Oxidative stress responses in Echinoderm embryos in response to ocean acidification.

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Abstract

Ocean acidification refers to the ongoing decrease in the pH of the Earth's oceans, caused by the uptake of carbon dioxide from the atmosphere. This ocean acidification is considered to be a threat to marine organisms and is suspected to act as a physiological stressor especially in Antarctic marine environments where the effects of ocean acidification will be first observed. There have been few studies that have investigated the effects of ocean acidification on key physiological processes. To address this gap in the knowledge base of climate change related physiological responses this thesis aimed to investigate the effects of ocean acidification on oxidative stress responses in the embryos of 2 polar echinoderm species, *Sterechinus neumayeri* and *Odontaster validus* and a temperate species *Patiriella regularis*. Embryos and early larval stages are investigated because these early life history stages are particularly vulnerable and are a potential bottleneck for wider population ecology.

Oxidative stress has been selected as a biomarker for stress because it is a highly conserved physiological process that is common in all life. Oxidative stress is the production and accumulation of reactive oxygen species beyond the capacity of an organism to quench those reactive species using antioxidants. Failure to quench these reactive oxygen species results in the damage of lipids, proteins and DNA. In this study I measured the concentration of lipid hydroperoxides, protein carbonyls and 8-OHdG in DNA as biomarkers for oxidative damage. In response to decreased seawater pH the antioxidant molecules superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPOX), glutathione S-transferase (GST), total glutathione and the % of reduced glutathione in embryos were measured.

To test the hypotheses that decreased seawater pH will cause oxidative damage and secondly that this damage will elicit an antioxidant response in *S. neumayeri*, *O.* validus and *P. regularis* embryos, freshly fertilised eggs were exposed to 3 seawater pH treatments, pH 8.1 (ambient seawater), pH 7.8 (IPCC 100 year scenario) and pH 7.6 (IPCC 200 year scenario) and left them to develop until the late blastula phase. It was found that development within seawater with a decreased pH significantly increased levels of protein carbonyls, lipid hydroperoxides and 8-OHdG, increased abnormality and the cross sectional area of *S. neumayeri* blastula. In response to our second hypothesis, *S. neumayeri* blastula had significant increases in SOD, CAT, GR, GPOX, GST and had a significant decrease in the total amount of glutathione. *O. validus* showed a significant increase in the amount of protein carbonyls and had a significant increase in the

amount of GR. The baseline levels of antioxidants were higher than those expressed by *S. neumayeri*. For *P. regularis* there was a significant increase in the proportion of abnormal blastula. The size of the blastula that developed in seawater with a decreased pH was also significantly smaller. *P. regularis* blastula showed significant increases in lipid hydroperoxides and protein carbonyls. As well as *O. validus*, *P. regularis* had high baseline levels of antioxidants. *S. neumayeri* embryos appear to be vulnerable to oxidative stress caused by decreased seawater pH whereas the high levels of antioxidants within *O. validus* and *P. regularis* appear to buffer these species to the effects of oxidative stress caused by decreased seawater pH.

Marine stressors seldom act on marine organisms in isolation and this thesis also investigates the interaction of ocean acidification and ultraviolet radiation (UV-R) on oxidative stress in *S. neumayeri* embryos. In response to our hypothesis that decreased seawater pH would will be additive with exposure to a secondary stressor of UV-R freshly fertilised eggs were exposed to 3 pH treatments (pH 8.1, 7.8 and 7.6) and after 7 days of development the blastula were exposed to a 1 hour light treatment that either blocked UV-A and UV-B light or exposed blastula to PAR, UV-A and UV-B that was similar to 1 hour on the surface of the sea ice near Ross Island, Antarctica. These were preserved to measure oxidative damage and antioxidant defence. It was observed that there was a significant increase in the size of embryos that were exposed to UV-R. Lipid hydroperoxides and 8-OHdG increased with exposure to both seawater with a decreased pH and UV-R. Protein carbonyls increased with exposure to decreased seawater pH, UV-R and there was a significant interaction where the increase in protein carbonyls was greater than expected with exposure to pH 7.6 and UV-R. The antioxidant response included significant increases in SOD, GR and GPOX with a significant interaction caused by a decrease in antioxidants in the pH 7.6/UV-R treatment. CAT significantly increased with exposure to UV-R with a significant interaction again in the pH 7.6/UV-R treatment. GST and total glutathione only increased with exposure to decreased seawater pH. The percentage of reduced glutathione increased with exposure to UV-R. Seawater with a decreased pH appears to increase the amount of oxidative stress inflicted when there is a short exposure to UV-R than either stressor causes individually.

In conclusion seawater with a decreased pH caused oxidative stress in *S. neumayeri* and the addition of UV-R stress was additive. There was some oxidative damage in *O. validus* and *P. regularis* however high baseline antioxidants may increase the resilience of these species.

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When I began the journey that is a Masters of Science I never fully realised the enormity of the task that was to become this thesis. It is probably for this reason that this degree has taken me longer than I anticipated to complete. This thesis and I has had a love hate relationship but I feel all the richer for finally beating this into submission, finally. I am so thankful for all those that have contributed to this research presented here. Without your help and support this thesis would have never been completed and I would like to give thanks.

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Chapter 1 General introduction

This thesis investigates the effects of ocean acidification on the production of reactive oxygen species and antioxidant defence responses of Echinoderm larvae from temperate and polar waters to assess how these are responding physiologically. The species used in this study are the Antarctic echinoid *Sterechinus neumayeri* and two asteroid species *Odontaster validus* from Antarctica, and *Patiriella regularis* from temperate New Zealand. Ocean acidification refers to the on-going decrease in the pH of the Earth's oceans, caused by the uptake of carbon dioxide from the atmosphere (IPCC, 2013). Using oxidative stress as a biomarker for stress is not a novel approach in the field of ecotoxicology and has been used in plants, fish, birds and invertebrates (Abele & Puntarulo, 2004; Hader et al., 1998; Janssens et al., 2000; Lesser et al., 2001; Lesser, 2006; Livingstone et al., 1992; Lister et al., 2010, 2015; Regoli et al., 2011; Viarengo et al., 1998). Oxidative stress, however, has seen limited use to measure the effects of ocean acidification in marine systems outside of coral species (Kaniewska et al., 2012). The motivation for the research responds to multiple review articles that point to a lack of knowledge on the physiological responses to ocean acidification and UV-R as singular and combined stressors (Pörtner, 2008; Dupont & Pörtner, 2013; Häder et al., 2014). By measuring the oxidative stress responses that can be elicited by environmental changes in the laboratory, it was possible to gain an understanding of how the organisms will be physiologically affected by climate change (Halliwell, 2007). This is particularly relevant for Antarctic species that are already living in a potentially pro-oxidative environment with of high levels of dissolved oxygen and more polyunsaturated fatty acids in cellular and mitochondrial membranes and a decreased mitochondrial functioning caused by the cold temperatures (Lesser, 2006; Regoli et al., 2011; Abele & Puntarulo, 2004).

Anthropogenic climate change

Benthic marine invertebrates live in an environment where multiple stressors are present and will continue to be exacerbated by anthropogenic climate change and societal pressures on ecosystems. Anthropogenic induced stressors include, but are not limited to, temperature changes, oxygen fluctuations, ultraviolet radiation, pollution, fertilizer loading in coastal environments and nutrient limited environments (Byrne, 2012; IPCC, 2013). The steady increase of greenhouse gases since the industrial revolution is driving increasing mean global temperatures and increasing the concentrations of carbon dioxide (CO_2) in the ocean (Meehl et al., 2007; IPCC, 2013; Fabry et al., 2009). Such stressors will reduce the survival of organisms, therefore understanding how organisms will respond to these changes and predicting the

potential ecosystem shifts that may result is of importance (Fabry et al., 2008, 2009; Feely et al., 2009; Byrne, 2012).

By using ocean acidification as our stressor we are able to measure the effects of the key physiological process of oxidative stress on the larvae and early embryos of *S. neumayeri*, *O. validus* and *P. regularis*. In an attempt to understand the effects of multiple stressors on physiological stress, the embryos from *Sterechinus neumayeri* were investigated for their responses to ocean acidification and UV light. Multiple stress experiments are recognised as the focus of ecotoxicology and there are limited studies on the effects of secondary stressors and the response to ocean acidification (Byrne, 2012; Fabry et al., 2009; Doney et al., 2009; Dupont et al., 2010; Lamare et al., 2011).

Biological effects of OA and UV-R

The effects of environmental stressors within marine ecosystems occur at all levels, for stressors such as OA and UV-R, the effects may be greater during certain stages in a species life-history. These effects are the most influential at the embryonic and larval stages for marine invertebrates (Fabry et al., 2009; Karentz et al., 2004; Lamare et al., 2011). The developmental stages of many marine species are particularly vulnerable to stress in the marine environment. This is linked to an inability to cope with singular and multiple stressors due to their limited metabolic resources during early development, direct exposure to the environment, small size, rapid cellular division, transparency (in respect of UV-R exposure) and a distribution in the upper surface waters (Dupont & Pörtner, 2013; Lamare et al., 2011; Johnsen & Widder, 2001).

The effects of UV-R and ocean acidification on polar marine species is of interest as it is these organisms which have been identified to be potentially at great risk with future climate change scenarios (Fabry et al., 2009). High latitude species may be at threat as they evolved in a low UV-R environment and Polar Regions will be the first to see the effects of ocean acidification. Polar species have therefore been referred to as a bellwether for the effects of ocean acidification (Tedetti & Sempere 2006, Fabry et al. 2009, Lamare et al. 2011). Polar organisms may have a limited ability to cope with these individual stressors and perhaps less so to cope with multiple stressors. Reasons for this inability include; their cold-adapted physiology and metabolic rates, small sizes, long development times, lack of a protective layer with no extracellular fluids to buffer against the environment, cold water and high oxygen concentrations (Viarengo et al., 1998; Dupont & Pörtner, 2013). Additionally the timing of the spawning of many polar

invertebrates coincides with phytoplankton blooms, increasing light levels and the opening of the ozone holes over the Polar Regions (Smith et al., 1992).

Ocean acidification

Ocean acidification will have an impact on the biota of the oceans (Fabry et al., 2008; Doney et al., 2009b; Kroeker et al., 2013) and forecasting the impacts of acidification on ecosystems, functional groups, species, individuals and physiological processes has received much recent attention (see reviews by Fabry et al., 2008; Byrne, 2012; Kroeker et al., 2013). These responses include but are not limited to reduced calcification rates in oysters (Talmage & Gobler, 2010), reduced growth, calcification and delayed development in echinoderms (Clark et al., 2009; Dupont et al., 2010; Byrne, 2011), impaired homing ability in fishes (Munday et al., 2009), bleaching and impaired calcification in corals (Ries et al., 2010; Anthony et al., 2008), alongside of positive growth responses in macroalgae (Hurd et al., 2009). Physiological effects like hypercapnia, a condition related to too much CO_2 in the blood or cellular fluids, have been shown to affect marine animals when grown in high ρCO_2 seawater conditions, altering the internal acid-base regulation and potentially disrupting normal cellular processes (Melzner et al., 2009; Siikavuopio et al., 2007). The effects of hypercapnia on other physiological processes, detected using transcriptomics, has been shown to depress metabolic rates and down regulate gene expression including genes that are responsible for the cellular stress response and skeletal growth in sea urchin larvae (Todgham & Hofmann, 2009). Not all studies, however, show the same down regulatory effects. An increase in the metabolic rate and gene expression in the Antarctic bivalve (*Laternula elliptica*, Cummings et al. 2011), oysters (*Crassostrea virginica*, Beniash et al. 2010) and the purple sea urchin (*Strongylocentrotus purpuratus*, Stumpp, Dupont, et al. 2011; Stumpp, Wren, et al. 2011) has been observed. The effects of hypercapnia on metabolic rates and cellular defences can be significant. For example, in hermatypic coral, the effects of high ρCO_2 can cause the loss of the symbiotic algae, generalised genetic down regulation, a lowering of metabolic rate, an increase in levels of oxidative damage caused by reactive oxygen species and increased apoptosis within coral polyps (Kaniewska et al., 2012).

There are only a few studies that have investigated the effects of exposure to future ocean acidification scenarios on the physiological stress effects and metabolic changes. What is clear, however, is that the effects include up and down regulation of specific gene complexes, changes in metabolic rate (this can vary among species), developmental delays (in larvae and juveniles), impacts on calcification processes and the loss of symbiotic cells in corals (Todgham & Hofmann,

2009; Stumpp et al., 2011a, 2011b; Beniash et al., 2010; Anthony et al., 2008; Kaniewska et al., 2012; Tomanek et al., 2011; Cummings et al., 2011). Transcriptomic responses to ocean acidification in the purple sea urchin larvae *Strongylocentrotus purpuratus* resulted in a 50-70% down regulation of genes associated with biomineralisation, cellular stress responses and energy metabolism when compared to those raised in the control treatments (Todgham & Hofmann, 2009). Stumpp et al. (2011a) and Stumpp et al. (2011b) have reported that seawater acidification results in metabolic suppression in *S. purpuratus* larvae before they reach the feeding stage during larval development. However, once the larvae reached the developmental phase where they began feeding, an upregulation of metabolic genes and metabolic rate was observed. This suggests that larvae have a high-energy demand in order to maintain cellular homeostasis and to begin calcification. An increase in the metabolic rate has also been observed in the oyster *Crassostrea virginica*, where juveniles raised in seawater in a pH of 7.5 consumed more O_2 than the control group. The proposed reasons for the increase in metabolism was that more energy was needed in order to maintain acid-base regulation and the cost associated with skeletogenesis (Beniash et al., 2010). An increase in metabolic rate was also seen in the Antarctic bivalve *Laternula elliptica* in response to lowered seawater pH with increased consumption of O_2 and an upregulation of the chitin synthase gene that helps with calcification. An increase in variation of heat shock protein (HSP70) expression was also observed within the low pH treatment in experiments (Cummings et al., 2011). Proteomic responses in the oyster *C. virginica* in response to a high ρCO_2 environment showed 12% of proteins on the proteome map changed significantly in adult oysters in response to a lowered pH. Proteins that were down regulated included collagen and Rab1b precursor. In addition, 14 proteins showed upregulation in lowered pH which included several antioxidant proteins such as peroxiredoxins 2, 4 and 5, neucleoredoxin and cytosolic Cu and Zn-superoxide dismutase. There was also an increase in mitochondrial malate dehydrogenase which is an important kerbs cycle enzyme, 40S ribosomal protein and proteasome α type 3 protein which are all involved in protein synthesis (Tomanek et al., 2011). What this shows is that this oyster species was upregulating their metabolism and their stress response mechanisms under a high ρCO_2 treatment where pro-oxidant conditions and potential oxidative stress is occurring within the cell. The effects of ocean acidification in some species of corals (*Acropora millepora*, *Acropora intermedia* and *Porolithon onkodes*) has been linked to bleaching and a loss of productivity (Kaniewska et al., 2012; Anthony et al., 2008). A 28-day exposure to reduced seawater pH affected *A. millepora* metabolism, membrane cytoskeleton interaction, signalling, transduction, transport, calcification, protein folding and increased levels of cellular apoptosis. Symbionts were lost from branches of the corals and the remaining symbionts were

less productive. These effects of lowered seawater pH resulted in less energy being transferred to the host. As a result, *A. millepora* showed a down regulation in its metabolism and a down regulation of genes involved in the tricarboxylic acid cycle and the mitochondrial electron transport chain (Kaniewska et al., 2012). Metabolic suppression due to acidosis is likely to have long-term fitness costs and will alter the ability of coral to employ the cellular stress response. Kaniewska et al. (2012) also observed an upregulation of genes responsible for protecting cells against oxidative stress through oxidoreducase activity alongside an upregulation of mitochondrial ATPase and a down regulation of apoptotic inhibitors which together disrupt the mitochondria and cause cell death. This suggests that prolonged environmental stress and a lack of cellular pH homeostasis or elevated maintenance costs to the organism. Kaniewska et al. (2012) suggest that exposure to high CO_2 is disrupting the symbiont and the host's mitochondria and causing increases in reactive oxygen species and reactive nitrogen species. There is much that is known about the effects of ocean acidification on the developmental and metabolic level but much less known at the biochemical level. To my knowledge, there are no recorded studies that have measured both the oxidative damage and the antioxidant responses to ocean acidification in any organism let alone its ability to cope with a second environmental stressor that is known to induce oxidative damage, such as UV-R exposure.

Echinoderms have been labelled as a group that are particularly vulnerable to ocean acidification. This had been attributed to having a skeleton that consists mostly of the highly soluble magnesium calcite (Byrne, 2011). When comparing the responses of echinoid larvae from polar, temperate, and tropical waters to near future ocean pH regimes, Clark et al. (2009) found that skeletal growth decreased in all three cases. The effects of ocean acidification on species from polar areas is of particular interest due to the expectation that polar waters are going to be the first places to experience under saturation of aragonite (Mcneil & Matear, 2008; Mcneil et al., 2010). The responses of polar organisms to this stress can serve as a bellwether for perspective impacts on organisms from mid to low latitudes in response to ocean acidification (Fabry et al., 2009).

UV-R

Ultraviolet radiation (UV-R) enters the ocean in the form of UV-A (320-400 nm) and UV-B light (280-320 nm). Once UV-R enters the ocean it attenuates as it travels through the water column. UV-B light can reach depths down to 16m and UV-A down to 46m (Tedetti & Sempere, 2006). Within these surface waters UV-R can play a crucial role in the lives organisms. UV-R is an

environmental stressor and can cause biological damage (Hader et al., 1998; Tedetti & Sempere, 2006; Lamare et al., 2011). The effects of UV-R within the marine environment vary temporally and spatially. The variations in UV-R irradiances changes with variations in atmospheric ozone (O_3) in the stratosphere. The depth within the ocean that UV-R can penetrate can also vary with latitude and the turbidity of the water column (Kerr & McElroy, 1993).

Polar organisms could be especially vulnerable to increases in the penetration UV-R through the sea ice (Lesser et al., 2004). Penetration of UV-R through the sea ice may increase spatially and temporally as a result of global warming and increases in mean sea temperature (Stroeve et al., 2007; Holland et al., 2006).

The effect of UV-R on biological organisms is significant in areas that receive high levels of UV-B radiation. Normally the majority of UV-B is absorbed in the presence of stratospheric ozone (Inn & Tanaka, 1953). UV-B and UV-A radiation form part of the light spectrum that is biologically damaging. Exposure to UV-B and UV-A can result in a range of detrimental effects including abnormal reproduction and development and increased mutation rates in phytoplankton, macro algae, larvae fish and eggs along with the larvae of other invertebrates (Reviewed by Häder et al., 2007). The early life history phases of marine organisms are particularly vulnerable to the effects of UV-R. They are often undergoing rapid cellular division and have limited energy resources to maintain homeostasis and repair the damage caused by UV-B and UV-A exposure (Adams et al., 2012). At a cellular level, UV-B exposure can directly cause DNA damage, resulting in the formation of cyclobutane pyrimidine dimers lesions and 6-4 photoproducts (Sinha & Häder, 2002). DNA damage has been linked to developmental delays in invertebrates and has been measured to occur *in situ* as well as in laboratory experiments (Lamare et al., 2007; Halliwell & Gutteridge, 1999). Not only does UV-B induce damage directly to DNA and proteins, it can indirectly cause damage by increasing the formation of reactive oxygen species (ROS) such as superoxide anion (O_2•), hydrogen peroxide (H_2O_2) and the hydroxyl radical (•OH) (Lesser et al., 2001; Lesser & Barry, 2003; Lister et al., 2010).

Introduction to oxidative stress and the relationship between oxidative stress OA and UV-R

The term ‘oxidative stress’ has been defined by Sies (1986) as a disturbance in the pro-oxidant – antioxidant balance in favour of the former which leads to potential damage. This is a serious imbalance between reactive species (RS) production and the antioxidant defences, the imbalance

often results in 'oxidative damage'. Oxidative damage is the biomolecular damage caused by the attack of RS upon the constituents of living organisms (Halliwell & Whiteman, 2004). Cells that are exposed to RS can show a wide range of responses ranging from increased proliferation, prevention of cell division, senescence, necrosis, apoptosis and cell death (Halliwell, 2007). Within the cell, if ROS are not controlled by antioxidant defences produced by the organism, or in the case of early non-feeding echinoderm larvae from antioxidant defences maternally inherited, they can cause oxidative stress which can ultimately lead to apoptosis or death if not controlled (Lesser, 2006; Halliwell & Gutteridge, 2007). These free radicals ultimately damage proteins, lipids and also DNA; **Table 1** highlights the most dominant ROS species and the macromolecules that they most commonly react with. It is because of these damaging properties and the ability to detect the upregulation of antioxidants in response to stress that makes oxidative damage and antioxidant defence useful biomarkers for an organism's ability to cope with environmental stress. There have been no studies that have investigated the effects of oxidative stress in response to ocean acidification and quantified the levels of oxidative damage and the antioxidant response despite this being a key physiological and conserved stress response.

Table 1: Common reactive oxygen species (ROS) in biological organisms, including a summary of the corresponding macromolecules that they most commonly react with.

ROS	Name	Target cellular structures		
		Protein	**Lipid**	**DNA**
$^1O_2$	Singlet oxygen	Trp, His, Tyr, Met, Cys	PUFAs	Mostly guanine
$O_2^{\bullet-}$	Superoxide	Fe-S centres	Rarely	No
H_2O_2	Hydrogen peroxide	Cys	Rarely	No
HO•	Hydroxyl radical	Trp, Phe, Tyr, Met, Cys	PUFAs	Guanine

Oxidative stress and ocean acidification

In relation to ocean acidification and oxidative stress, it is has been shown that there is an upregulation in some genes responsible for protecting the cells against oxidative stress in coral that may be caused by a disruption in the symbiotic cell, the coral host mitochondria or both (Kaniewska et al., 2012). Todgham & Hofmann (2009) found that the purple sea urchin, *S. purpuratus*, under development in a sea water acidified with CO_2 down regulated mRNA transcripts for cellular stress responses that included some oxidative defence proteins. In other biological systems such as the bacterium *Escherichia coli,* atmospheric increases in CO_2 (causing

hypercapnia) increased death rates due to H_2O_2 stress and this depended on the dose of CO_2 in which the cultures were grown (Ezraty et al., 2011). In a study investigating the proteomic effects of elevated pCO_2 on *Crassostrea virginica* elevated mRNA transcripts coding for antioxidant proteins and enzymes were observed (Tomanek et al., 2011). There are three hypotheses as to why this upregulation in antioxidants was observed and these are: that CO_2 is interacting with peroxynitrate ($ONOO^-$), a highly reactive nitrogen species that is formed through the interaction between superoxide and nitric oxide (Tomanek et al., 2011; Dean, 2010). High pCO_2 or lower seawater pH may be affecting the functioning of the mitochondria which is resulting in an increase of radical species (Tomanek et al., 2011; Kaniewska et al., 2012; Starkov, 2006; Murphy, 2009). Finally, a decrease in intracellular pH may cause a release of chelated transition metals like that of Fe^{2+} inducing Fenton reactions (Stohs & Bagchi, 1995; Halliwell & Gutteridge, 2007; Tomanek et al., 2011). Because of these 3 hypotheses it is highly relevant that I investigate how a decrease is seawater pH is affecting what cellular compounds and if echinoderm embryos are able to upregulate antioxidants in response.

