict the good performing genotypes based on performance at fertilisation and early development. This is challenging for species with very long generational times, i.e. *S. neumayeri*, so *H. erythrogramma* represents a perfect model system for assessing effects across the life cycle.

6.3 Winners and losers in a changing ocean

Future ocean warming and acidification is going to result in range shifts, extinctions and invasions affecting overall ecosystem function (Brierley & Kingsford, 2009; Burrows et al., 2014; Sunday et al., 2014). Across polar, tropical and temperature sea urchins, the mechanisms that may facilitate persistence in a changing ocean differ, revealing the potential winners and losers (see Table 6.1 for summary). For *S. neumayeri*, no additive genetic variation was present in the response of early embryos to ocean change stressors. Phenotypic plasticity through maternal effects, and non-additive sire-dam effects will be essential in buffering development as the ocean increases in temperature and acidification. However in the long term, adaptation may be required for population persistence (Hoffmann and Parsons, 1991). Thus *S. neumayeri* represents the most vulnerable species examined.

Pseudoboletia indiana shows a great susceptibility to decreased pH. Ocean acidification could change normal fertilisation dynamics rendering compatible pairs incompatible. These effects could be due to a sensitive egg jelly coat but this remains to be examined. Investigating the effects of stressors on the egg cell should be a priority to determine factors that can drive reduction in fertilisation success in a changing ocean.

Pseudoboletia indiana covers the largest latitudinal distribution and therefore largest temperature gradients, a factor likely to influence its resilient response to increased temperature. *Pseudoboletia indiana* was the only species examined here that showed heritable genetic variation in responses to stressors. Increases in temperature

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Table 6.1. Summary of quantitative genetics experiments with *Sterechinus neumayeri*, *Pseudoboletia indiana* and *Heliocidaris erythrogramma*. As studies differed in the developmental stage they were collected, results have been separated into prezygotic and postzygotic development. The table contrasts the responses of the difference species regarding: (1) overall effects of increased temperature and decreased pH on prezygotic development, (2) whether there were significant contribution of sire and dam to performance at prezygotic development, (3) whether sire and dam compatibility was important for fertilisation success, (4) overall effects of increased temperature and decreased pH on postzygotic development, (5) whether there were significant sire x stressor interactions and hence the presence of additive genetic variance, (6) whether there were significant dam x stressor interactions and lastly (7) whether sire x dam interactions affected postzygotic development.

	Sterechinus neumayerii	*Pseudoboletia indiana*	*Heliocidaris erythrogramma*
	Prezygotic development		
Effects of pH/temperature	Decreased pH decreased % cleavage stage embryos with a significant interactive effect with increased temperature	Decreased pH decreased % fert	Increased temp decreased % fert
Contributions of sire and dam	Significant dam contribution	Significant sire and dam contributions	Significant sire contributions
Sire/dam compatibility	Sire/dam compatibility important	Sire/dam compatibility important in stressor scenarios	Sire/dam compatibility **not** important
	Postzygotic development		
Effects of pH/temperature	Decreased pH decreased % blastulae	Increased temperature increased the % of normal gastrulae	Decreased pH decreased % larvae with no effects of stressors at metamorphosis
Sire x stressor interactions	None	Sire x temp	None
Dam x stressor interactions	Dam x temperature	Dam x temp	Dam x temp x pH
Sire x dam interactions	None	Sire x dam x temp Sire x dam x pH Sire x dam x temp x pH	Sire x dam x pH

and its alleviation of the negative effects of decreased pH will allow persistence and expansion of populations in NSW.

For *H. erythrogramma*, inherent resilience is likely due to preadaptation to a habitat which highly fluctuates in temperature and pH levels. The extracellular jelly coat of *H. erythrogramma* was unaffected by a decrease in pH showing a strong resilience of the egg to ocean change. Strong maternal effects found throughout the entire life cycle will be an important mechanism in persisting through a changing ocean, especially for lecithotrophic species like *H. erythrogramma* that have a significant maternal investment in production of large eggs, a trait already shown to have buffered species through past climate change extinction events (Uthicke et al., 2009). The robust response of *H. erythrogramma* to end of century predictions suggest that this species will be a winner, with persistence of populations across Australia and potential for northern populations to supplement populations at the southern end of its range (Byrne et al., 2010).