Oxidative stress and UV-R

UV-R is able to induce direct cellular damage as it is absorbed by macromolecules such as DNA and proteins, and indirectly it increases the formation of reactive oxygen species (ROS) (Lesser et al., 2001; Lesser & Barry, 2003). Lister et al. (2010) have shown that the levels of oxidative stress in *Sterechinus neumayeri* embryos was greater in areas of open ocean compared to areas covered with sea ice when using *in situ* experiments. The results suggest that the production of ROS is a serious future threat if Antarctic animals are to undergo development without UV-R protect from the sea ice.

Pro-oxidative polar environments

Marine animals that live in cold-water environments and in Polar Regions typically have lower metabolic rates compared to temperate and tropical counterparts. If only lower metabolic rates are taken into consideration then there should be decreases in the production of ROS as a by-product of cellular respiration. Polar marine organisms, however, are living in an environment which can contain up to 40% more oxygen than temperate waters (when comparing sea water between 15 and 0°C) and contain cells than often have higher mitochondrial volume densities and peripheral localisation of mitochondria (Abele & Puntarulo, 2004; Lurman et al., 2010b, 2010a). The polar organisms also have an increased unsaturation of cellular membranes which increases the fluidity of these membranes in the cold (Cummings et al., 2011). This increase of unsaturated

lipids in cellular membranes can promote lipid peroxidation, unless antioxidant resources are available (Lesser, 2006). This combination of low metabolic rates and a potential high basal rate of oxidation may decrease the ability of polar organisms to respond to environmental stressors that causes increases in oxidation (Abele & Puntarulo, 2004). Below is a summary of the different types of oxidative damage and some of the enzymes that are responsible for maintaining homeostasis in a pro-oxidative environment.

Lipid damage

Lipid peroxidation affects polyunsaturated fatty acids (PUFAs) that contain groups, which have reactive hydrogen bonds. Lipid peroxidation is initiated in the presence of hydroxyl radicals (OH•) that in turn create a lipid radical that can easily react with oxygen to form a lipid peroxyl. This lipid peroxyl radical can then start the process again by reacting with another polyunsaturated fatty acid to form lipid peroxide. This process is described graphically in Figure 1. Due to this it is possible for a single lipid peroxidation event to convert hundreds of fatty acid side chains into lipid hydroperoxide (Halliwell & Chirico, 1993). It is this ROS damage to lipids that is one of the most common mechanisms of cellular injury (Lesser, 2006). Increases in lipid peroxides can have an effect on enzyme activity, ATP production and initiate apoptosis depending on the sites of ROS activity (Lesser, 2006; Green & Reed, 1998).

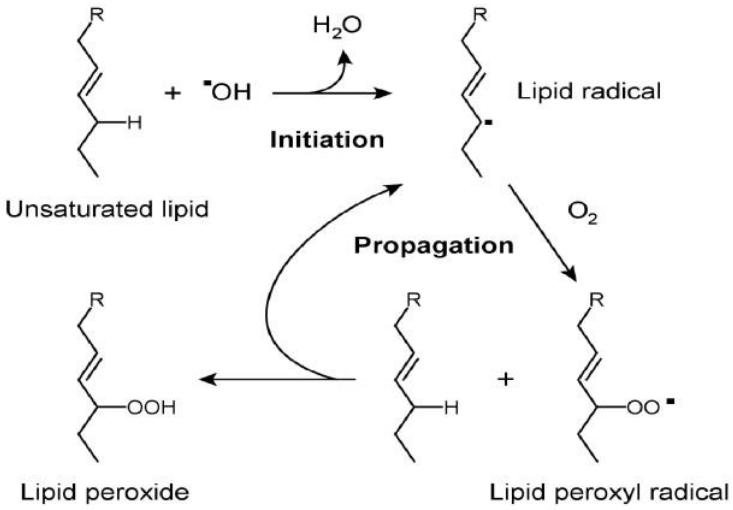

Figure 1: Lipid peroxidation pathway showing the propagation of lipid radicals from unsaturated lipids. Adapted from Young and McEneny (2001)

Protein damage

The effect of an oxidative attack on proteins can result in site-specific amino acid modifications, fragmentation of peptide chains, aggregation of cross-linked reaction products, altered electrical charges and an increased chance of protein removal and degradation (Lesser, 2006). ROS often causes the formation of carbonyl groups such as ketones and aldehydes (Dalle-Donne et al., 2003). Protein carbonylation can affect the function of receptors, enzymes and transport molecules and contribute to secondary damage to biomolecules such as enzymes responsible for DNA repair (Halliwell, 1978; Dalle-Donne et al., 2003). Protein carbonyls are commonly used as an indication of oxidative damage due to the products of protein side chain modification being relatively stable and the ability of sensitive assays available to detect their presence within tissues (Dalle-Donne et al., 2003).

DNA damage

The generation of ROS can cause lesions to form within the DNA strand, which in turn can cause deletions, mutations, and other lethal genetic effects. Both the sugar and the functional amino acid base of DNA are prone to oxidisation which can degrade the base, cause single strand breakage and cross-linking to proteins (Imlay & Linn, 1987). The effects of H_2O_2 and O_2^- are unable to cause strand breaks on their own but it is likely that the damage is caused by Fenton reactions with transition metals that are present and when the toxicity is high enough (Imlay & Linn, 1987). It is the balance between damage and repair that determines the fate of oxidatively damaged cells. There are repair enzymes present in both prokaryotes and eukaryotes (Lesser, 2006).

Enzymatic antioxidants

The function of enzymatic antioxidants is to prevent the O_2 radical cascades and to stop lipid peroxidation chain reactions (Ahmad, 1995). This chain reaction begins with superoxide dismutase scavenging superoxide radicals, then catalase and hydroperoxidases decompose H_2O_2, peroxidases then break down ROOH's and finally glutathione reductase regenerates reduced glutathione (GSH) from its essential but undesirable oxidised form (GSSH) which is a by-product from the peroxidase reactions, see **Table 2** for a brief summary (Ahmad, 1995; Halliwell & Gutteridge, 2007). This process is outlined in more detail below with equations relating the specific enzymes, and the specific reactions.

Table 2: Summary of the major antioxidants, the ROS they react with and the reactive by product of their reaction if there is one.

Antioxidant	ROS that is being reacted with	By product of reaction
Superoxide dismutase	Superoxide (O_2^-)	Hydrogen peroxide (H_2O_2) + O_2
Catalase	Hydrogen peroxide ($2H_2O_2$)	Water + O_2
Glutathione peroxidase	Hydrogen peroxide (H_2O_2) and reduced glutathione (2GSH)	Glutathione disulfide (GSSG) and water
Glutathione	O_2, O_2^- and $HO^\bullet$	

Superoxide dismutase

Superoxide dismutase (SOD) protects cells by catalysing the dismutation of the super oxide radical O_2^-:

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

Catalase and peroxidises

One of the resulting products form SOD activity is H_2O_2. Catalase is able to convert this by-product into H_2O and O_2 as expressed below:

$$2H_2O_2 \xrightarrow{Catalase} 2H_2O + O_2$$

The activity of catalase can largely depend on the rate of protein turn over which can be affected by osmotic stress, along with cold and heat shock (Lesser, 2006; Halliwell, 2007)

Peroxidases remove H_2O_2 by using it to oxidise another substrate (expressed as SH_2 below):

$$SH_2 + H_2O_2 \xrightarrow{Peroxidase} S + 2H_2O$$

(S refers to the substrate to be oxidised)

There are many peroxidase enzymes that are known but glutathione peroxidase (GPOX) is specific to the breakdown of H_2O_2. GPOX oxidises reduced glutathione (GSH) to produce glutathione disulfide (GSSG) and water (Lesser, 2006; Halliwell & Gutteridge, 2007) as described below:

$$H_2O_2 + 2GSH \xrightarrow{GPX} GSSG + 2H_2O$$

Glutathione reductase

The ratios of reduced glutathione to oxidised glutathione in cells are high. The enzyme glutathione reductase (GR) is crucial as it catalyses the reduction of GSSG (See above equation) back into GSH (See below equation):

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADPH^+$$

GR is critical to this reaction as it completes the peroxidase/glutathione cycle (Halliwell & Gutteridge, 1999). Having a high ratio of GSH to GSSG is important in cell redox maintenance in almost all cells. It is because of this function that GR is widely distributed in high levels throughout all cells (Ahmad, 1995).

Glutathione S-transferase

Glutathione S-transferases (GST) are a broad family of enzymes that are best known to catalyse the reduced form of GSH to xenobiotic substrates that are water soluble metabolites. An example of this is the ability of GST enzymes to reduce the reactivity of lipid peroxides (Halliwell & Gutteridge, 2007).

Non enzymatic antioxidants

Glutathione

Glutathione (GSH) is a tripeptide found in animals and plants. When GSH becomes oxidised it forms a thiol radical that will react with a second oxidised glutathione forming a disulphide bond (GSSG) (Halliwell & Gutteridge, 2007). The ratio of GSH/GSSG can be used as an indicator of stress within cells. GSH also functions as an antioxidant by reacting with O_2, O_2^- and $HO^\bullet$ (Lesser, 2006). Glutathione can be thought of acting as a chain breaker of free radical reactions and is required for GPOX. It is crucial for cellular heath that GSH levels are maintained and that the internal cellular environment is not a place of reduction (Halliwell & Gutteridge, 2007).

Ascorbic acid

Ascorbic acid or vitamin-C acts as a reluctant source for ROS. All plants and animals can synthesise ascorbic acid except humans, animals can also obtain ascorbic acid through their diet (Halliwell & Gutteridge, 2007). Ascorbate works as an antioxidant by scavenging H_2O_2, O_2^-, and $HO^\bullet$ without

any enzyme catalysts. It can also facilitate the scavenging of ROS by aiding in the recycling of α-tocopherol from its oxidised to its reduced form (Lesser, 2006; Ahmad, 1995; Halliwell & Gutteridge, 2007).

Ecology of species in this study

The three species examined belong to the phylum Echinodermata. In many ecosystems sea urchins are suggested to either be key stone species or ecosystem engineers (McClintock, 1994; Dupont et al., 2010). The organisms that were selected for this study are found in relatively high abundances in their respective habitats, were easy to capture, store in captivity and are relatively easy to spawn with the lifecycles and developmental schedules being well studied. To assess the effects of ocean acidification and UV-R exposure on oxidative stress levels, the Antarctic sea urchin, *Sterechinus neumayeri Meissner* (1900) (Echinoidea: Echinidae) was used. The effects of ocean acidification on oxidative stress were also tested on the Antarctic sea star *Odontaster validus* Koehler (1906) (Asteroidea: Odontasteridae) and the temperate sea star *Patiriella regularis* Verrill (1867) (Asteroidea: Asterinidae).

S. neumayeri is the most abundant sea urchin in the shallow coastal Antarctic waters and occurs to a depth of 400m (Brey et al., 1995). *S. neumayeri* is an omnivore that has been observed to feed on macro algae, seal faeces, carrion and other invertebrates (Reviewed by McClintock, 1994). *S. neumayeri* is a broadcast spawner with gamete release from late spring through to midsummer. Larval development times are shown in Table 3, with *S. neumayeri* taking 115 days to reach metamorphosis (Pearse & Giese, 1966; Bosch et al., 1987).

Table 3: Number of days from fertilisation for *Sterechinus neumayeri* to reach each major developmental stage. Table adapted from Bosch et al. (1987).

Developmental stage	Time (Days)
Blastula	2.1
Hatching	5.1
Gastrula	10
Prism	16
Early pluteus	21
Six-arm pluteus	43
Eight-arm pluteus	56
Juvenile	115

O. validus is found in high densities surrounding the Antarctic coast in intertidal areas down to 940m, although it is most commonly found between 15 and 200 m (McClintock et al., 1988). *O. validus* is a generalist feeder and is known to feed on a wide range of invertebrates, detritus and diatom film (Reviewed by McClintock, 1994). Due to the abundance of *O. validus* around the Antarctic shelf and its ability to influence community structure, this sea star may be a keystone species of the Antarctic benthic habitat (McClintock et al., 1988). *O. validus* spawn during the austral winter with larvae taking at least 167 days to reach settlement. Table 4 shows the time from fertilisation to each key developmental phase. This species has the longest known larval development time of all echinoderms (Bosch et al., 1987).

Table 4: Time from fertilisation for *Odontaster validus* and *Patiriella regularis* to reach each key developmental stage. Table adapted from Pearse and Bosch (1986) for *O. validus* and Byrne and Barker (1991) for *P. regularis.*

Developmental stage	Odontaster validus	Patiriella regularis
First cleavage	17 hours	40-60mins
Blastula	3 days	3.5-5 h
Gastrula	8 days	25 h
Bipinnaria	42 days	45-55 h
Brachiolaria	140 days	28-42 days
Rudiment with 5 lobes	162 days	56 days
Metamorphosis	167 days	63-70 days

Patiriella regularis is the most commonly found sea star around the coast of New Zealand and can be found grazing over coralline turf in rock pools or underneath stones. The developmental schedule of *P. regularis* is shown in Table 4 with spawning commencing during the summer and the larvae take approximately 63-70 days to reach the juvenile stage in 18-20°C water (Byrne & Barker, 1991).

I have chosen to use the embryos of these Echinoderms as it is the embryonic and larval stages that have been identified as some of the most sensitive life history stages (Byrne, 2012). Environmental stressors like those of ocean acidification and UV-R, during these early life history phases may have detrimental synergistic effects. This is of concern because of the "developmental domino effect" (See Byrne, 2012) that could create developmental bottle necks for species and limit population growth (Ericson et al., 2011).

Thesis outline

Future climate change scenarios will have important effects on the biota of the oceans. It is important to investigate how the effects of these stress at the cellular and molecular level. Oxidative stress assays will be employed to determine the impact of ocean acidification on the early, non-feeding blastula of *S. neumayeri*, *O. validus* from Antarctica and *P. regularis* from New Zealand temperate waters. The Antarctic sea urchin, will be investigated to see if there are any interactions between ocean acidification and the effects of UV light using oxidative stress assays. All three species are common in their respective habitats and play key roles in their ecosystems.

In response to the limited knowledge of the physiological effects of ocean acidification the following hypotheses are proposed and will be answered in the following chapters of this thesis:

Hypothesis 1: Seawater with a decreased pH caused by increased ρCO_2 will cause oxidative damage during the early embryonic development of *S. neumayeri*, *O. validus* and *P. regularis*.

Hypothesis 2: An increase in cellular damage caused by ROS in response to seawater with a decreased pH will result in an upregulation of antioxidant enzymes in *S. neumayeri*, *O. validus* and *P. regularis*. These two hypotheses will be addressed in Chapter 2.

Hypothesis 3: The physiological stress response of *S. neumayeri* embryos to development within seawater with a decreased pH will be additive with exposure to a secondary stressor of UV-R. This will result in higher levels of oxidative damage with limited antioxidant upregulation in response. This third hypothesis will be addressed in Chapter 3.

Chapter 4 will provide an overview of the results presented in chapters 2 and 3 and add a general discussion surrounding ocean acidification, UV-R and oxidative stress caused by these stressors.

Chapter 2 Ocean acidification and Oxidative stress

This chapter investigates the effects of ocean acidification on the production of reactive oxygen species and antioxidant defence in the echinoderm embryos of *Sterechinus neumayeri*, *Odontaster validus* and *Patiriella regularis* through experimentally increasing the ρCO_2 of seawater to decrease the pH. Measuring oxidative stress has been widely used in the field of ecotoxicology but has seen limited use in the field of ocean acidification outside of coral species (Kaniewska et al., 2012; Livingstone et al., 1992; Hader et al., 1998; Viarengo et al., 1998; Lesser, 2006; Lister et al., 2015). By measuring oxidative stress through these laboratory experiments it will be possible to gain an understanding of how these organisms will respond to this key physiological process in response to ocean acidification. This chapter will address our first two hypotheses that decreased seawater pH will result in an increase in oxidative damage and secondly that there will be an upregulation of antioxidant molecules in response to this increase in oxidative damage.

Introduction

Since the industrial revolution in the mid-19th century the concentration of atmospheric CO_2 has increased from 280ppm to just over 400ppm in November 2015 (Lüthi et al., 2008; Monastersky, 2013; Tans & Keeling, 2015). Atmospheric CO_2 levels have not been this high for the last 800,000 years and 50% of this increase has occurred within the last three decades (Feely et al., 2009; Lüthi et al., 2008). The ocean provides a sink for this inorganic carbon and absorbs 25-40% of anthropogenic carbon emissions from the atmosphere, this has helped moderate the levels of carbon in the atmosphere but is decreasing the pH of the oceans (Monastersky, 2013; Le Quéré et al., 2010; Orr et al., 2005).

The oceanic uptake of CO_2 results in carbonic acid (HCO_3^-) forming as the carbon dioxide reacts with the water. Hydrogen ions (H^+) are released through the dissociation of carbonic acid that releases bicarbonate ions (HCO_3^-) and ultimately carbonate ion (CO_3^{2-}) and hydrogen ions (H^+). This reaction reduces the availability of carbonate ions in the water as well as calcium carbonate (equation 1) (Orr et al., 2005; Caldeira & Wickett, 2003). This additional CO_2 has changed the oceans carbonate buffering system and is shifting the equilibrium of carbon dioxide towards a less alkaline ocean. As a result of this increased CO_2 in the world's oceans, a drop in pH of 0.1 units has been observed since the industrial revolution (Orr et al., 2005; Caldeira & Wickett, 2003). The average oceanic pH is currently 8.1 and is projected to decrease to pH 7.6 in the next 200 years if we continue releasing emissions at the present rate and as a worst case scenario

(representative concentration pathway 8.5) set by the IPCC (2013). It is expected that the pH of the oceans will decrease by 0.14 to 0.4 and 0.3 to 0.7 pH units, by the year 2100 and 2300, respectively (Caldeira & Wickett, 2003; Doney et al., 2009; IPCC, 2013).

CO_2 enters the oceans through diffusion as part of the natural carbon cycle and are buffering the increase in atmospheric CO_2. Once the CO_2 has entered the seawater it interacts as described in equation 1 below:

$$\mathrm{CO_{2(atmospheric)}} \rightleftharpoons \mathrm{CO_{2(aqueous)}} + \mathrm{H_2O} \rightleftharpoons \mathrm{H_2CO_3} \rightleftharpoons \mathrm{H^+} + \mathrm{HCO_3^-} \rightleftharpoons 2\mathrm{H^+} + \mathrm{CO_3^{2-}} \text{ (eq.1)}$$

For seawater with a pH of approximately 8.1, 90% of the inorganic carbon occurs as bicarbonate ions, 9% carbonate ion and 1% dissolved CO_2. If the amount of CO_2 within water is increased this has the effect of increasing the amounts of aqueous CO_2, bicarbonate, and hydrogen ions. Thus decreasing the pH of the water as seen in equation 2:

$$\mathrm{pH} = -\log_{10}[\mathrm{H^+}] \text{ (eq.2)}$$

The increasing acidity of the oceans projected for the 21st century of a drop in pH of 0.3-0.4 units is the same as an increase of 150% of H^+ ions and a 50% decrease in bicarbonate ions (Orr et al., 2005).

The oceans ability to absorb CO_2 from the atmosphere depends on the extent of calcium carbonate ($CaCO_3$) dissolution in the water or within the sediments. Equation 3 shows what is required to create calcium carbonate.

$$\mathrm{CaCO_3} \rightleftharpoons \mathrm{CO_3^{2-}} + \mathrm{Ca^{2+}} \text{ (eq.3)}$$

The current saturation states of calcium carbonate vary spatially across the oceans (see Figure 2). They are the highest in the warm shallow waters of the tropics and the lowest in cold high latitude areas and in the deep ocean, these observations by Feely et al. (2004) are consistent with what is expected with $CaCO_3$ solubility with decreasing temperature and increasing pressure and shows the vulnerability of the polar environments to near future ocean acidification.

Ocean acidification is a major threat to calcifying marine organisms (Fabry et al., 2008; Dupont et al., 2010; Kroeker et al., 2013). It decreases the availability of carbonate ions which are required for skeletogenesis and exerts a direct pH effect upon the cells of the individual (Kroeker et al., 2013). Hypercapnia has a narcotic effect that can suppress the immune system and metabolism (Fabry et al., 2008; Dupont & Pörtner, 2013; Doney et al., 2009; Melzner et al., 2009; Kaniewska et al., 2012).

What is of particular concern is how the larvae and the early life history stages of invertebrates cope with ocean acidification and more generally climate change due to their sensitivities to stress. The vulnerabilities of early life history stages of animals may cause ecological bottlenecks for species that are unable to either acclimate or adapt to the changes that will occur (Byrne, 2012). For this reason, the effects of ocean acidification on the early embryos of the three echinoderm species, *Sterechinus neumayeri*, *Odontaster validus* and *Patiriella regularis* are being investigated in this chapter.

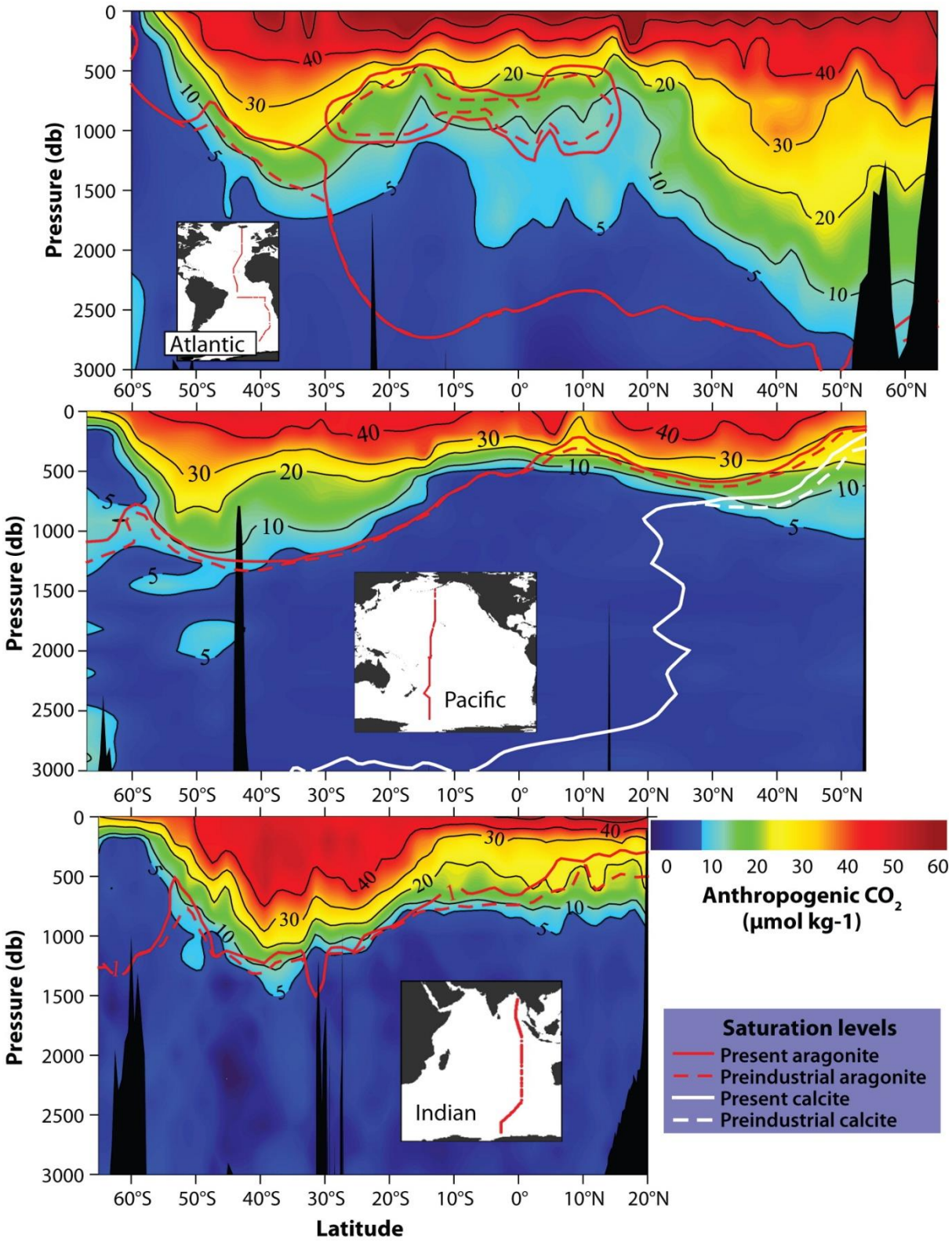

Figure 2: Vertical distributions of anthropogenic CO_2 concentrations (µmol kg^{-1}) and the saturation state Ω=1.0 horizons for aragonite (red) and calcite (white) for present (solid line) and pre-industrial (dashed line) conditions along north-south transects in the Atlantic, Pacific, and Indian oceans. Originally published by Feely et al., (2004) and adapted by Doney et al., (2009b).

Previous studies show that fertilisation was only slightly reduced in reduced seawater pHs for *O. validus* (Gonzalez-Bernat et al., 2013) and had no effect on fertilisation in *P. regularis* and *S. neumayeri* (Byrne et al., 2010, 2013a; Ericson et al., 2011). Larval survival was not affected by seawater with a pH of 7.8 for *O. validus* but was at pH 7.6, which is within the range of pH conditions expected surrounding the Antarctica continent over the coming decades (Doney et al., 2009; Gonzalez-Bernat et al., 2013). Larval survival was impacted at pH 7.6 for *P. regularis* 3-day old bipinnaria larvae and in the long term, development with seawater with a lowered pH reduced larval size and the survival of feeding larvae (Byrne et al., 2013a). Decreased seawater pH also depressed the growth rate in *S. neumayeri* and can act as a teratogen, increasing asymmetry and abnormality in the early and later stages of development (Clark et al., 2009; Byrne et al., 2013b; Ericson et al., 2011; Yu et al., 2013; Byrne et al., 2013c). Later in development, seawater with a reduced pH may shorten the post-oral arm length in *S. neumayeri* (Byrne et al., 2013b; Yu et al., 2013; Byrne et al., 2013c). The larvae of *S. neumayeri* are relatively robust to mild changes in pH (pH 7.8) and short term heat shock events showing a mild resilience to ocean stressors (Kapsenberg & Hofmann, 2014).

Information investigating the molecular influence of high ρCO_2 on early, pre-skeletal, larval marine invertebrates is mostly focused on transcriptomic gene responses (Todgham & Hofmann, 2009; Stumpp et al., 2011a; Kaniewska et al., 2012; Dilly et al., 2015). The effects of future climate change scenarios have been shown to extend further than the gene expression involved in biomineralisation but are also affecting the cellular stress response, metabolism and apoptosis (Todgham & Hofmann, 2009; Stumpp et al., 2011a). Todgham & Hofmann (2009) found that when *Strongylocentrotus purpuratus* larvae were exposed to experimentally lowered pH seawater conditions, the larvae expressed lower mRNA transcript levels for a number of genes involved in the cellular stress response, specifically genes that are important for protein integrity and defending against oxidative stress. This suggests that those larvae are either unable to upregulate antioxidants in response to oxidative stress, or, development with seawater with a decreased pH is not causing oxidative damage. Contrary to this finding Stumpp, Dupont, et al. (2011) found that is the same species the timing of sampling affects mRNA expression and that there was an upregulation in mRNA transcripts associated with oxidative stress. This finding of mRNA expression that codes for antioxidant enzymes being upregulated in response to low pH seawater with a high ρCO_2 in *Crassostrea virginica* (Tomanek et al., 2011). There is very limited knowledge of what oxidative damage is occurring and the specific response of key antioxidants in response to seawater with a lowered pH.

In this thesis, the effects of ocean acidification on oxidative stress and the antioxidant response within *S. neumayeri*, *O. validus* and *P. regularis* embryos are quantified. The effects of oxidative damage on the larval stages of Antarctic invertebrates is of particular interest as the animals of the Antarctic have the potential to act as a bellwether to ocean acidification because of the high solubility of gasses into the cold waters (Fabry et al., 2009). This high solubility of gasses not only increases the amount of CO_2 able to enter the water but also increases the levels of O_2 making the polar coastline an area in which easy formation of ROS causing a highly pro-oxidative environment (Lesser, 2006; Lister et al., 2010). Polar animals have compensated for the cold conditions by having tissues that contain higher densities of mitochondria (Peck, 2002; Lurman et al., 2010a, 2010b). The mitochondria in polar animals contain a high internal surface area to help compensate in cold environments which can increase the amount of oxygen free radicals that leak out of the mitochondria (Peck, 2002; Lesser, 2006).

To assess the impact of ocean acidification on the formation of oxidative damage and the ability for *S. neumayeri*, *O. validus* and *P. regularis* to regulate damage through the up regulation of antioxidants, the early embryos were exposed to the acidified conditions of pH 7.8 and pH 7.6 and measured their oxidative stress response. This will allow us to test our hypotheses that seawater with a decreased pH caused by increased ρCO_2 will cause oxidative damage during the early embryonic development of *S. neumayeri*, *O. validus* and *P. regularis*. Secondly, the increase in cellular damage caused by ROS in response to seawater with a decreased pH will result in an upregulation of antioxidant enzymes in *S. neumayeri*, *O. validus* and *P. regularis*.

Methods

Animal collection

Antarctic sea urchins, *Sterechinus neumayeri* Meissner (Echinidae), were collected from Cape Evans (77.38°S, 166.24°E) by divers and a remotely operated vehicle (ROV). Urchins were kept in a flow through system with seawater at ambient -1.9°C temperature until spawned. The Antarctic sea star *Odontaster validus* Koehler (Odontasteridae) were collected from Cape Evans (77.38°S, 166.24°E) by ROV and stored in tanks at Scott Base temporarily in a flow through system with seawater at ambient -1.9°C temperature until they were air transported to the University of Otago's Portobello Marine Laboratory in Dunedin New Zealand, where there were maintained within freezer units set to -1°C until their winter spawning period. Animals were kept in 5 L tanks at a density of 10 animals per tank, with water changed every week, and the animals feed squid

once per month. The temperate cushion star *Patiriella regularis* Verrill (Asterinidae) was collected from South Beach Portobello (45.49°S, 170.38°), Otago Harbour, New Zealand by divers from the shallow intertidal bay and were kept in flow through tanks at ambient temperature (16°C) before spawning.

Spawning

Reproductively mature *S. neumayeri* were induced to spawn by intracoelomic injection of 2 mL of 0.5M potassium chloride (KCl). Injected animals were inverted over appropriate sized beakers that contained filtered seawater to collect the gametes. Once gamete release had stopped, the animals were removed and the gametes were washed by several partial water changes. Eggs were checked for health and the sperm for activity. In order to gain sufficient material for experiments the eggs from 13 females were combined and fertilised in filtered seawater of ambient pH and temperature using several drops of sperm diluted with filtered seawater from 3 ripe males (final concentration $10^5 – 10^6$ sperm mL). Fertilisation was determined by the appearance of the fertilisation envelope, with fertilisation success observed to be greater than 95%. After five minutes the freshly fertilised eggs were washed with fresh filtered seawater to remove excess sperm.

Mature *O. validus* were induced to spawn by the injection of 1ml of 10^{-5}M 1-methylanine into the gonad of each individual. Individuals were placed into glass bowls, covered with filtered seawater and left to release gametes in the freezer at a temperature of -1°C in ambient pH seawater. Once gamete release had stopped, animals were removed and the eggs washed by serial partial water changes. Eggs and sperm were visually checked for good quality. The eggs of 10 females were combined with several diluted drops of sperm from 4 ripe males (final concentration $10^5 – 10^6$ sperm mL). Fertilisation was observed to be greater than 95%. After 5 minutes, the fertilised eggs were washed with fresh filtered seawater to remove excess sperm.

For *P. regularis*, the gonads from 8 ripe females and 4 ripe males were dissected and placed into filtered seawater and 10^{-5} M 1-methylalanine to induce maturation of gametes. Once sufficient eggs had accumulated they were checked for quality then pooled then washed with fresh filtered seawater. Several diluted drops of sperm were added to the eggs. Fertilisation was greater than 95%. After 5 minutes the fertilised eggs were washed to remove excess sperm.

Acidification of seawater for all experiments

The pH of the 0.22µm filtered seawater was adjusted by bubbling pure CO_2 gas into ambient seawater ($pH_{(NBS)}$ 8.1) to achieve three pH levels ($pH_{(NBS)}$ 8.1, 7.8 and 7.6). The pH was checked using a pH meter (Mettler Toledo MP220) and calibrated to buffers of pH 4.0, 7.0 and 9.2 (Labserv Pro-analysis, Biolab New Zealand). Once calibrated to the buffers a TRIS seawater buffer was used to calibrate the pH meter to the conductivity of the filtered seawater. All pH measurements were adjusted for differences in seawater temperature. All pH levels were ± 0.03 of the desired pH units. Once stabilisation of the seawater at the desired pH was achieved, the water containers were sealed to prevent pH shift due to atmospheric exposure. pH was then measured at the conclusion of the experiment and was found to be stable during the duration of these experiments. 1L Seawater samples were collected from each treatment and fixed with saturated mercuric chloride for later analysis of carbonate parameters at the beginning of the experiments.

Table 5: Temperature (°C), salinity (PSU), $pH_{(NBS)}$, total alkalinity (µmol kg^{-1} $soln^{-1}$), partial pressure of CO_2 (ρCO_2), $CaCO_3$ saturation for calcite (Ω_C) and aragonite (Ω_A) for ambient and treated seawater for (a) *Sterechinus neumayeri*, (b) *Odontaster validus*, (c) *Patiriella regularis*.

(a) *Sterechinus neumayeri*, McMurdo Sound, Ambient T = -1°C, ambient salinity = 34.8			
$pH_{(NBS)}$	8.10	7.81	7.62
Alkalinity (A_T)	2351	2328	2388
ρCO_2	411	856	1389
$\Omega_{Calcite}$	2	1.1	0.7
$\Omega_{Aragonite}$	1.2	0.7	0.4
(b) *Odontaster validus,* Otago Harbour, Ambient T = -1°C, ambient salinity = 34.1			
$pH_{(NBS)}$	8.10	7.80	7.61
Alkalinity (A_T)	2275	2268	2270
ρCO_2	423	886	1400
$\Omega_{Calcite}$	2.2	1.2	0.8
$\Omega_{Aragonite}$	1.4	0.7	0.5
(c) *Patiriella regularis*, Otago Harbour, Ambient T = 15.7°C, ambient salinity = 34.1			
$pH_{(NBS)}$	8.15	7.83	7.73
Alkalinity (A_T)	2271	2269	2260
ρCO_2	412	944	1206
$\Omega_{Calcite}$	3.8	2	1.6
$\Omega_{Aragonite}$	2.4	1.3	1

Sterechinus neumayeri exposure to OA

Larvae were spawned using the methods above and placed into 20 litre containers at a density of 21 larvae per ml and exposed for 7 days in sealed containers to 3 pH treatments; (1) the control

(ambient seawater pH 8.1, and (2) moderately reduced pH 7.8 and (3) low pH 7.6 treatments). There were 3 replicates for each treatment. Treatments were kept cold (-1°C) by placing buckets in a flow through seawater system with seawater being pumped directly from the ocean at Scott Base. The seawater within each bucket was mixed each day by inversion and rolling. After 7 days the embryos were at the late-blastula stage and the experiment concluded. Larvae were filtered out of the water using a sieve with a 50µm filter mesh and pipetted into micro centrifuge tubes in lots of 20,000 larvae. The tubes were spun down in a Minispin centrifuge for 20 seconds and the excess seawater removed. Preservation was by freezing with liquid nitrogen. Samples were placed into storage at -80°C until the extractions were performed.

Odontaster validus exposure to OA

Larvae were spawned using the methods above and placed into 10 litre containers at a density of 12 larvae per ml and exposed for 7 days in sealed containers to 3 pH treatments; (1) the control (ambient seawater (pH (8.1)), (2) moderately reduced pH (pH 7.8) and (3) low pH (pH 7.6) with 3 replicates for each treatment. Seawater for this experiment was taken from the Otago Harbour, filtered then chilled to -1°C for 24 hours before acidification. All experimental treatments were kept in a freezer at -1°C for the duration of the experiment. The seawater within each bucket was mixed each day by inversion and rolling each container. After 7 days the embryos were at the gastrula stage and the experiment concluded. Larvae were filtered out of the water using a sieve with a 50 µm filter mesh and pipetted into micro centrifuge tubes in lots of 20,000 larvae. The tubes were spun down in a Minispin centrifuge for 20 seconds and the excess seawater removed then preserved by freezing with liquid nitrogen and placed into storage at -80°C until the extractions were performed.

Patiriella regularis exposure to OA

Larvae were spawned using the methods above and placed into 10 litre containers at a density of 12 larvae per ml and exposed for 3 days in sealed containers to 3 pH treatments; (1) the control (ambient seawater (pH (8.1)), (2) moderately reduced pH (pH 7.8) and (3) low pH (pH 7.7) with 3 replicates for each treatment. The pH 7.7 treatment was higher than the other two exposures due to an issue with the volume of CO_2 that was able to be dissolved in the experimental sea water treatments. The ambient seawater temperature was maintained by placing containers into a controlled temperature room at 15°C. The seawater within each container was mixed each day by inversion and rolling. After 3 days the embryos were at the gastrula stage and the experiment concluded. Larvae were filtered out of the water using a sieve with a 50µm filter mesh and

pipetted into micro centrifuge tubes in lots of 20,000 larvae. The tubes were spun down in a Minispin centrifuge for 20 seconds and the excess seawater removed then preserved by freezing with liquid nitrogen and placed into storage at -80°C until the extractions were performed.

Larval morphology

A sample of larvae was fixed in a 10% solution of buffered formalin and seawater to be photographed at a later date. Once photographed 15 embryos from each sample of *S. neumayeri* and 20 embryos from each sample of *O. validus* and *P. regularis* were scored for abnormality and their cross sectional area measured. Cross sectional area was measured using the Image J magic wand function to calculate the number of pixels. The measurements were calibrated using a calibration bar photographed at the same magnification and the pixel size converted to µm. Embryos were scored as abnormal if they showed one or more of the following traits; pronounced thickening of the blastoderm in combination with a reduction of the blastocoels, abnormal development of primary mesochyme cells, occlusion of the blastocoel by cellular debris, exogastrulation or any other forms of aberrant archenteron development, examples of these can been seen in Figure 3 (See Lamare et al., 2007; Lister et al., 2010; Gonzalez-Bernat et al. 2012).

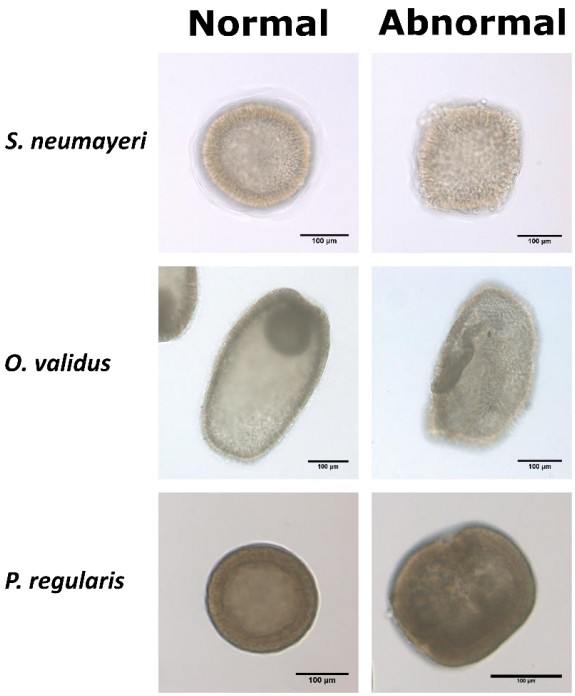

Figure 3: Examples of normal and abnormal embryos for *Sterechinus neumayeri*, *Odontaster validus* and *Patiriella regularis* photographed under 10x magnification. The scale bar represents 100 µm.

Biochemical assays

Enzymes, protein and protein carbonyls

For protein levels, superoxide dismutase, catalase and glutathione reductase 900 µl of 100 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM Na_2 EDTA, 1 µM PMSF and 0.5% TritonX-100 was added to each sample and mixed. Samples were then centrifuged at 13,000 g for 15 minutes at 4°C. The supernatant was pipetted off into micro centrifuge tubes and stored at -80 °C. Protein extracts were purified to remove small molecules that could interfere with subsequent assays by pipetting 200 µl of extract into Vivaspin 500 ultrafiltration units (Sartorius Stedim Biotech, Germany). 200 µl of 100 mM potassium phosphate buffer (pH 7.0) was then added to the filtration unit and extracts were centrifuged for 20 minutes at 13,000 rpm. This step last step was repeated 3 times. After the third centrifugation the protein extract was reconstituted to volume of 400 µl with 100 mM potassium phosphate buffer (pH 7.0), pipetted into a micro centrifuge tube and placed into storage at -80 °C for later analysis. The protein content of each extract was determined using a modified Lowry protein assay (Fryer et al., 1986) using bovine serum albumin (BSA) as a standard.

Superoxide dismutase (SOD)

Superoxide dismutase was assayed using the microplate assay described by Banowetz et al. (2004) with minor modifications. Standards were prepared using bovine liver SOD (Sigma-Aldrich, St. Louis, MO, U.S.A.) where one unit of SOD corresponded to the amount of enzyme that inhibited the reduction of cytochrome *c* by 50% in a coupled system with xanthine oxidase at pH 7.8 and 25 °C. 50 µl aliquots of larval extract, diluted extract or standard were mixed with 125 µl of freshly prepared reaction solution (containing piperazine 1, 4-bis(2-ethanesulfonic acid) (Pipes) buffer at pH 7.8, 0.4 mM *o*-dianisidine, 0.5 mM diethylenetriaminepentaacetic acid (DTPA) and 26 µM riboflavin). The absorbance at 450 nm (A_{450}) was measured immediately (t=0 min). The samples were then illuminated with an 18 W florescent light placed 12 cm above the plate for 30 min (t=30 min) and the A_{450} was measured again. A regression analysis was used to prepare a standard line relating SOD activity to the change in A_{450}. SOD activities in the extracts, calculated with reference to the standard line and were expressed as units SOD per milligram of total protein.

Catalase (CAT)

The chemiluminescent method of Maral et al., (1977), as described by Janssens et al. (2000) for 96-well microplates, was used to assay catalase (CAT) activity. 50 µl of larval extract, diluted

extract or a standard of purified bovine liver catalase in homogenisation buffer (Sigma-Aldrich, St Louis, MO, U.S.A.) was mixed with 100 µl of 100 mM phosphate buffer (pH 7.0) containing 100 mM NaEDTA and 10^{-6} M H_2O. Samples were then incubated at 25 °C for 30 minutes after which 50 µl of a solution containing 20 mM luminal and 11.6 units mL^{-1} of horseradish peroxidase (Sigma-Aldrich, St Louis, MO, U.S.A.) was injected into each well. Light emission, which was proportional in intensity to the amount of H_2O_2 remaining in the mixture, was measured. A regression analysis was used to prepare a standard line relating standard CAT activities to the intensity of light emission. CAT activities were then calculated with reference to the standard line and expressed as µM of H_2O_2 consumed per milligram of total protein.

Glutathione reductase (GR)

Glutathione reductase was assayed using the method described by Cribb et al. (1989) with minor modifications. 50 µl of larval extract, diluted extract or a standard obtained from wheat germ in homogenisation buffer (Sigma-Aldrich, St Louis, MO, U.S.A.) was mixed with 150 µl of 100 mM sodium phosphate buffer (pH 7.6) containing 0.1mM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and 10 µl of NADPH (10 mg/ml; 12mM). The reaction was initiated by the injection of 10µl of oxidised glutathione (GSSG) (1 mg/ml; 3.25mM) and the absorbance at 415 nm (A_{415}) was measured every 30 seconds for 3 minutes, with the plate shaken automatically before each reading. The rate of increase in A_{415} per minute was calculated and a regression analysis was used to prepare a standard line relating standard GR activities to the change in the A_{415}. GR activities in the extracts were then calculated with reference to the standard line and expressed as µ moles or n moles of oxidised glutathione reduced per min per milligram of total protein.