Results from this thesis suggest that animals from fluctuating intertidal environments and broad latitudinal distribution across a range of thermal or pH environments are likely to have an in-built capacity to persist in changing oceans (Bradshaw and Holzapfel, 2001; Pespeni et al., 2013). On the other hand, polar species may show a reduced capacity to persist in changing oceans.

Studies which investigate the contributions of genetic and environmental variance to progeny performance are important in identifying resilient genotypes for genetic rescue. The main purposes of genetic rescue are to restore genetic diversity in populations that are small and isolated (Whiteley et al., 2015). Free spawning marine invertebrates provide an ideal model for quantitative genetics using separate male x female pairs to determine the potential that more resilient pairs could seed future populations in a changing ocean (Foo et al. 2012; Schlegel et al., 2012; Foo et al. 2014. Genetic improvement of many plants and animals has been utilised for many years, and could help augment the capacity of corals and other ecologically and economically significant species to endure a changing climate (Van Oppen et al., 2015).

The results of this thesis show that considering only genetic variation in adaptive capacity studies might not reveal true population persistence, and effects of stressors may be overestimated (Sunday et al., 2011; Thor and Dupont, 2015). Phenotypic change

in a population might not always involve adaptive evolution and be entirely mediated by non-genetic factors (Bonduriansky and Day, 2009). Plastic responses, as seen for *H. erythrogramma*, can allow populations to persist in a changing environment, especially those changing too fast for genetic adaptation (Lloyd Morgan 1896; Merilä, 2012).

6.4 Future directions

As highlighted by Chapter Two, the jelly coat of the egg can be greatly affected by ocean change stressors with potential flow on effects for fertilisation. Therefore this is an important aspect to consider in future climate change studies. As exemplified by Chapters Three to Five, discerning variation into paternal, maternal and environmental effects is challenging. The field of quantitative genetics was originally developed for agriculture with high numbers of replicates and therefore applying the same statistical tests may mean that important effects remain undetected as the tests may not be able to account for much lower sample sizes. Furthermore, the computation of heritability estimates is not so straightforward, and the appropriate statistics need to be developed to consider multistressor environments.

Future research is moving towards multistressor, long term studies which transcend generations. Parental exposure to climate change can cause transgenerational changes that allow offspring to endure stressors, with carry over effects persisting over later life-history stages and multiple generations. To estimate the adaptive potential of marine species to a changing climate, there is a need for long term, multigenerational experiments which capture developmental plasticity, genetic variation and transgenerational effects. It will be essential to measure multiple traits related to morphology and physiology, lethal and sub lethal effects and to quantify corresponding changes in the transcriptome, proteome and metholome. Ultimately, a combined approach will be most informative in making informed predictions of how the seascape, marine communities and ecosystems will be altered by climate change. This is needed to empower development of effective management strategies to protect marine resources.

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APPENDIX ONE: CONTRIBUTION OF EACH AUTHOR TO MANUSCRIPT

Experimental design

Shawna Foo, Symon Dworjanyn, Alistair Poore and Maria Byrne all contributed to the design of the experiment.

Materials

Reagents, materials and analysis tools were provided by Maria Byrne, Alistair Poore and Mehar Khatkar.
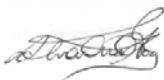
Experiment execution

The experiments were performed by Shawna Foo at the Sydney Institute of Marine Science. This involved single dam-sire crosses of 2 female and 4 male sea urchins, with embryos fertilised in 6 different pH/temperature treatments. This was repeated 2 times, with embryos collected at two different developmental stages. At the end of the experiment, we had data on the percentage of normally developing embryos at two different stages for 16 different genotypes across 6 different treatments.

Statistical analysis

Data on development for each time point were analysed by Shawna Foo using analysis of variance (ANOVA) conducted in the PERMANOVA routine of Primer V6. Variance components were calculated by Alistair Poore in R to allow calculations of genetic correlations. Heritability estimates were calculated by Mehar Khatkar.

Manuscript

The paper was written by Shawna Foo with contributions from Alistair Poore, Symon Dworjanyn, Maria Byrne and Mehar Khatkar. All authors contributed to editorial revisions.

_____   25th August 2015

Shawna A. Foo   Date

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