Glutathione peroxidase (GPOX)

GPOX was quantified using the method described by Paglia & Valentine (1967), adapted for use with a microplate reader. 20 µl of extract or standard was mixed with 170 µl of assay buffer containing 50 mM Tris-HCl buffer (pH 7.6), 5 mM Na2EDTA, 0.14 mM NADPH, 1 mM GSH and 3 units ml^{-1} glutathione reductase (from wheat germ, Sigma–Aldrich, St. Louis, MO, U.S.A.; EC 1.6.4.2). The reaction was initiated by the addition of 20 µl t-butyl hydroperoxide to give a final concentration of 0.2 mM. The consumption of NADPH was monitored at 340 nm (A_{340}) every 30 seconds for 3 minutes, with the plate shaken automatically before each reading. GPOX activities in the extracts were calculated with reference to a standard line constructed with GPOX purified from bovine erythrocytes (Sigma–Aldrich, St. Louis, MO, U.S.A.) in extraction buffer. GPOX activity was expressed as nmol per min/mg of total protein.

Glutathione-S-transferase (GST)

Total glutathione-S-transferase (GST) activity (cytosolic and microsomal) was determined using the method of Habig et al. (1974), modified by Brogdon & Barber (1990) for use in a microplate reader, by measuring the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) to reduced glutathione, which is by an increase in absorbance at 340 nm. The rate of increase is directly proportional to the GST activity in the sample. GST was assayed using the photometric 1-chloro-2, 4-dinitrobenzene (CDNB). GST activities in extracts were calculated with reference to a standard line constructed with GST purified from equine liver (Sigma–Aldrich, St. Louis, MO, U.S.A.) in extraction buffer. GST activity was expressed as nmol per min/mg of total protein.

Protein carbonyls

Protein carbonyls were determined by a reaction with 2.4-dinitrophenylhydrazine (DNPH) as described by Reznick and Packer (2005) with adaptations for measurement in a microplate reader and after the removal of potential interfering substances as detailed above. Levels were determined by measuring the absorbance at 370 nm. Protein carbonyl content was determined by using the extinction coefficient of the DNPH at 370nm (0.022/µM/cm), corrected for the calculated path length of the solution (0.6 cm). Protein carbonyl content was expressed as nmol protein carbonyl per milligram of total protein.

Lipid hydroperoxides

Pellets of cell debris remaining after protein extraction, as detailed above, were homogenised in 600 µl of methanol:chloroform (2:1 v/v) and left for 1 minute. 400 µl of chloroform was then added and the sample mixed for 30 seconds. 400 µl of deionised water was added to each sample then mixed for 30 seconds. Samples were stored until the phases had separated, at which point 50 µl of the chloroform layer was transferred to 1mL reaction box for lipid hydroperoxide analysis. Lipid hydroperoxides were determined using the ferric thiocyanate method described by Mihaljevic et al., (1996), with adaptations for measurement in a microplate reader. Levels were determined by measuring the absorbance at 480 nm using a glass microtiter plate. A calibration curve with t-butyl hydroperoxide was used and the lipid hydroperoxide calculated as nmol lipid hydroperoxide per mg protein.

Glutathione

500 µl of 5% sulfosalicylic acid was added to each sample, mixed and then centrifuged at 13,000 g for 5 minutes at 4 °C. The supernatant was removed and stored at -80 °C before analysis. Total glutathione levels (GSH + GSSG) and the % of total glutathione present as GSH were measured

using the enzymatic recycling method using the microplate assay described by Rahman et al. (2006) and expressed as µg mg-1 protein.

8-hydroydeoxyguanosine determination (8-OHdG)

DNA extractions were carried out with a Qiagen DNeasy Blood and Tissue kit, according the instructions, and stored in micro centrifuge tubes at -80 °C. DNA purity was determined by the A260/A280 ratio, with all values being within the range: 1.7–1.9 Sambrook et al. (1989).

A sample of extracted DNA was precipitated by the addition of 0.1 volume of 4M NaCl and 2.5 volumes of cold ethanol and digested as per Shigenaga et al. (1994) with minor modifications. The precipitated DNA was re-dissolved in 200 µl of sterile DNA hydrolysis buffer (1 mM deferoxamine, 20 mM sodium acetate, pH 5). Nuclease P1 was added (4 µl; 3.3 mg ml-1) and samples were incubated at 65°C for 15 minutes. Alkaline phosphatase (4 U in 1 M Tris-HCl (pH 8)) was added and the samples were incubated at 37°C for 60 minutes. Finally, 20 µl 3 M sodium acetate was added to each sample, followed by 20 µl of chelating solution (50 mM EDTA, 10 mM deferoxamine). The solutions were filtered through a 30 kDa cut-off filter-membrane and the filtered solutions, containing the nucleotides, were collected for 8-OHdG analysis.

Digested DNA samples were analysed using high-performance liquid chromatography (HPLC) followed by UV detection of 8-OHdG and electrochemical detection (coulometric) of 8-OHdG. The procedure was performed essentially as described by Shigenaga et al. (1994), using a C18 reverse-phase (5 mm, 4.6 mm x 250 mm) column (JASCO, Ishikawa-cho, Hachioji-shi, Tokyo, Japan), a Perkin-Elmer HPLC system (Boston, U.S.A.) and an electrochemical detector (model 5100, ESA, Chelmsford, MA). The oxidation potentials of the analytical cell of the electrochemical detector were set to 150 mV and 350 mV for electrodes 1 and 2, respectively, with the guard cell potential set at 400 mV. Unmodified nucleotides were detected by their absorbance at 260 nm. Separation, of 50 ml of digested DNA, was achieved using an isocratic mobile phase consisting of 50 mM potassium phosphate (pH 5.5) and 10% methanol, at a flow rate of 1 ml min^{-1}, with the column maintained at 30°C. Peak data were collected and analysed using a Data Centre 4000 general-purpose laboratory data interface, and Delta chromatography data acquisition and analysis software (DataworkX, Brisbane, Australia). The retention times for guanosine (G) and 8-OHdG were 12 and 17 min, respectively. Solutions of 8-OHdG and G (Sigma, Chemical Co, St Louis, MO, USA), prepared in HPLC-grade water (Merck, Darmstadt, Germany) and sterilised by passage through 0.22 mm filters (Millipore, Bedford, MA, USA), were used as standards. For each sample,

the amount of DNA injected onto the column was estimated using the signal for G and 8-OHdG was quantified by comparison to external standards.

Statistical analysis

All data, except the abnormality and the % reduced glutathione data, was ln(x) transformed to ensure normally of the data to help meet the assumptions of the ANOVA. The abnormality and the %reduced glutathione were arcsine square root transformed as this data is expressed as a proportion. The residuals were graphed and checked for normality and homogeneity of variances and were normally distributed. Data sets were checked for normality and met the criteria of the ANOVA test. One-way ANOVAs comparing the measured variable against the experimental variable of varied pH were used to look for significant differences among treatments and the nominal significance cut off was set at $\alpha = 0.05$. *Post hoc* differences among the pH treatments were determined using Tukey's 95% simultaneous confidence intervals with all pairwise comparisons among levels of pH. All statistical analysis was carried out using Minitab® 16.2.1.

Results

Carbonate chemistry

For *S. neumayeri*, the ρCO_2 was 411 µatm under ambient conditions. For the treated water the ρCO_2 increased two fold to 856 µatm for the pH 7.8 treatments and increased more than fourfold to 1389 µatm. Calcite and aragonite were both saturated in the pH 8.1 treatment ($\Omega_C = 2$; $\Omega_A = 1.2$). Calcite was saturated but aragonite was under saturated for the pH 7.8 treatment ($\Omega_C = 1.1$; $\Omega_A = 0.7$) and for the pH 7.6 treatment both aragonite and calcite were under saturated ($\Omega_C = 0.7$; $\Omega_A = 0.4$) (Table 5). The pH was stable over the course of the experiment.

For *O. validus* the ambient seawater conditions had a ρCO_2 of 423 µatm. For the pH 7.8 treatment the ρCO_2 more than doubled to 886 µatm. The ρCO_2 for the pH 7.6 treatment was 1400 µatm. The calcite and aragonite were both saturated in the ambient treatments ($\Omega_C = 2.2$; $\Omega_A = 1.4$. For the pH 7.8 treatment calcite was saturated and aragonite was under saturated ($\Omega_C = 1.2$; $\Omega_A = 0.7$). In the pH 7.6 treatment, both calcite and aragonite were under saturated ($\Omega_C = 0.8$; $\Omega_A = 0.5$) (Table 5). The pH was stable over the course of the experiment.

In the *P. regularis* experiment the ambient seawater had a pH of 8.15 and a ρCO_2 of 412 µatm and was saturated with calcite and aragonite ($\Omega_C = 3.8$; $\Omega_A = 2.4$). The pH 7.8 treatment had a ρCO_2 of

944 µatm and was saturated with both calcite and aragonite (Ω_C = 2.0; Ω_A = 1.3). The pH 7.7 treatment had a ρCO_2 of 1206 µatm and was saturated for calcite and aragonite (Ω_C = 1.6; Ω_A = 1) (Table 5). The pH was stable over the course of the experiment.

Oxidative damage and abnormal development

There was a significant increase in the percentage of abnormally developed *S. neumayeri* embryos between the pH 8.1 and the pH 7.6 treatments. The percentage of abnormal embryos varied between 16% (± se=5.4%) (pH 8.1) and 60% (± se=7.3%) (pH 7.6) (Figure 4, Table 6). *P. regularis* also showed a significant increase in the percentage of abnormal larvae between pH treatments. This varied between 2% (± se=1.6%) (pH 8.1) and 18% (± se=6%) (pH 7.7) (Figure 4, Table 6). There were no significant differences between pH treatments for *O. validus* (Figure 4, Table 6).

There was a significant difference in blastula size, measured in cross sectional area for *S. neumayeri* (Figure 4, Table 6). Development in lowered pH caused blastula to range in size between 1.858 x 10^5 µm^2 (± se=0.170 x 10^5 µm^2) (pH 7.6) and 1.713 x10^5 µm^2 (± se=0.159 x 10^5 µm^2) (pH 8.1) with a Tukey's post-hoc significant test showing the treatments exposed to seawater with a lower pH being larger than the control (Figure 4, Table 6). Low pH also had a significant effect on the cross sectional area of *P. regularis* by decreasing the size from 3.157x 10^4 µm^2 (± se=629 µm^2) (pH 8.1) to 4.003.x 10^4 µm^2 (± se=0.192 x 10^4 µm^2) (pH 7.7) with a difference being observed between the pH 8.1 and the 7.7 treatments (Figure 4, Table 6). There was no significant effect of low pH on the cross sectional area of *O. validus* (Figure 4, Table 6).

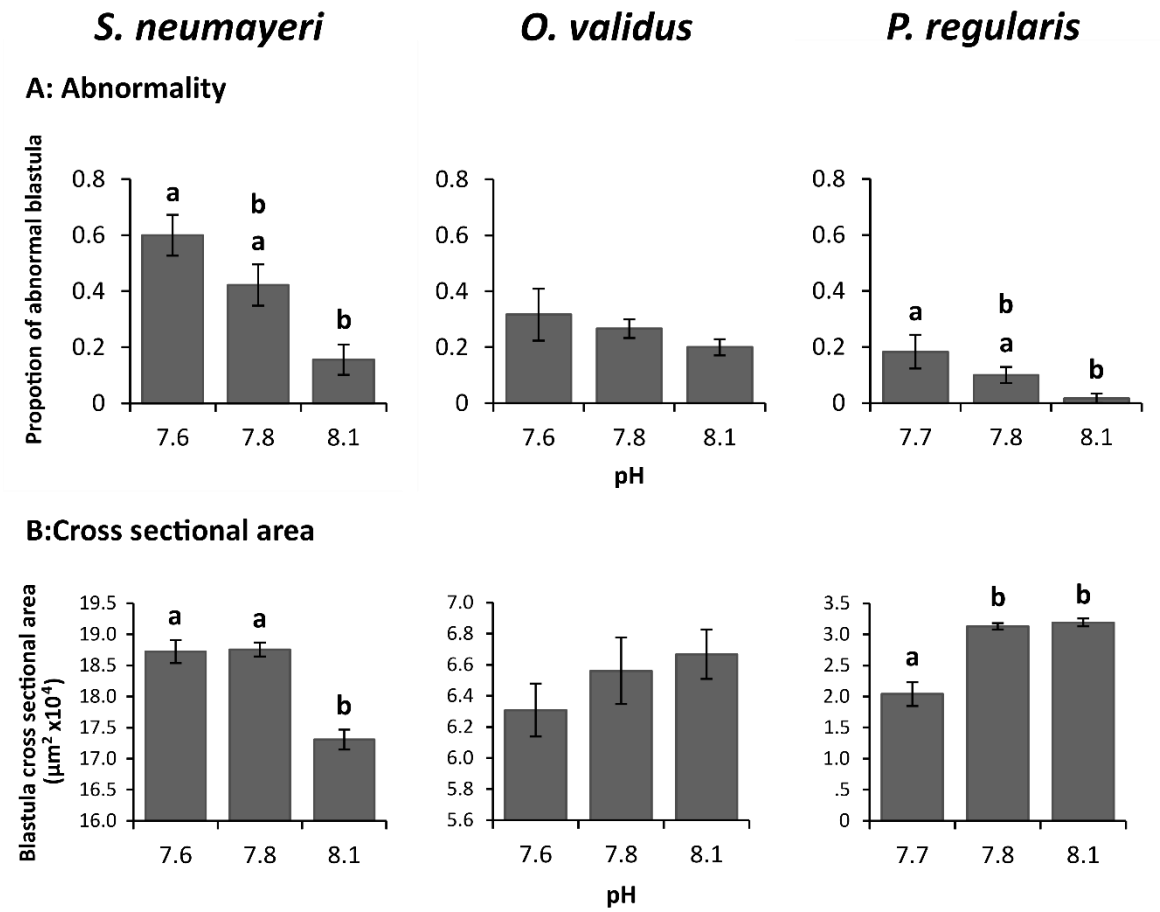

Figure 4: A: Mean proportion of abnormal blastula ± the standard error for *Sterechinus neumayeri* (n=9), *Odontaster validus* (n=9) and *Patiriella regularis* (n=9) for 3 seawater pH treatments. pH 8.1 is the ambient seawater in each experiment. B: Mean cross sectional area ± the standard error for *Sterechinus neumayeri* (n=135, *Odontaster validus* (n=180) and *Patiriella regularis* (n=180) for 3 seawater pH treatments. Statistical significance was determined with a 1-way ANOVA with a Tukey's post-hoc test. Within each graph, columns sharing the same letter are not significantly different at a significance level of $p \leq 0.05$.

Table 6: ANOVA on abnormality and cross sectional area of embryos that have developed in seawater with lowered pH in *Sterechinus neumayeri*, *Odontaster validus* and *Patiriella regularis*. All analyses were 1-way ANOVA with seawater pH as a fixed factor. Abnormality proportions were transformed using an arcsine square root transformation. The cross sectional area measurements were transformed with a natural log to increase normality of the data.

Parameter	Source	DF	Sum of Squares	Mean Squares	F	p- value
Sterechinus neumayeri **Abnormality**	pH	2	0.399	0.1997	7.3334	**0.0245**
	Error	6	0.1633	0.0273		
	Total	8	0.5626			
Sterechinus neumayeri **cross-sectional area**	pH	2	0.1869	0.0934	4.24	**0.016**
	Error	132	2.9112	0.0221		
	Total	134	3.0981			
Odontaster validus **Abnormality**	pH	2	0.0255	0.0127	1.013	0.4177
	Error	6	0.0755	0.012575		
	Total	8	0.1009			
Odontaster validus **cross-sectional area**	pH	2	0.0652	0.0326	0.73	0.484
	Error	132	5.9087	0.0448		
	Total	134	5.9740			
Patiriella regularis **Abnormality**	pH	2	0.1995	0.0998	7.1319	**0.026**
	Error	6	0.0829	0.01399		
	Total	8	0.2834			
Patiriella regularis **cross-sectional area**	pH	2	169.17	84.59	30.29	**<0.001**
	Error	177	494.28	2.79		
	Total	179	663.45			

8-OHdG concentration varied significantly among pH treatments in *S. neumayeri* with the concentration of 8-OHdG ranging from 1.37 8-OHdG per 10^6 dG (± se=0.108) (pH 8.1) to 4.35 8-OHdG per 10^6 dG (± se=0.118) (pH 7.6) (Figure 5, Table 7). Tukey's post-hoc significance testing shows that the pH 7.6 treatments have significantly higher concentrations of 8-hydroxydeooxyguanosine/10^6 than the pH 7.8 and pH 8.1 seawater treatments Figure 5. Lowered seawater pH had no significant effect on 8-OHdG per 10^6 dG concentration for *O. validus* and *P. regularis* (Figure 5, Table 7).

There were significant increases in oxidative damage to lipids (nmol of lipid hydroperoxide per 2 x 10^3 larvae) among pH treatments for *S. neumayeri* and *P. regularis*. Lipid hydroperoxides levels ranged between 2.48 (nmol of lipid hydroperoxide per 2 x 10^3 larvae) (± se=0.062) (pH 8.1) and 6.74 (± se=0.442) (pH 7.6) nmol of lipid hydroperoxide per 2 x 10^3 larvae for *S. neumayeri.* Lipid hydroperoxides levels for *P. regularis* ranged between 2.99 (± se=0.156 (pH 8.1)) and 3.82 (± se=0.062 (pH 7.7)) nmol of lipid hydroperoxide per 2 x 10^3 larvae with Tukey's post-hoc

significance testing showing significantly higher concentrations of lipid hydroperoxides in the pH 7.7 treatments compared to both pH 7.7 and pH 8.1 treatments. There was no significant difference in lipid hydroperoxides among pH treatments for *O. validus* (Figure 5, Table 7).

Oxidative damage to proteins generally increased with decreasing seawater pH treatments for all species (Figure 5, Table 7). *S. neumayeri* protein carbonyls ranged between 1.02 (± se=0.054) (pH 8.1) and 1.42 (± se=0.084) (pH 7.6) nmol mg^{-1} protein with a Tukey's post-hoc significance test showing that the pH 7.6 treatments were significantly different from both pH 7.8 and pH 8.1 treatments. *O. validus* protein carbonyl levels ranged between 1.42 (± se=0.032) (pH 8.1) and 1.85 (± se=0.185) (pH 7.6 nmol mg^{-1} protein. Tukey's post-hoc significance testing shows the pH 7.8 treatments being significantly different to the pH 7.6 treatment and the pH 8.1 treatment not being significantly different from either the pH 7.6 or the pH 7.8 treatments (Figure 5, Table 7). The concentrations of proteins carbonyls for *P. regularis* varied between 1.21 (± se=0.101) (pH 8.1) and 1.76 (± se=0.051) (pH 7.7) nmol mg^{-1} protein with the pH 7.7 treatments being significantly higher than both the pH 7.8 and the pH 8.1 treatments with Tukey's post-hoc significance testing (Figure 5, Table 7).

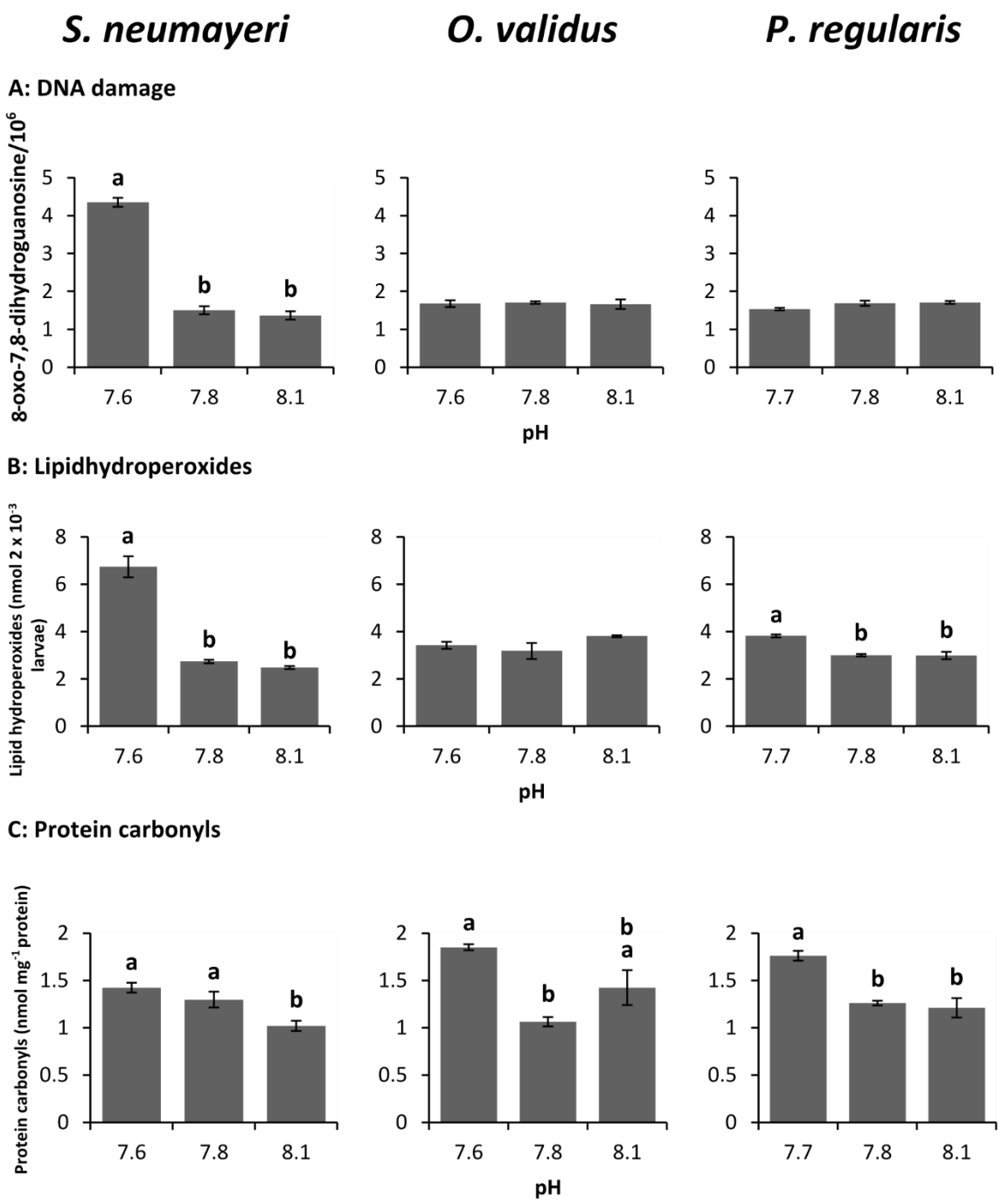

Figure 5: A: DNA damage measured in 8-hydroydeoxyguanosine/10^6 dG ± the standard error for *Sterechinus neumayeri* (n=9), *Odontaster validus* (n=9) and *Patiriella regularis* (n=9) for 3 seawater pH treatments. B: Lipid hydroperoxide levels per 20,000 larvae (nmol) ± the standard error for *Sterechinus neumayeri* (n=9), *Odontaster validus* (n=9) and *Patiriella regularis* (n=9) for 3 seawater pH treatments. C: Protein carbonyls measured in nmol mg^{-1} protein ± the standard error for *Sterechinus neumayeri* (n=9), *Odontaster validus* (n=9) and *Patiriella regularis* (n=9) for 3 seawater pH treatments. pH 8.1 is the ambient seawater in each experiment. Statistical significance determined with a 1-way ANOVA with a Tukey's post-hoc test. Within each graph, columns sharing the same letter are not significantly different at a significance level of $p \leq 0.05$.

Table 7: ANOVA on 8-hydroydeoxyguanosine/ 10^6 dG, lipid hydroperoxides and protein carbonyls of embryos that have developed in seawater with lowered pH for *Sterechinus neumayeri*, *Odontaster validus* and *Patiriella regularis*. All analyses were 1-way ANOVA with seawater pH as a fixed factor. All data was ln transformed for statistical testing.

Parameter	Source	DF	Sum of Squares	Mean Squares	F	p- value
Sterechinus neumayeri	pH	2	2.5016	1.2508	98.71	**<0.001**
8-hydroydeoxyguanosine/ 10^6 dG	Error	6	0.0760	0.0127		
	Total	8	2.5776			
Sterechinus neumayeri	pH	2	1.80844	0.90422	166.36	**<0.001**
lipid hydroperoxides	Error	6	0.03261	0.00544		
	Total	8	1.84106			
Sterechinus neumayeri	pH	2	0.05264	0.02632	10.14	**0.012**
protein carbonyls	Error	6	0.01558	0.00260		
	Total	8	0.06822			
Odontaster validus	pH	2	0.00147	0.00074	0.09	0.919
8-hydroydeoxyguanosine/ 10^6 dG	Error	6	0.05139	0.00856		
	Total	8	0.05286			
Odontaster validus	pH	2	0.0537	0.0269	2.14	0.199
lipid hydroperoxides	Error	6	0.0754	0.0126		
	Total	8	0.1291			
Odontaster validus	pH	2	0.15698	0.07849	12.51	**0.007**
protein carbonyls	Error	6	0.03763	0.00627		
	Total	8	0.19461			
Patiriella regularis	pH	2	0.02095	0.01047	3.97	0.80
8-hydroydeoxyguanosine/ 10^6 dG	Error	6	0.01585	0.00264		
	Total	8	0.03679			
Patiriella regularis	pH	2	0.11877	0.05939	18.78	**0.003**
lipid hydroperoxides	Error	6	0.01898	0.00316		
	Total	8	0.13775			
Patiriella regularis	pH	2	0.25906	0.12953	15.55	**0.005**
protein carbonyls	Error	6	0.05340	0.00890		
	Total	8	0.31245			

Enzymatic antioxidant activities

For *S. neumayeri* the activity of the enzymatic antioxidant SOD increased significantly among pH treatments. The SOD levels varied between 120 (± se=1.15) (pH 8.1) and 366 (± se=21.26) (pH 7.6) units^{-1} protein min^{-1} (Figure 6, Table 8). Tukey's post-hoc significance testing shows that the SOD concentration for the pH 7.6 treatment was significantly higher than the pH 7.8 and the pH 8.1 treatments. There was no significant difference among pH treatments for the two starfish species *O. validus* and *P. regularis* (Figure 6, Table 8). The SOD levels for both *O. validus* and *P. regularis* were 2.8 fold higher than *S. neumayeri* in the pH 8.1 treatments (Figure 6).

There was a significant difference among pH treatments on CAT levels in *S. neumayeri*. These ranged between 132 (± se=5.77) (pH 8.1) and 182 (± se=2.89) (pH 7.6) µmol H_2O_2 mg^{-1} protein min^{-1} with the significant difference between the pH 7.6 and the pH 8.1 treatments shown by Tukey's post-hoc significance testing (Figure 6). There was no significant effect of pH on CAT levels for *O. validus* or *P. regularis*. The CAT baseline enzyme levels for *O. validus* and *P. regularis* were 1.4 times higher than *S. neumayeri* in the pH 8.1 treatments (Figure 6, Table 8).

Glutathione reductase levels showed a significant difference among pH treatments for *S. neumayeri* and *O. validus*. GR levels ranged between 1.46 (± se=0.052) (pH 8.1) and 2.96 (± se=0.087) (pH 7.6) µmol mg^{-1} protein min^{-1} for *S. neumayeri* and 2.27 (± se=0.026) (pH 8.1) and 2.91 (± se=0.113) (pH 7.6) µmol mg^{-1} protein min^{-1} for *O. validus*. Tukey's post-hoc significance testing shows that the pH 7.6 treatment was significantly different from the pH 7.8 treatment which were both significantly higher than the pH 8.1 treatment for *S. neumayeri* (Figure 7). For *O. validus* the pH 7.6 treatment was significantly higher than both the pH 7.8 and the pH 8.1 treatments (Figure 7). There was no significant difference among pH treatments for *P. regularis* (Figure 7, Table 8).

Glutathione peroxidase levels were significantly different among pH treatments for *S. neumayeri* with levels ranging between 17.52 (± se=1.53) (pH 8.1) and 35.41 (± se=1.062) (pH 7.6) nmol mg^{-1} protein min^{-1}. Tukey's post-hoc significance testing showing that the pH 7.6 treatments were significantly different from the pH 7.8 and the pH 8.1 treatments. There was no significant difference among pH treatments on GPOX levels for *O. validus* and *P. regularis* (Figure 7, Table 8).

There was a significant difference in GST levels among pH treatments for *S. neumayeri*. GST levels ranged between 13.89 (± se=0.16) (pH 8.1) and 21.41 (± se=0.89 (pH 7.6) nmol min^{-1} mg protein^{-1}.

Tukey's post-hoc significance testing showing that the pH 7.6 treatments were significantly different from the pH 7.8 and the pH 8.1 treatments. There was no significant difference among pH treatments for GST levels for *O. validus* and *P. regularis* (Figure 7, Table 8).

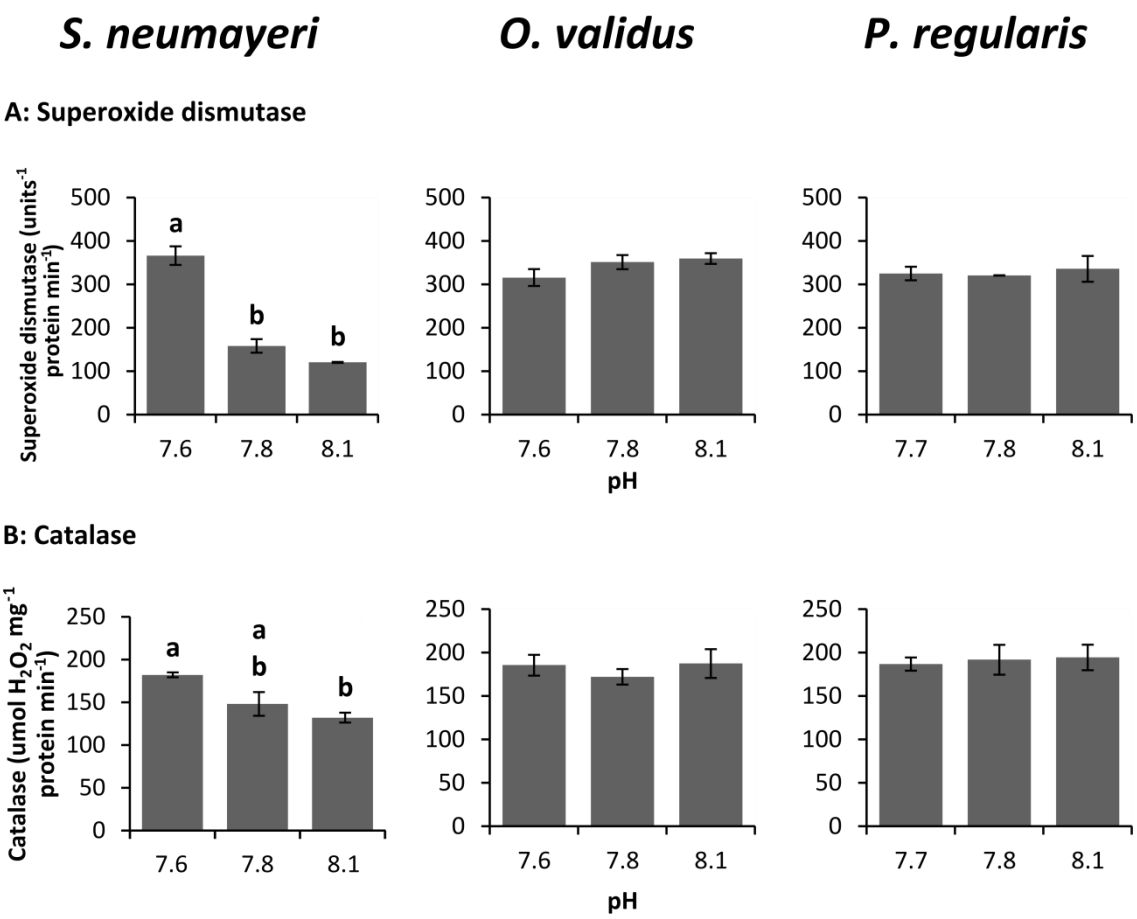

Figure 6: A: Superoxide dismutase activity (units^{-1} protein min^{-1}) ± the standard error for *Sterechinus neumayeri* (n=9), *Odontaster validus* (n=9) and *Patiriella regularis* (n=9) for 3 seawater pH treatments. B: Catalase activity (µmol H_2O_2 mg^{-1} protein min^{-1}) ± the standard error for *Sterechinus neumayeri* (n=9), *Odontaster validus* (n=9) and *Patiriella regularis* (n=9) for 3 seawater pH treatments. pH 8.1 is the ambient seawater in each experiment. Statistical significance was determined with a 1-way ANOVA with a Tukey's post-hoc test. Within each graph, columns sharing the same letter are not significantly different at a significance level of $p \leq 0.05$.

Table 8: ANOVA on superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPOX) and glutathione S-transferase (GST) levels for *Sterechinus neumayeri*, *Odontaster validus* and *Patiriella regularis* embryos that have developed in seawater with lowered pH. All analyses were 1-way ANOVA with seawater pH as a fixed factor. All data was ln transformed for statistical testing.

Parameter	Source	DF	Sum of Squares	Mean Squares	F	p- value
Sterechinus neumayeri	pH	2	2.0250	1.0125	71.37	**<0.001**
SOD	Error	6	0.0851	0.0142		
	Total	8	2.1101			
Sterechinus neumayeri	pH	2	0.1624	0.0812	7.51	**0.023**
CAT	Error	6	0.0648	0.0108		
	Total	8	0.2272			
Sterechinus neumayeri	pH	2	0.79423	0.39712	104.07	**<0.001**
GR	Error	6	0.02290	0.00382		
	Total	8	0.81713			
Sterechinus neumayeri	pH	2	0.9446	0.4723	24.00	**0.001**
GPOX	Error	6	0.1181	0.0197		
	Total	8	1.0626			
Sterechinus neumayeri	pH	2	0.32642	0.16321	41.08	**<0.001**
GST	Error	6	0.02384	0.00397		
	Total	8	0.35026			
Odontaster validus	pH	2	0.02990	0.01495	2.16	0.196
SOD	Error	6	0.04146	0.00691		
	Total	8	0.07136			
Odontaster validus	pH	2	0.0118	0.0059	0.41	0.678
CAT	Error	6	0.0855	0.0143		
	Total	8	0.0973			
Odontaster validus	pH	2	0.09961	0.04981	23.42	**0.001**
GR	Error	6	0.01276	0.00213		
	Total	8	0.11238			
Odontaster validus	pH	2	0.0079	0.0040	0.34	0.722
GPOX	Error	6	0.0692	0.0115		
	Total	8	0.0771			
Odontaster validus	pH	2	0.0031	0.0016	0.13	0.884
GST	Error	6	0.0740	0.0123		
	Total	8	0.0771			
Patiriella regularis	pH	2	0.00238	0.00119	0.12	0.887
SOD	Error	6	0.05826	0.00971		
	Total	8	0.06064			
Patiriella regularis	pH	2	0.0019	0.0010	0.06	0.945
CAT	Error	6	0.1005	0.0167		
	Total	8	0.1024			
Patiriella regularis	pH	2	0.0191	0.0096	0.59	0.582
GR	Error	6	0.0967	0.0161		
	Total	8	0.1158			
Patiriella regularis	pH	2	0.02975	0.01487	2.12	0.201
GPOX	Error	6	0.04208	0.00701		
	Total	8	0.07183			
Patiriella regularis	pH	2	0.0045	0.0023	0.15	0.860
GST	Error	6	0.0878	0.0146		
	Total	8	0.0924			

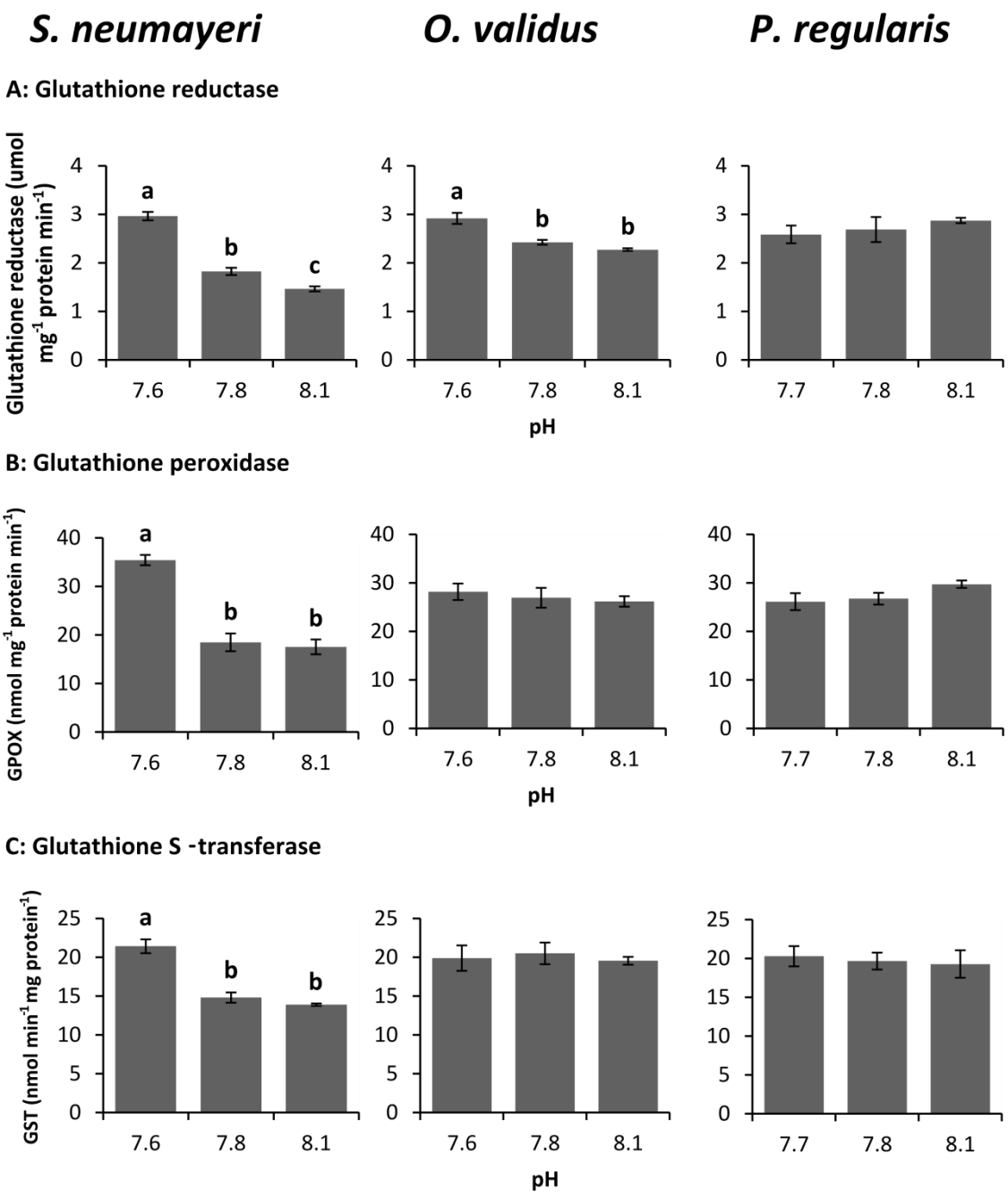

Figure 7: A: Glutathione reductase (GR) activity (µmol mg^{-1} protein min^{-1}) ± the standard error for *Sterechinus neumayeri* (n=9), *Odontaster validus* (n=9) and *Patiriella regularis* (n=9) for 3 seawater pH treatments. B: Glutathione peroxidase (GPOX) measured in nmol mg^{-1} protein min^{-1} ± the standard error for *Sterechinus neumayeri* (n=9), *Odontaster validus* (n=9) and *Patiriella regularis* (n=9) for 3 seawater pH treatments. C: Glutathione S-transferase measured in nmol mg^{-1} protein min^{-1} ± the standard error for *Sterechinus neumayeri* (n=9), *Odontaster validus (*n=9) and the *Patiriella regularis* (n=9) for 3 seawater pH treatments. pH 8.1 is the ambient seawater in each experiment. Statistical significance was determined with a 1-way ANOVA with a Tukey's post-hoc test. Within each graph, columns sharing the same letter are not significantly different at a significance level of $p \leq 0.05$.

Non-enzymatic antioxidant activities

There was a significant difference in the total amount of glutathione among treatments for *S. neumayeri* (Table 9). Total glutathione levels decreased from 86.61 (± se=2.71) (pH 8.1) to 68.85 (± se=3.03) (pH 7.6) μg^{-1}protein^{-1}. There was no significant difference among pH treatments for *O. validus* and *P. regularis* (Figure 8, Table 9). The percentage of reduced glutathione did not vary significantly among pH treatments for *S. neumayeri* with p=0.0501 (Table 9). The percentage of reduced glutathione decreased from 94% (± se=4.04%) (pH 8.1) to 77% (± se=2.31%) (pH 7.6) for *S. neumayeri*. The percentage of reduced glutathione did not vary significantly for *O. validus* and *P. regularis* (Figure 8, Table 9).

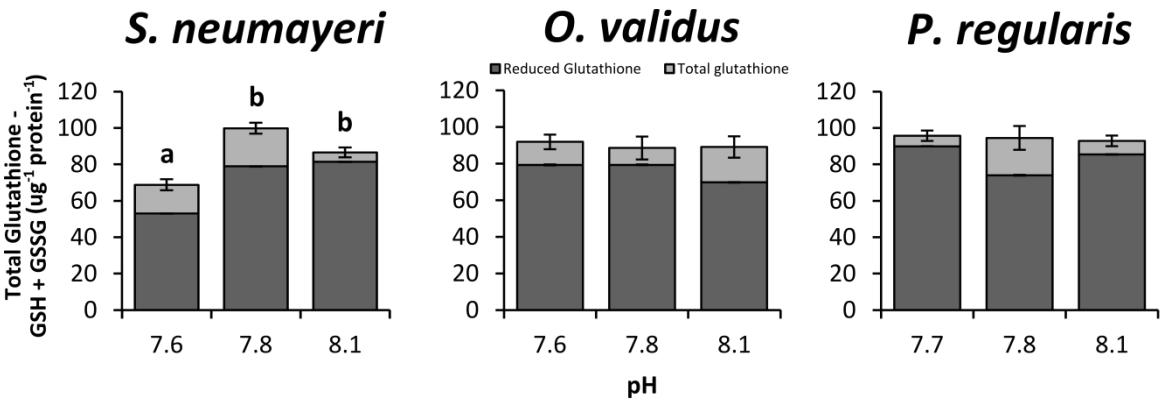

Figure 8: Mean total glutathione – GSSH + GSSG levels (μg^{-1} protein^{-1}) ± the standard error for *Sterechinus neumayeri* (n=9), *Odontaster validus* (n=9) and *Patiriella regularis* (n=9) for 3 seawater pH treatments. pH 8.1 is the ambient seawater in each experiment. Levels are divided into reduced (bottom segment of each bar) and oxidised forms (top segment of each bar). Statistical significance was determined with a 1-way ANOVA with a Tukey's post-hoc test. Within each graph, columns sharing the same letter are not significantly different at a significance level of $p \leq 0.05$.

Table 9: ANOVA on total glutathione and %reduced glutathione for *Sterechinus neumayeri*, *Odontaster validus* and *Patiriella regularis* embryos that have developed in seawater with lowered pH. All analyses were 1-way ANOVA with seawater pH as a fixed factor. Total glutathione data was ln transformed and the % reduced glutathione was arcsine square root transformed for statistical testing.

Parameter	Source	DF	Sum of Squares	Mean Squares	F	p- value
Sterechinus neumayeri	pH	2	0.21271	.10635	28.03	**0.001**
Total Glutathione	Error	6	0.02276	0.00379		
	Total	8	0.23547			
Sterechinus neumayeri	pH	2	0.1486	0.0743	5.1372	**0.0501**
% Reduced Glutathione	Error	6	0.0868	0.0145		
	Total	8	0.2353			
Odontaster validus	pH	2	0.0027	0.0014	0.12	0.887
Total Glutathione	Error	6	0.0669	0.0112		
	Total	8	0.0697			
Odontaster validus	pH	2	0.047	0.0223	1.4176	0.3132
% Reduced Glutathione	Error	6	0.0945	0.0157		
	Total	8	0.1391			
Patiriella regularis	pH	2	0.0011	0.0006	0.06	0.944
Total Glutathione	Error	6	0.0583	0.00972		
	Total	8	0.0594			
Patiriella regularis	pH	2	0.115	0.0575	3.285	0.1087
% Reduced Glutathione	Error	6	0.105	0.0175		
	Total	8	0.2200			

Discussion

This chapter has investigated the oxidative stress responses of echinoderm embryos from the species; *Sterechinus neumayeri*, *Odontaster validus* and *Patiriella regularis*, that have developed in seawater that has had the pH reduced by increasing the ρCO_2 within the seawater. In response to the first hypothesis: That seawater with a decreased pH caused by increased ρCO_2 will cause oxidative damage during the early embryonic development of *S. neumayeri*, *O. validus* and *P. regularis.* I have observed that there was an increase in oxidative stress as observed by increases in DNA damage through 8-OHdG per 10^6 dG to *S. neumayeri*, an increase in lipid hydroperoxide concentrations for *S. neumayeri* and *P. regularis* embryos and an increase in protein carbonyls for all three species. This demonstrated that although not all species showed increases in all of the oxidative damage markers that were measured, they all showed some form of oxidative damage in response to seawater with a lowered pH.

The second hypothesis proposed that, the increase in cellular damage caused by an increase in ROS in response to seawater with a decreased pH will result in an upregulation of antioxidant enzymes in *S. neumayeri*, *O. validus* and *P. regularis*. *S. neumayeri* showed an increase in SOD,

CAT, GR, GST and a decrease in total glutathione showing that for this species, our hypothesis was correct. For *O. validus* there was an increase in the concentration of glutathione reductase in response to the pH 7.6 treatments. *P. regularis* did not upregulate antioxidants in response to a decrease in seawater pH treatments.

Previous studies that have examined the effect of ocean acidification on oxidative stress have indicated that the mRNA that codes for antioxidant and molecular defence proteins are being regulated in response to ocean acidification (Todgham & Hofmann, 2009; Dilly et al., 2015). These techniques, however, do not specifically quantify the levels of antioxidants, the activities of enzymes associated with antioxidant metabolism, and hence possible post-transcriptional or translational regulation, or measure key indicators of oxidative damage. In the present study, quantifiable measurements of key oxidative damage indicators and the levels of antioxidants within the blastula were made, which gives a more direct indication to how the effects of reduced seawater pH on oxidative processes.

Oxidative damage caused by ocean acidification

In response to future ocean acidification scenarios, a reduction in seawater pH from the ambient pH 8.1 down to 7.6 causes a significant increase in the amount of DNA damage in the form of 8-OHdG formation, and increased levels of lipid hydroperoxides. For *S. neumayeri*, pH 7.8 and 7.6 resulted in an increase in the formation of protein carbonyls. This is the first documentation of oxidative damage being caused to marine invertebrate larvae in response to ocean acidification and alongside these measures of oxidative stress, the decrease in pH caused a significant increase in signs of abnormal development. *S. neumayeri* blastula that were reared in the lowered pH treatments were all larger. The increased abnormal development that was observed in this species is similar to other studies (Clark et al., 2009; Ericson et al., 2011; Byrne et al., 2013b). This study however focused on the blastula developmental phase where as other studies have made their measurements much later in development.

The Antarctic sea star species, *O. validus,* did not show any significant oxidative damage or any effect on blastula development within the pH treatments. Gonzalez-Bernat et al. (2012) found a significant reduction in larval survival in *O. validus* at pH 7.8 and 7.6. Interestingly they note that a difference in larval survival became apparent after 9 days of development in the same temperature as our experiment in the pH 7.6 treatment. It is suspected that if this experiment was run over a longer time period that 7 days a difference may have been observed for *O. validus*

in this study. They also noted that larvae in the pH 7.8 and 7.6 treatments were smaller than those raised in the ambient seawater with a pH of 8.1 (Gonzalez-Bernat et al., 2013).

For *P. regularis, d*evelopment in reduced seawater pH did cause an increase in the levels of lipid hydroperoxides and protein carbonyls when raised at pH 7.7. *P. regularis* showed increased levels of abnormal development at pH 7.7 treatment compared to the control. The blastula were also comparatively smaller suggesting that development has been impaired or slowed to deal with the increased stress of ocean acidification (Byrne et al. 2013). The decrease in larval size and increases in abnormal development in response to development in seawater with a reduced pH is consistent with studies by Byrne et al. (2013) on *P. regularis*.

To date, there are no ocean acidification studies that examine oxidative damage in *S. neumayeri*. However, it is possible to compare the effects of ocean acidification on oxidative damage in *S. neumayeri* against studies that have used UVR as their stressor. UVR is an important environmental stressor in which the oxidative damage has been measured as a biomarker of stress. The amount of damage to proteins and lipids at the most extreme pH treatment of pH 7.6 in this study were similar to that of the most extreme UV-R treatment in Lister et al. (2010). In addition, the levels of lipid hydroperoxide formation in blastula reared in pH 7.6 were similar to larvae exposed to a high dose of UVR. Importantly the experimental control treatments show similar amount of oxidative damage to proteins and lipids. For example, the control in Lister et al. (2010) had very similar levels to the present ambient treatment (ph 8.1) for lipid hydroperoxides.

For *S. neumayeri* and *P. regularis,* oxidative damage levels to proteins, lipids and DNA suggest that ocean acidification can cause oxidative stress at levels that are similar to those experienced under other environmental stressors such as UV-R.

Antioxidant responses to ocean acidification

Todgham & Hofmann (2009) suggest that development in seawater with a decreased pH either has little to no effect on cellular integrity, or, that the larvae of the purple sea urchin, *S. purpuratus*, do not have the capacity to maintain protective mechanisms and the cellular stress response pathway was impaired at a molecular level. This suggestion is supported by transcriptomic results that found lower levels of mRNA involved in antioxidant defences compared to their control in ambient seawater. The present results for *S. neumayeri* and to a lesser extent in *P. regularis* suggest that development in seawater with a decreased pH is having

an effect on cellular integrity. *S. neumayeri* did, to a limited extent, upregulate some antioxidants in response to increases in oxidative damage. However these increases were not sufficient to ameliorate the negative impacts of acidification on ROS for *S. neumayeri*. However, *O. validus* and *P. regularis* both had higher base line levels of antioxidants. These high baseline levels of antioxidants would help these two species buffer the effects of slight increases in oxygen free radicals caused by stressor events due to the already high presence of antioxidants (Halliwell & Gutteridge, 2007).

A decrease in seawater pH from the ambient 8.1 to 7.6 caused *S. neumayeri* to upregulate SOD and CAT. What is interesting here is the lack of change in SOD and CAT antioxidant enzyme levels in *O. validus* and *P. regularis*. These enzymes did not change significantly across the treatment groups and were also expressed in naturally high levels in the control groups. The levels for the *O. validus* and *P. regularis* were very similar to the levels seen in *S. neumayeri* in the pH 7.6 treatment where they were significantly stressed. These initially high enzyme concentration levels may offset any increase in free radical species while they are developing in low pH seawater. This effect has been seen before within different seaweed species that existed in environments of varying stress tolerances. Seaweeds that lived in high stress environments were able to respond to stress events faster than those from low stress habitats (Collen & Davison, 1999). In tropical corals, mRNA levels coding for genes that initiate the oxidoreductase pathway (including catalase) were upregulated in reduced seawater pH (Kaniewska et al., 2012). This is important as it shows that a hypercapnia environment does not just suppress metabolism as some have suggested, but metabolic suppression may be an additional mechanism to maintain protein integrity (Todgham & Hofmann, 2009; Stumpp et al., 2011b; Kaniewska et al., 2012).

For *S. neumayeri*, the enzymatic antioxidants GR, GPOX and GST increased in response to the lowest pH 7.6 treatment. GR also increased significantly in response to pH 7.8 compared to the control. This response along with the increased levels of CAT suggests that they are using both the metabolism of glutathione and CAT to help breakdown H_2O_2. Typically, invertebrates use catalase to dismutate H_2O_2 and the levels measured in this study are slightly higher than observed in some invertebrates (see Livingstone et al. (1992), however this could have been due to their use of partially purified protein extracts for the assays as opposed to the more commonly used approach of using crude protein extracts. *O. validus* had a significant increase in GR levels in the pH 7.6 treatment compared to the pH 7.8 and ambient seawater treatment. The levels of GR, GPOX and GST in all experimental treatments were expressed in concentrations greater than that

of *S. neumayeri*. This suggests that these species are able to utilize both the CAT and the glutathione antioxidant defence systems which would increase their tolerance to a stressor that potentially increases the cellular levels of H_2O_2 (Halliwell & Gutteridge, 2007; Ezraty et al., 2011).

Measuring total levels of glutathione, GSH (the reduced form that can be oxidised) and GSSG (the oxidised form of GSH) can be used as a measure of the effect low of pH on oxidative responses (Halliwell & Gutteridge, 2007). In *S. neumayeri* in response to seawater pH 7.8, high levels of total glutathione were maintained by the blastula, and there was no significant difference in the amount of glutathione that is still able to be oxidised. In the pH 7.6 treatments, there was significantly less glutathione in *S. neumayeri* blastula compared to controls and there is significantly less GSH available to be oxidised by free radical molecules. This is of interest as not only are the blastula not producing as much antioxidant as the control group, but much more of what is available has been oxidised already. This indicates that the blastula may not be coping in seawater with a pH of 7.6 or lower as the larvae were not restoring the GSSG back to GSH, leaving them vulnerable to more oxidative damage (Halliwell & Gutteridge, 2007).

These high levels of enzymatic antioxidants, and in particular, CAT and GPOX suggests that the higher baseline levels of antioxidants in the sea stars are allowing these species to cope with the increased physiological stress of a lowered pH environment. *P. regularis* is a shallow intertidal species that can be found within harbours and is a known invasive species around the coasts of Australia that can cope with high environmental fluctuations of temperature, salinity and light (Byrne, 2012). In other biological systems red seaweed species that are more stress tolerant typically contained higher levels of antioxidant enzymes and suffered less oxidative damage in experimental stress experiments. Species that are not as stress tolerant show higher amounts of oxidative damage and are not able to produce high levels of antioxidants to respond adequately to the stressor (Collen & Davison, 1999). This indicates that a species ability to react to a stress event is highly linked to the habitat and the normal stress regimes that it can tolerate. The Antarctic benthic habit is considered to be environmentally stable and the biotic factors that influence the species there are predation and competition and the fauna that inhabit these water have been isolated since the opening of the Drake passage and the development of the circumpolar current between 47 and 17 million years age (Arntz et al., 1994). Due to this stability, many Antarctic species have a low tolerance to environmental change and coupled with the chemistry of cold water which hold more CO_2 than tropical regions, gives them less time to adapt to these changing conditions (Fabry et al., 2009; Doney et al., 2009). This means that any stress

response and ability to cope will likely have to come from evolutionary conserved responses. The results here show that the antioxidant response of *O. validus* is more likely to be able to survive future climatic change that inflicts oxidative damage compared to *S. neumayeri*.

Effects of Ocean Acidification on oxidative stress

Ocean acidification inflicts molecular damage and affects protein integrity and this is a real problem with future climate change scenarios predicting decreases in ocean pH. Todgham & Hofmann (2009) have observed in the larvae of the purple sea urchin, *Strongylocentrotus purpuratus*, suppression in the mRNA that codes for the genes responsible for maintaining protein integrity and those that code for defence against oxidative damage. Specifically this included mRNA that codes for GST and GPOX which are both responsible for the detoxification of H_2O_2 and lipid hydroperoxides in the case of GST (Lesser, 2006; Todgham & Hofmann, 2009). This study demonstrates that understanding posttranscriptional and post translation regulation could be as important as transcriptomic analysis in our understanding of marine animals to OA. Todgham & Hofmann (2009) note that in *S. purpuratus* the cellular stress response has been impaired at a molecular level when exposed to lowered seawater pH and that this will impair the organism to respond to other stressors which require similar defence mechanisms, like that of UV-R (Adams et al., 2012).

A high CO_2 environment exacerbated the toxicity of H_2O_2 and OH^-, which resulted in an increase in DNA damage caused through 8-oxoguanine oxidation and increased levels of protein carbonyls in *E. coli* bacteria (Ezraty et al., 2011). If a similar mechanism is occurring in seawater this would help explain one of the mechanisms behind the oxidative damage observed in *S. neumayeri* and *P. regularis* in this study. The ability of *O. validus* to produce high levels of catalase and glutathione would offset this effect and could explain why these two enzymes responsible for H_2O_2 removal are expressed in high quantities. This effect of increased CO_2 causing more oxidative damage to DNA is worrying because the experimental treatment levels of atmospheric CO_2 that were used in the study on bacteria are within the same time frame of our experimental treatments in which a decrease of seawater pH to 7.6 is expected. Low seawater pH and or high ρCO_2 may also be affecting the functioning of the mitochondria causing the production of more radical species as well disrupting some of the pH specific enzyme functions that occur within the mitochondrial election transport chain (Tomanek et al., 2011; Starkov, 2006; Murphy, 2009). Cold related membrane adaptations such as higher proportions of polyunsaturated fatty acids may increase the “leakage” of ROS from the mitochondria into the cellular environment in the

Antarctic species. This would make them more vulnerable to oxidative stress (Lesser, 2006; Abele & Puntarulo, 2004; Lurman et al., 2010b, 2010a).

Impacts of future ocean acidification on *S. neumayeri*, *O. validus* and *P. regularis*

S. neumayeri may be able to cope with the oxidative damage caused by near-future changes. However the mid-future scenario of seawater pH 7.6 caused a high level of oxidative stress. In a more realistic multi-stressor environment involving increases in ocean temperature and increased UVR exposure with early sea ice break out in Polar Regions, *S. neumayeri* and *O. validus* may not be able to cope with the increased environmental pressures. *S. neumayeri* shows a limited ability to respond to the increases in oxidative stress and cellular damage at pH 7.6. Byrne et al. (2013) and Davis et al. (2013) have both showed that organism responses to multiple stressors do not just act cumulatively but can have interactive effects. In some cases the effects of slight increases in temperature can offset the negative effects of OA (Byrne et al. 2013). However the effects of ocean warming offsetting the negative effects of ocean acidification are more prevalent in later developmental stages than we have tested in this chapter. Many of the interactions among the stressors relevant to the marine environment are not fully understood and their influence on physiological and molecular stress levels is largely unknown (Häder et al., 2014).

The sea stars in the present experiments appear to have a high tolerance towards environmental stressors based on the higher basal levels of antioxidant enzymes and glutathione based antioxidants. For *P. regularis* these high basal levels of antioxidants may be passed to the larvae maternally as the adult specimens are found in the highly variable intertidal zone of the Otago Harbour. The instability of conditions within intertidal environments like light, salinity, pH, oxygen and temperatures may help precondition the organisms found in these environments to be preconditioned to stress events (Tomanek & Elmuth, 2002). This preconditioning to stress in adult individuals may allow females to produce more resilient offspring. This stress preconditioning has been seen in gametes from *S. neumayeri* that are produced from mothers that come from highly polluted sites compared to those from pristine sites. The offspring from the polluted sites have been shown to have higher levels of antioxidants than those that come from pristine sites (Lister et al., 2015). This may help larvae survive in a stressful environment but can come with the resource trade off of decreased fecundity in *S. neumayeri* and *Evechinus chloroticus* (Lister et al., 2015). This could provide an opportunity for these species to continue to reproduce successfully in stressful near future environments but for how long will these species be able to increase the resilience of their offspring?

Summary

- Ocean acidification does cause oxidative stress in *S. neumayeri*. There was an increase in the number of abnormal larvae in the acidified treatments and an increase in the cross sectional area of the blastula. 8-OHdG formation was observed in response to the pH 7.6 treatment alongside higher levels of lipid hydroperoxides and protein carbonyls.
- The increase in oxidative stress also caused limited increases in the activities of the antioxidant enzymes SOD, CAT, GR, GPOX and GST in *S. neumayeri*. The non-enzymatic antioxidant glutathione (GSH + GSSG) decreased in response to pH 7.6.
- Ocean acidification did not cause any significant effect on the development of *O. validus*. There was a significant increase in the number of abnormal larvae for *P. regularis* and a decrease in the mean cross sectional area. But, the increase in abnormal larvae was small. Both species showed little increases in the amount of 8-OHdG and lipid hydroperoxide levels. *P. regularis* did show an increase in the concentration of protein carbonyls in the pH 7.7 treatment.
- Antioxidant enzymes in *P. regularis* and *O. validus* both showed higher basal levels of SOD, CAT, GR, GPOX and GST, than *S. neumayeri*, but levels did not increase consistently in response to acidification. The higher basal levels of these enzymes could aid in limiting the effects of a high ρCO_2 environment. Hence it appears that these two starfish species are better equipped to deal with acidification than *S. neumayeri*.

Chapter 3 The effect of ocean acidification and UV-R exposure on oxidative stress in *Sterechinus neumayeri*

This chapter investigates the effects of ocean acidification and ultraviolet radiation (UV-R) exposure on the production of oxygen free radicals and antioxidants in the early developing embryos of the Antarctic echinoid, *Sterechinus neumayeri*. UV-R is a biological stressor that is well known to cause oxidative damage in *S. neumayeri* and the previous chapter documents that ocean acidification can cause oxidative damage and elicit cellular stress response by increasing antioxidants enzymes (Lesser, 2006; Lesser & Barry, 2003; Lister et al., 2010; Lamare et al., 2011). This chapter will address that the development of *S. neumayeri* embryos in seawater with a decreased pH will be additive with exposure to a second stressor UV-R. This will result in high levels of oxidative damage with limited antioxidant upregulation in response.

Introduction

Climate change processes include a number of important physiological stressors interacting within ecosystems. The world's oceans are already a place of multiple stressors with interactions influencing the biota within these systems. These stressors include, but are not limited to, temperature changes, oxygen fluctuations, ultraviolet radiation, pollution, fertilizer loading in coastal environments and nutrient limited environments (Byrne, 2012; IPCC, 2013). Investigating the potential effects of future climate change on biology therefore requires combining multiple stressors experimentally to investigate potential interactions to determine if they are synergistic, additive or cancel one another out (Byrne, 2012).

The larval stages of marine invertebrates are a potential ecological bottleneck as these life history stages have been identified as the one of the most vulnerable. Small decreases in fitness and developmental delays can have large implications for populations to be able to maintain their size and structure (Eckman, 1996). These life history phases are the most vulnerable to environmental changes. Larval stages and early developing embryos are vulnerable because of their small size, rapid cellular division and limited maternal resources to deal with both a stressful environment and progressing growth (Lamare et al., 2011; Byrne, 2012).

The interactive effects of multiple environmental stressors on invertebrate larvae have been largely examined in laboratory experiments, and the responses have varied between invertebrate phyla and species. For example, a 3°C increase in temperature can diminish the negative effects

of hypercapnia on growth in the echinoid, *Tripneustes gratilla* (Sheppard Brennand et al., 2010). The effects of increased salinity and temperature stress on the mortality and developmental abnormalities in the molluscan larvae *Bembicium nanum* and *Siphonaria denticulata* was exacerbated by exposure to UV-R (Przeslawski et al., 2005). A temperature increase of 2 and 4°C and a decrease in pH of 7.8 to 7.6 resulted in unshelled larvae and abnormal juveniles in *Haliotis coccoradiata* (Byrne et al., 2011). In the same study, Byrne et al. (2011) found that the echinoid species *Heliocidaris erythrogramma* showed abnormal development in response to both of the stressors, and while a 2°C increase in water temperature negated the effects of low pH, a 4°C increase breached the developmental thermal tolerance and resulted in significant levels of stress.

A metadata study by Przeslawski et al. (2015) on multiple stressor studies for marine embryos and larvae has shown that the larvae are more vulnerable than the embryos to thermal and pH stress. The response to specific stressors vary across ontogenetic stages and species, however there are some more consistent responses to the stressors between phyla. Ultimately the analysis suggested that the effect of OA is greater for calcifying larvae than for non-calcifying (Przeslawski et al., 2015).

The response of *S. neumayeri* larvae to ocean acidification has been documented by an increasing number of studies. When *S. neumayeri* larvae are reared in seawater with a decreased pH, their ability to develop normally has been mixed. Some studies have found no decreases in the normal development of early larvae in their study (Clark et al., 2009; Yu et al., 2013) but others have found a negative response to a lowered pH (Ericson et al., 2011; Byrne et al., 2013b). The lowering of seawater pH to around 7.6 has typically caused larvae to be smaller than the control treatments and can increase the length of time it takes embryos to hatch (Clark et al., 2009; Ericson et al., 2011; Byrne et al., 2013b; Yu et al., 2013). Yu et al. (2013) suggest that this delay in hatching is indicative of an environmental stress response. Multiple stressor responses of *S. neumayeri* to ocean acidification and warming show that the increase in temperature of 2°C did not offset the effects of ocean acidification and decreased pH acted as a teratogen and disrupted developmental planning (Byrne et al., 2013b).

UV-R can influence the lives of organisms that live within the surface waters of the ocean (Häder et al., 2014). It has the potential to act as a major biological stressor that can cause direct biological damage (Tedetti & Sempere, 2006; Häder et al., 2007; Lamare et al., 2011). For *S. neumayeri*, increased UV-R through the development an ozone hole can increase the penetration

of damaging light through the sea ice each Austral spring. The development of the ozone hole coincides with the beginning of the spawning season for *S. neumayeri* (Bosch et al., 1987; Solomon, 1999; Lesser, 2006; Lamare et al., 2011). Coupled with decreases in stratospheric ozone is the threat of sea ice loss. This loss may further increase UV-R exposure as sea ice cover has a high albedo effect (Lesser et al., 2004; Overpeck et al., 2006). When *S. neumayeri* is exposed to UV-R there is an increase in oxidative stress through the accumulation of reactive oxygen species and an increase in direct DNA damage through the accumulation cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (Lesser, 2006; Lister et al., 2010; Lamare et al., 2011). This exposure to UV-R can also cause larvae and embryos to develop abnormally and slow their development (Lesser et al., 2004; Lister et al., 2010).

The majority of marine climate change experiments use isolated stressors and investigated the responses. The results of these studies are useful, however they are difficult to extrapolate into the ecosystem as they do not take into account the interactions among multiple stressors. These stressors do not act alone in the coastal environment and can have greater synergistic effects (Lamare et al., 2011; Byrne, 2012) so it important to understand how these stressors will interact in the future.

UV-R is a stressor that can act synergistically with other environmental variables, including decreases in seawater pH. Exposure to UV-R may exacerbate the detrimental effects of reduced seawater pH on marine algae (Gao et al., 2009; Gao & Zheng, 2010; Xu & Gao, 2015). Ocean acidification increased developmental aberrations and mortality in the molluscan larvae which was offset with increased temperature. Increased temperature also increased the rate of development as expected, however UV slowed development but had minimal impact of the development of the larvae (Davis et al., 2013). This shows that the interactions between stressors is complex, species specific and not always as expected, even when a stressor that is known to cause direct damage to cellular components as well as increasing oxidative damage is involved (Lesser, 2006).

To assess the interactive effects of ocean acidification and UV-R exposure on oxidative damage and the antioxidant response of *S. neumayeri* larvae, embryos were exposed post-fertilisation to a range of seawater pH treatments and then, after 7days of development, the larvae were exposed to a single dose of environmentally relevant UV-R. This chapter will investigate our hypothesis that the development of *S. neumayeri* embryos in seawater with a decreased pH will be additive

with exposure to a second stressor UV-R. This will result in high levels of oxidative damage with limited antioxidant upregulation in response.

Methods

Animal Collection

The Antarctic sea urchin *Sterechinus neumayeri* Meissner (Echinidae), were collected from Cape Evans (77.38°S, 166.24°E) by divers and a remotely operated vehicle (ROV). Urchins were kept in a flow through system with seawater at ambient -1.9°C temperature until they were spawned at Scott Base, Antarctica.

Spawning

Reproductively mature *S. neumayeri* were induced to spawn by an intracoelomic injection of 2 mL of 0.5M potassium chloride (KCl). Injected animals were inverted over appropriate sized beakers that contained filtered seawater to collect the gametes. Once gamete release had stopped, the animals were removed and the gametes were washed by several partial water changes. Eggs were checked for health and the sperm for activity. In order to gain sufficient material for experiments the eggs from 13 females were combined and fertilised in filtered seawater of ambient pH and temperature using several drops of sperm diluted with filtered seawater from 3 ripe males (final concentration $10^5 – 10^6$ sperm mL). Fertilisation was determined by the appearance of the fertilisation envelope, with fertilisation success observed to be greater than 95%. After five minutes the freshly fertilised eggs were washed with fresh filtered seawater to remove excess sperm.

Acidification of seawater

The pH of the 0.22µm filtered seawater was adjusted by bubbling pure CO_2 gas into ambient seawater ($pH_{(NBS)}$ 8.1) to achieve three pH levels ($pH_{(NBS)}$ 8.1, 7.8 and 7.6). Once stabilisation of the seawater at the desired pH was achieved, the water containers were sealed to prevent re-equilibrilisation with the atmosphere. The pH was checked using a pH meter (Mettler Toledo MP220) and calibrated to buffers of pH 4.0, 7.0 and 9.2 (Labserv Pro-analysis, Biolab New Zealand). Once calibrated to the buffers, we used a TRIS seawater buffer to calibrate the pH meter to the conductivity of the seawater. All pH measurements were adjusted for differences in seawater temperature. 1L Seawater samples were collected from each treatment and fixed with saturated mercuric chloride for later analysis of carbonate parameters at the end of the

experiment Table 10. All pH levels were ± 0.03 of the desired pH units at the beginning and conclusion of the experiment.

Table 10: Temperature (°C), salinity (PSU), pH $_{(NBS)}$, total alkalinity (μmol kg^{-1} $soln^{-1}$), partial pressure of CO_2 (ρCO_2), $CaCO_3$ saturation for calcite (Ω_C) and aragonite (Ω_A) for ambient and treated seawater for *Sterechinus neumayeri*.

(a) *Sterechinus neumayeri*, McMurdo Sound, Ambient T = -1°C, ambient salinity = 34.8			
pH $_{(NBS)}$	8.10	7.81	7.62
Alkalinity (A_T)	2351	2328	2388
ρCO_2	411	856	1389
Ω $_{Calcite}$	2	1.1	0.7
Ω $_{Aragonite}$	1.2	0.7	0.4

Exposure to OA conditions

Larvae were placed into 20 litre containers at a density of 21 larvae per ml and allowed to develop for 7 days in sealed containers within 3 pH treatments; (1) A control (ambient seawater pH 8.1), and (2) moderately reduced seawater pH (pH 7.8) and (3) low seawater pH (pH 7.6) treatments, with 3 replicates for each treatment. Treatments were kept cold (-1°C) by placing buckets in a flow through seawater system with seawater being pumped directly from the ocean at Scott Base. The carbonate chemistry is presented in Table 10. This experiment was run concurrently with the OA experiment with *S. neumayeri* in Chapter 2.

UV-R exposure

Seven-day old embryos were split into two irradiance treatments and placed under two FS20 UV lamps for a 1 hour exposure in 2 litre containers with a larval density of 50 larvae per ml. There were 3 replicates for each light treatment. Containers were placed into a water bath within a flow through seawater system to keep embryos cool and at the ambient seawater temperature. The 2 UV lamps were suspended 30cm above to top of the 2 litre containers. Containers were placed under Plexiglas UV filters, one blocking wavelengths smaller than 290nm, exposing embryos to UV-B, UV-A and PAR (Full UV-R treatment). The second filter blocked wavelengths smaller than 400nm exposing embryos to PAR only (UV-R blocked treatment. Embryos were exposed for one hour underneath the lamps, the dosage of light is given in Table 11. The spectral irradiance was measured using a scanning spectral radiometer (LiCor 1800UW).

Larval preservation morphological measurements

A sample of larvae were fixed in a 10% solution of buffered formalin and seawater to be photographed at a later date. Once photographed 15 embryos from each sample of *S. neumayeri* were scored for abnormality and their cross sectional area measured using Image J using the magic wand tool to select the outside edges of the blastula. The measurements were calibrated using a calibration bar photographed at the same magnification and the number of pixels converted to μm^2. Embryos were scored as abnormal if they showed one or more of the following traits; pronounced thickening of the blastoderm in combination with a reduction of the blastocoels, abnormal development of primary mesochyme cells, occlusion of the blastocoel by cellular debris, exogastrulation or any other forms of aberrant archenteron development, an example of this can been seen in Figure 9 (See Lamare et al., 2007; Lister et al., 2010).

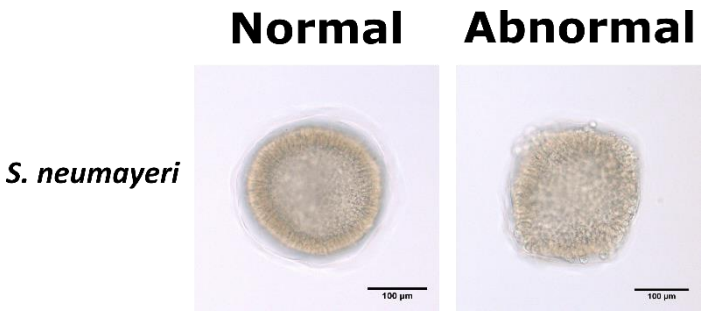

Figure 9: An example of a normal and an abnormal embryo for *Sterechinus neumayeri* photographed under 10x magnification.

Larval preservation for biochemical assays

After the exposure to the light treatments larvae were filtered out of the water using a sieve with a 50µm filter mesh and pipetted into micro centrifuge tubes in lots of 20,000 larvae. The tubes were spun down in a Minispin centrifuge for 20 seconds and the excess seawater removed. Preservation was by freezing with liquid nitrogen. Samples were placed into storage at -80 °C until the extractions were performed.

Biochemical assays

All methodologies are as detailed in Chapter 2.

Statistical analysis

All data points were transformed using natural logs except the abnormality and the % reduced glutathione data which was arcsine square root transformed so as to meet the assumptions of

ANOVA. Statistically significant differences in oxidative stress measurements among pH and UV levels, and their interactions, were tested using a 2-way ANOVA using Minitab statistical software with the independent variables of pH and UVR. pH has 3 levels; pH 8.1, pH 7.8 and pH 7.6. UVR has 2 levels, full UV spectrum and UV blocked. The residuals were graphed and checked for normality and homogeneity of variances and were normally distributed. Significance levels were set at 0.05.

Results

For S. neumayeri, the $\rho CO2$ was 411 µatm under ambient conditions. For the treated water the $\rho CO2$ increased two fold to 856 µatm for the pH 7.8 treatments and increased more than fourfold to 1389 µatm. Calcite and aragonite were both saturated in the pH 8.1 treatment (ΩC = 2; ΩA = 1.2). Calcite was saturated but aragonite was under saturated for the pH 7.8 treatment (ΩC = 1.1; ΩA = 0.7) and for the pH 7.6 treatment both aragonite and calcite were under saturated (ΩC = 0.7; ΩA = 0.4) (Table 10).

UV-R dose

The UV-B irradiance and the dose received by the embryos in this experiment were ~3-fold greater than that at the surface of McMurdo Sound on the 6 November 2012 (Table 11), while the UV-A irradiance and dose was similar underneath our experimental UV lamps to ambient surface doses recorded for McMurdo Sound. The total UV-R irradiance and dose was similar between the experiment and ambient levels. The maximum irradiance and total dose of visible light were 4.8 and 3.6 fold lower respectively in the experimental conditions compared to the ambient levels (Table 11).

Table 11: Experimental irradiances underneath experimental lamps and at Ross Island, McMurdo Sound, comparing maximum irradiances received and the total dose of UV-A, UV-B and visible light for 1 hour at midday on the 6th of November.

Experimental irradiances and springtime radiances				
	Max. Irradiance (W m^{-2} s^{-1})		**Total dose (kJ m^{-2})**	
	Laboratory lights	Ambient 6 Nov. 2012	Laboratory lights	Ambient 6 Nov. 2012
UV-B	2.96	0.84	10.64	3.02
UV-A	17.75	18.39	63.90	66.19
UV-R	20.71	19.23	74.55	69.22
VIS	23.90	115.59	189.52	675.16
UV-B:PAR	0.12	0.007		
UV-A, ultraviolet-A light (320-400 nm); UV-B, ultraviolet-B radiation (300-320 nm); VIS, visible light (400-600 nm); UV-B:PAR, ratio of ultraviolet-B radiation to visible light.				

Oxidative damage and abnormal development

There was no significant difference in the proportion of abnormally developed blastula among pH or UV treatments (Figure 10, Table 12).

Blastula size was significantly smaller in UV-R exposed blastula with UV-R blocked blastula 2.023 x 10^5 (± se= 0.587 x 10^5) μm^2 compared to the UV-R exposed blastula being 1.731 x 10^5 (± se= 0.359 x 10^5) μm^2 for the pH 8.1 treatment (Figure 10, Table 12). Exposure to sea water with a lowered pH had no significant effect among treatments (Figure 10, Table 12). There was no significant interaction between pH and UV-R treatments on the cross sectional area of the blastula (Table 12).

Oxidative damage caused to DNA through 8-OHdG formation significantly increased with exposure to the full UV spectrum, and with development in a lower pH (Figure 11, Table 13). 8-OHdG formation increased from 1.52 (± se= 0.108) to 5.11 (± se= 0.334) 8-OHdG per10^6 dG for the pH 8.1/PAR-only treatment to the pH 8.1 treatment exposed to the full UV spectrum. For the pH 7.6 treatment damage increased from 4.35 (± se= 0.118) to 12.90 (± se= 0.888) 8-OHdG per 10^6 dG for the treatment exposed to PAR compared to the treatment exposed to the full UV spectrum. There was no significant interaction on oxidative damage caused to DNA between pH and UV treatments (Figure 11, Table 13).

There were significant increases in the amount of oxidative damage caused to lipids for those treatments reared in lowered pH sea water and exposed to UV-R (Figure 11, Table 13). The

concentration of lipid hydroperoxides increased from 3.15 (± se= 0.195) to 9.84 (± se= 0.811) nmol of lipid hydroperoxide per 20,000 blastula for the pH 8.1 treatment only exposed to PAR compared to the pH 8.1 treatment exposed to the full UV spectrum. For the pH 7.6 treatment the damage ranged between 7.98 (± se=0.602) and 22.71 (± se=1.402) nmol of lipid hydroperoxide per 20,000 blastula for the treatment exposed to PAR compared to the treatment exposed to the full UV spectrum (Figure 11). There was no significant interaction effect between pH and UV treatment on lipid hydroperoxide levels (Table 13).

Protein carbonyls were significantly greater in those treatments exposed to low pH seawater and UV-R. The increase in damage as pH decreased with the additional stressor caused the concentration of protein carbonyls to range between 1.261 (± se=0.010) and 1.931 (± se=0.110) nmol per mg of protein for embryos exposed to sea water with a pH of 8.1 and exposed to PAR or UV-R, respectfully. For sea water with a pH of 7.6 the concentrations of protein carbonyls ranged between 2.382 (± se=0.118) and 4.366 (± se=0.139) nmol per mg of protein for embryos exposed to sea water with a pH of 7.6 and exposed to PAR or UV-R respectfully. There was a significant interaction between those treatments in lowered pH conditions and those exposed to UV-R (Figure 11, Table 13).

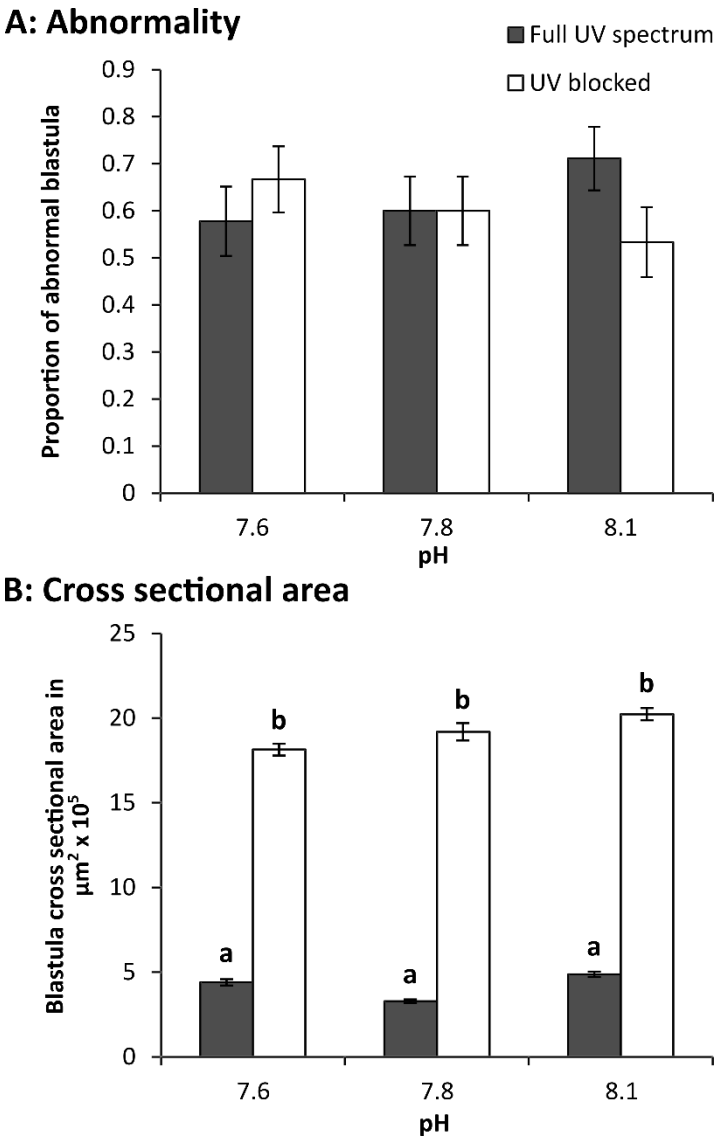

Figure 10 A: Mean proportion of abnormal blastula ± SE (n=18), for 3 pH treatments and two irradiance treatments. pH 8.1 is the ambient sea water in each experiment. **B:** Mean cross sectional area ± SE (n=302). Grey bars represent blastula that have been exposed to artificial light underneath filters that allowed wavelengths greater than 280nm (UV-B, UV-A and PAR) to pass through. White bars represent blastula that have been exposed to wavelengths greater than 400nm (PAR only) underneath UV filters. Statistical significance determined with a 2-way ANOVA with a Tukey's post-hoc test. Within each graph, columns sharing the same letter are not significantly different at a significance level of $p \leq 0.05$.

Table 12: Two-way ANOVA of abnormality and cross sectional area of embryos that have developed in seawater with lowered pH and have been exposed to two artificial light treatments underneath filters that allowed wavelengths greater than 280nm (UV-B, UV-A and PAR) or wavelengths greater than 400nm (PAR only). Statistical significance determined with a two-way ANOVA.

Parameter	Source	DF	Sum of Squares	Mean Squares	F	p- value
Abnormality	pH	2	0.06877	0.03439	0.455	0.6449
	UV Treatment	1	0.00653	0.00653	0864	0.7738
	Interaction	2	0.06064	0.03032	0.4012	0.6782
	Error	12	0.90683	0.07557		
	Total	17	0.9996			
Blastula cross sectional area	pH	2	0.614	0.307	6.91	**0.001**
	UV Treatment	1	142.525	142.525	3209.28	**<0.001**
	Interaction	2	0.008	0.004	0.09	0.917
	Error	297	13.190	0.044		
	Total	302	163.511			

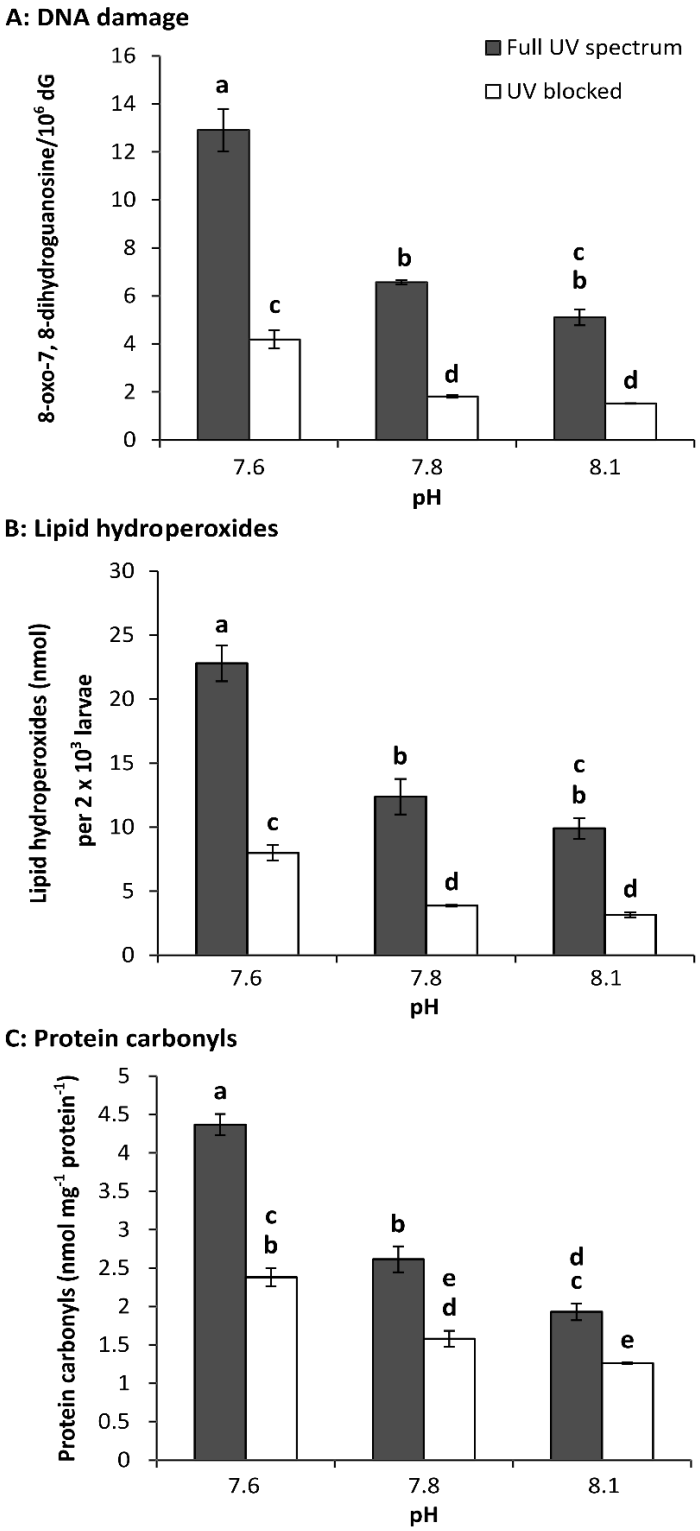

Figure 11 A: DNA damage measured in 8-hydroydeoxyguanosine/ 10^6 dG ± SE (n=18), for 3 pH treatments and two irradiance treatments. pH 8.1 is the ambient sea water in each experiment. **B:** nmol lipid hydroperoxide levels per 20,000 larvae ± SE (n=18). **C:** Protein carbonyls (nmol mg^{-1} protein) ± SE (n=18). Grey bars represent blastula that have been exposed to artificial light underneath filters that allowed wavelengths greater than 280nm (UV-B, UV-A and PAR) to pass through. White bars represent blastula that have been exposed to wavelengths greater than 400nm (PAR only) underneath UV filters. Statistical significance determined with a 2-way ANOVA with a Tukey's post-hoc test. Within each graph, columns sharing the same letter are not significantly different at a significance level of $p \leq 0.05$.

Table 13: Two-way ANOVA of DNA damage, lipid hydroperoxides and protein carbonyls of embryos that have developed in seawater with lowered pH and have been exposed to two artificial light treatments underneath filters that allowed wavelengths greater than 280nm (UV-B, UV-A and PAR) or wavelengths greater than 400nm (PAR only). Statistical significance determined with a two-way ANOVA.

Parameter	Source	DF	Sum of Squares	Mean Squares	F	p- value
8-hydroydeoxyguanosine/ 10^6 dG	pH	2	3.0906	1.5453	163.32	**<0.001**
	UV Treatment	1	6.5825	6.5825	695.67	**<0.001**
	Interaction	2	0.0195	0.0098	1.03	0.386
	Error	12	0.1195	0.0098		
	Total	17	9.8061			
Lipid hydroperoxides	pH	2	2.5438	1.2719	80.85	**<0.001**
	UV Treatment	1	5.5920	5.5920	355.46	**<0.001**
	Interaction	2	0.0095	0.0047	0.30	0.745
	Error	12	0.1888	0.0157		
	Total	17	8.3341			
Protein carbonyls	pH	2	0.7875	0.3937	109.63	**<0.001**
	UV Treatment	1	0.5586	0.5586	155.53	**<0.001**
	Interaction	2	0.0318	0.0159	4.42	**0.036**
	Error	12	0.0431	0.0036		
	Total	17	1.4209			

Enzymatic antioxidant activities

Superoxide dismutase (SOD) significantly increased with decreasing seawater pH and with exposure to UV-R. SOD concentrations were lower in the pH 7.6 full UV spectrum (271.51 units^{-1} protein min^{-1} ± se=7.506) than the pH 7.6 UV was blocked treatment (367.97 units^{-1} protein min^{-1} ± se=2.887) (Figure 12, Table 14). There was a significant interaction in the response of SOD between pH and UV treatment caused by the level of SOD decreasing in the pH 7.6 full UV spectrum treatment (Figure 12, Table 14).

Catalase (CAT) did not significantly increase in response to pH. CAT did however, increase significantly in response to UV-R exposure and there was a significant interaction between pH and UV treatment (Figure 12, Table 14). CAT was significantly higher in pH 8.1 and pH 7.8 treatments that were exposed to the full UV spectrum, compared to those that were in the UV blocked treatment (Figure 12).

Glutathione reductase (GR) significantly increased in response to reduced seawater pH and UV-R exposure. There was a significant interaction between treatments caused by a decrease in GR

concentration in the pH 7.6 full UV spectrum treatment that was less that the pH 7.6 UV blocked treatment (Figure 13, Table 14).

Glutathione peroxidase (GPOX) significantly increased in response to reduced seawater pH and UV-R treatment. There was also a significant interaction between pH and UV treatment. This interaction between pH and UV treatment can be seen in Figure 13 with the pH 7.6 full UV spectrum treatment in which the mean is lower than that of the pH 7.6 UV blocked treatment.

Glutathione S-transferase (GST) significantly increased with exposure to pH but did not vary significantly between the UV treatments. There was no significant interaction between pH and UV treatment affecting GST (Figure 13, Table 14).

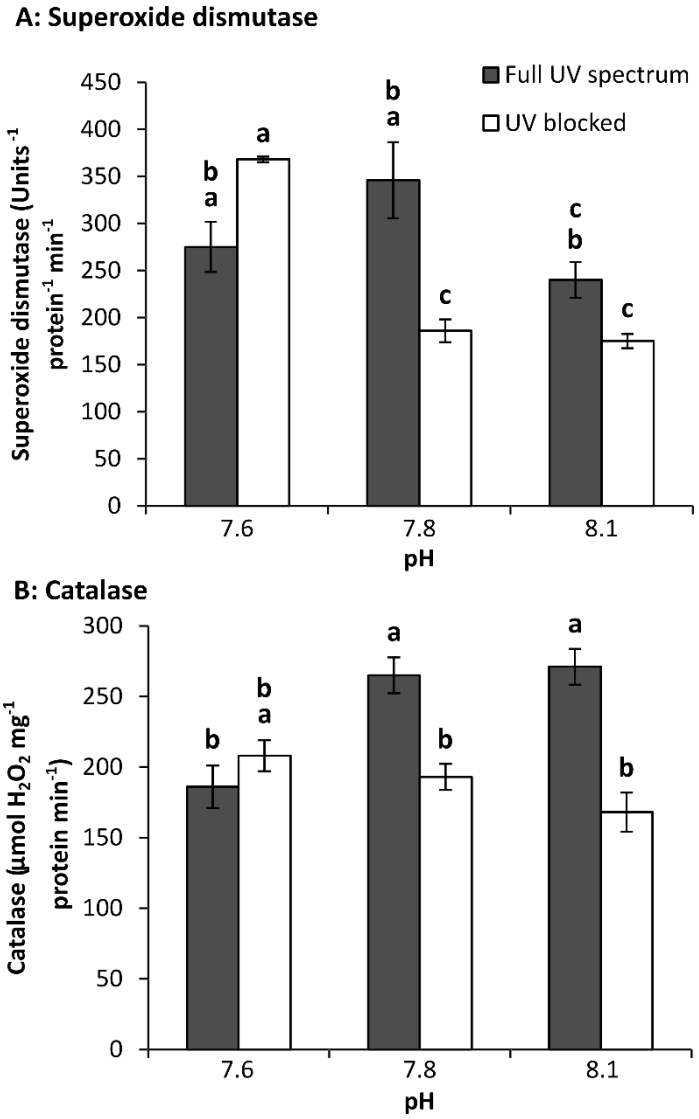

Figure 12: A: Superoxide dismutase (units^{-1} protein min^{-1}) ± SE (n=18), for 3 pH treatments and two irradiance treatments. pH 8.1 is the ambient sea water in each experiment. **B:** Catalase (µmol H_2O_2 mg^{-1} protein min^{-1}) ± SE (n=18). Grey bars represent blastula that have been exposed to artificial light underneath filters that allowed wavelengths greater than 280nm (UV-B, UV-A and PAR) to pass through. White bars represent blastula that have been exposed to wavelengths greater than 400nm (PAR only) underneath UV filters. Statistical significance determined with a 2-way ANOVA with a Tukey's post-hoc test. Within each graph columns sharing the same letter are not significantly different at a significance level of $p \leq 0.05$.

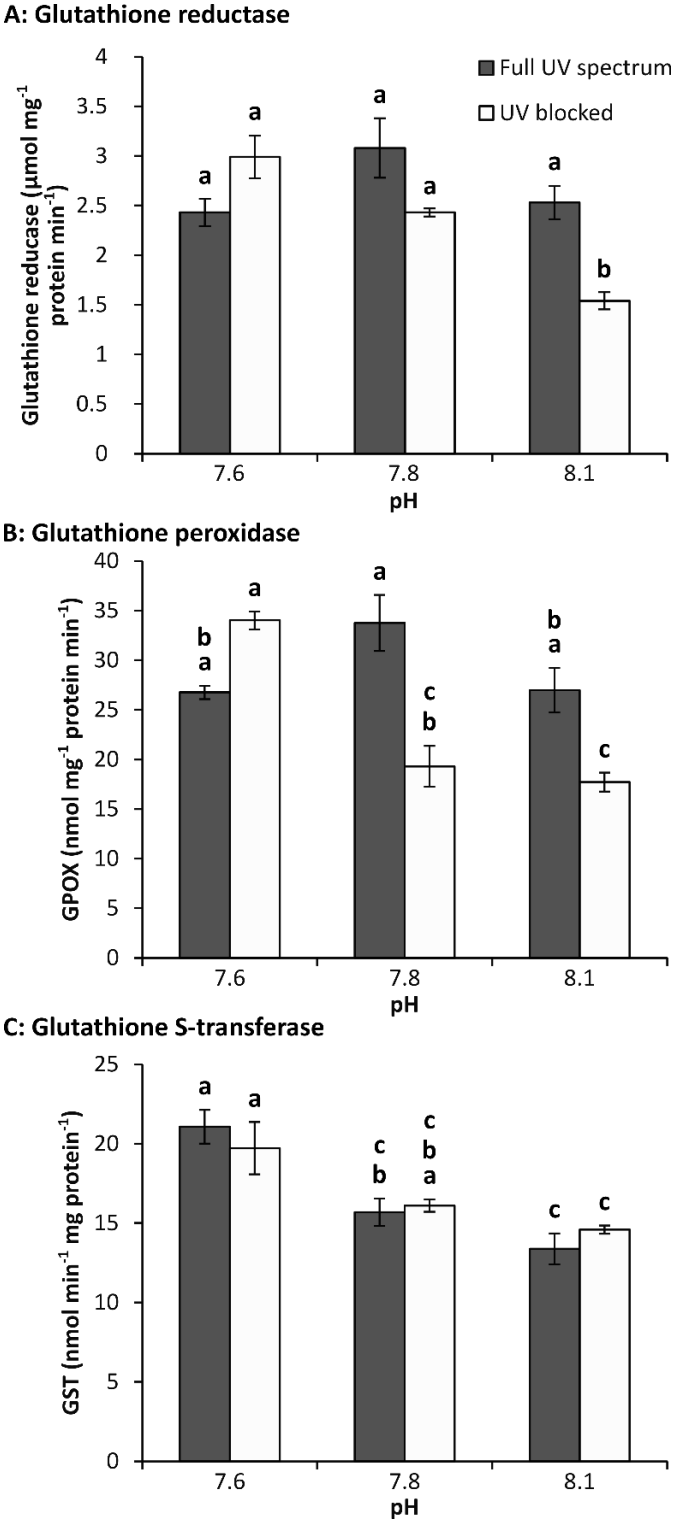

Figure 13: A: Glutathione reductase (GR) (µmol mg^{-1} protein min^{-1}) ± SE (n=18), for 3 pH treatments and two light treatments. pH 8.1 is the ambient sea water in each experiment. **B:** Glutathione peroxidase (GPOX) (nmol mg^{-1} protein min^{-1}) ± SE (n=18). **C:** Glutathione S-transferase (nmol mg^{-1} protein min^{-1}) ± SE (n=18). Grey bars represent blastula that have been exposed to artificial light underneath filters that allowed wavelengths greater than 280nm (UV-B, UV-A and PAR) to pass through. White bars represent blastula that have been exposed to wavelengths greater than 400nm (PAR only) underneath UV filters. Statistical significance determined with a 2-way ANOVA with a Tukey's post-hoc test. Within each graph columns sharing the same letter are not significantly different at a significance level of $p \leq 0.05$.

Table 14: 2-way ANOVA on superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase and glutathione S-transferase of embryos that have developed in seawater with lowered pH and have been exposed to two artificial light treatments underneath filters that allowed wavelengths greater than 280nm (UV-B, UV-A and PAR) or wavelengths greater than 400nm (PAR only). Statistical significance determined with a two-way ANOVA.

Parameter	Source	DF	Sum of Squares	Mean Squares	F	p- value
Superoxide dismutase	pH	2	0.57615	0.28808	15.04	**0.001**
	UV Treatment	1	0.19078	0.1978	9.96	**0.008**
	Interaction	2	0.65233	0.32616	17.03	**<0.001**
	Error	12	0.22982	0.01915		
	Total	17	1.64908			
Catalase	pH	2	0.06365	0.03183	2.73	**0.106**
	UV Treatment	1	0.23355	0.23355	20.01	**0.001**
	Interaction	2	0.28730	0.14365	12.31	**0.001**
	Error	12	0.14007	0.01167		
	Total	17	0.72457			
Glutathione reductase	pH	2	0.40411	0.20205	16.44	**<0.001**
	UV Treatment	1	0.13444	0.13444	10.94	**0.006**
	Interaction	2	0.38100	0.19050	15.50	**<0.001**
	Error	12	0.14750	0.01229		
	Total	17	1.06704			
Glutathione peroxidase	pH	2	0.32049	0.16025	10.04	**0.003**
	UV treatment	1	0.27346	0.27346	17.13	**0.001**
	Interaction	2	0.54914	0.27457	17.20	**<0.001**
	Error	12	0.19160	0.01597		
	Total	17	1.33468			
Glutathione S-transferase	pH	2	0.436149	0.218075	22.44	**<0.001**
	UV treatment	1	0.001218	0.001218	0.13	0.729
	Interaction	2	0.020261	0.010131	1.04	0.383
	Error	12	0.116639	0.009720		
	Total	17	0.574268			

Non-enzymatic antioxidants

Glutathione levels were significantly different among pH levels, with the greatest difference seen in the pH 7.6 treatment (Figure 14, Table 15). The UV treatment did not significantly decrease the total amount of glutathione present in the samples. There was no significant interaction between pH and UV treatment on the total glutathione levels (Figure 14, Table 15).

The percentage of reduced glutathione varied significantly with exposure to UV, but did not vary significantly among pH treatments (Figure 14, Table 15). There was no significant interaction between pH and the UV treatment on the percentage of reduced glutathione (Figure 14, Table 15).

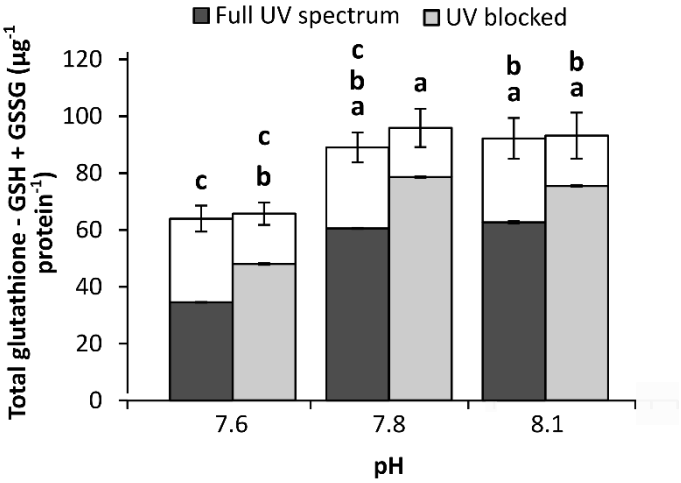

Figure 14: Mean total glutathione – GSSH + GSSG (μg^{-1} $protein^{-1}$) ± SE (n=18) for 3 pH treatments and two irradiance treatments. pH 8.1 is the ambient sea water in each experiment. Light grey bars represent blastula that have been exposed to artificial light underneath filters that allowed wavelengths greater than 280nm (UV-B, UV-A and PAR) to pass through. Dark grey bars represent blastula that have been exposed to wavelengths greater than 400nm (PAR only) underneath UV filters. Within each bar the white section represents the percentage of GSSG of the total glutathione content. Statistical significance determined with a 2-way ANOVA with a Tukey's post-hoc test. Within each graph columns sharing the same letter are not significantly different at a significance level of $p \leq 0.05$.

Table 15: Two-way ANOVA on glutathione and the percentage of reduced glutathione of embryos that have developed in seawater with lowered pH and have been exposed to two artificial light treatments underneath filters that allowed wavelengths greater than 280nm (UV-B, UV-A and PAR) or wavelengths greater than 400nm (PAR only). Statistical significance determined with a two-way ANOVA.

Parameter	Source	DF	Sum of Squares	Mean Squares	F	p- value
Glutathione	pH	2	0.50361	0.25180	15.39	**<0.001**
	UV Treatment	1	0.00552	0.00552	0.34	0.572
	Interaction	2	0.00320	0.00160	0.10	0.907
	Error	12	0.19628	0.019636		
	Total	17	0.70861			
% reduced glutathione	pH	2	0.02097106	0.01048553	1.1051	0.3627
	UV Treatment	1	0.06656827	0.06656827	7.0156	**0.0212**
	Interaction	2	0.00270936	0.00135468	0.1428	0.8684
	Error	12	0.11386366	0.009488638		
	Total	17	0.5114			

Discussion

In this chapter the oxidative stress response for echinoderm embryos from the species *Sterechinus neumayeri* that have developed at low pH and then exposed to a short dose of UV-R was investigated. In response to the third hypothesis: development of *S. neumayeri* embryos in

seawater with a decreased pH will be additive with exposure to a second stressor UV-R. The results show that development within seawater with a lowered pH increased the amount of oxidative damage with increases in the concentrations of 8-OHdG per 10^6 dG, lipid hydroperoxides and protein carbonyls. The antioxidant response was increased to compensate but appears to be nearing the maximum ability for these organisms to defend against oxidative damage in the most stressful experimental treatment of pH7.6/full UV-R treatment. In the instances where this is occurring there is a decrease in the activity of some antioxidant enzymes causing a significant interaction between the UV-R and OA.

Damage caused by ocean acidification and UV light

Abnormality rates were not greatly affected by either pH or UV-R. UV-R usually increases the proportion of abnormal embryos for *S. neumayeri* (Lesser, 2006; Lamare et al., 2007; Lister et al., 2010). The cross sectional area of *S. neumayeri* decreased with exposure to a lowered pH regime but this decrease in size was small compared to the difference between those exposed to UV-R and those only exposed to PAR. The embryos that were exposed to UV-R were on average 4.2-times smaller than those that were only exposed to PAR. This decrease in size is typical of an exposure to UV-R with reduced development being a key feature of embryos after exposure (Lamare et al., 2011; Adams et al., 2012). This reduction in size could be caused by two responses. Larvae may be reducing their metabolism as a short term stress response and they may be exogastrulating damaged cells (Lister et al., 2010). A similar reduction of size in sea urchin larvae in response to reduced pH has also been observed (Byrne et al. 2013). Larvae in lower pH environments may be halting their growth as they need to shift metabolic resources away from growth and into the regulation of intercellular pH levels and managing increased oxidative damage (Stumpp et al., 2011b; Todgham & Hofmann, 2009). Development within sea water with a lowered pH and then a 1 hour exposure to UV-R caused a large increase in oxidative damage to DNA through the formation of 8-OHdG, lipid hydroperoxides and protein carbonyls compared to the control treatment of ambient sea water that had a pH of 8.1 that was exposed to UV-R. It appears that the effects of pH and UV-R on the oxidative stress biomarkers are additive for lipid hydroperoxides and 8-OHdG concentration. This is an interesting result as the stressors are not exacerbating each other or interacting, and are suspected here to be both influencing oxidative stress through different molecular pathways for these two biomarkers.

Damage caused to DNA through the accumulation of 8-OHdG increased with exposure to pH and UV-R did not increase proportionally with decreasing pH. In the pH 8.1/full UV spectrum

treatment, the concentrations of lipid hydroperoxides were at a similar level to that of embryos that were left to develop for 4 days in open seawater just under the surface during an ozone hole event in the Antarctic (Lister et al. 2010). Levels were only slightly lower for the pH7.6/UV blocked treatment. The lipid hydroperoxide levels in the pH 7.8/full UV treatment was greater than the most stressful treatment conducted by Lister et al. (2010) and the most stressful treatment, pH 7.6/full UV treatment accumulated more than twice the amount of damaged lipids. Baseline protein carbonyl levels were similar to that of Lister et al. (2010), but protein carbonyl formation was not affected to the same degree under ocean acidification and UV-R exposed scenarios . In this respect, the pH 7.6 treatment appears to receive similar levels of damage as their full UV exposure when ozone was coving the Ross Sea with embryos developing in open water.

The levels of damage observed in this experiment and the trend of increasing damage with exposure to both reduced pH seawater and UV-R suggest that there will be a point is where the levels of oxidative damage and assault on the cellular components are exceeding the blastula's capacity to respond by upregulating antioxidants and cellular protective molecules. The results here suggest this point is reached between the pH 7.8 and 7.6 treatments. Once a cell reaches this point the ROS can cause damage that could end in apoptosis or cell death (Halliwell & Gutteridge, 2007; Lesser, 2006).

Antioxidant responses to ocean acidification and UV-R

In response to the cellular damage and abnormalities caused by sea water with a decreased pH and UV-R exposure, the enzymatic antioxidants GR, GPOX, SOD and CAT all followed the same trend, namely an upregulation in response to decreasing pH treatments and even more so in response to the UV-R treatment. For the enzymes SOD, CAT, GR, GPOX and GST the most extreme experimental treatment, pH 7.6/UV-R, the antioxidant levels that were measured in this experiment were less than the pH 7.6/UV blocked treatment although not statistically significant this decrease is the likely cause the interaction factor to be significant in our models. This decrease in antioxidant levels in the most stressful experimental is likely to be caused by the embryonic cells being unable to mitigate the level of damage due to the exhaustion cellular defence metabolites such as glutathione. This cascade could lead to apoptosis and death as shown in Figure 15 (Adams et al., 2012). In less stressful situations as seen in the OA only treatments, or the pH 8.1/UV-R and the pH 7.8/UV-R treatments, the increase in oxidative damage, although not lethal, still comes at a metabolic cost to the individual. During early development and before larvae begin feeding this will draw from the stock of antioxidants and

energy reserves that have been maternally given to each egg to protect it during early development, metabolic reserves that would be otherwise dedicated to growth and development (Bosch et al., 1987; Halliwell & Gutteridge, 2007; Lister et al., 2015).

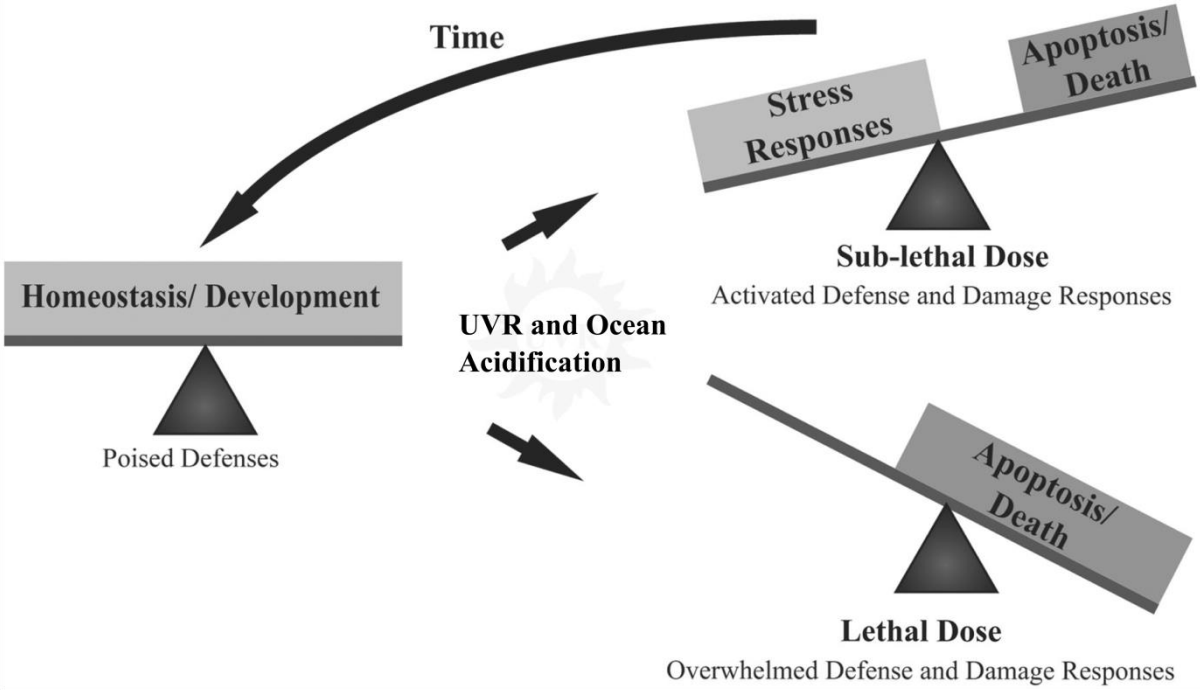

Figure 15: Model of cellular responses of *Sterechinus neumayeri* embryos exposed to future ocean acidification and a secondary stressor of ultraviolet light. Embryos that are protected from stress are able to progress through development while maintaining cellular homeostasis and retaining some defence proteins and molecules. During a sub-lethal stress event homeostasis is temporarily lost and the cellular stress response is activated to protect against further cellular damage. Cellular stress responses include upregulation of protective enzymes and antioxidants and may include apoptosis. However after a lethal dose of environmental stress, the embryo is unable to overcome the disruption to homeostasis and the macro-molecule damage and apoptotic cascades are initiated leading to death. Figure adapted from Adams et al. (2012).

Total glutathione levels decreased significantly in response to decreasing pH but UV-R exposure had no effect on these levels. The percentage of reduced glutathione (GSSG) was lowest in the pH 7.6 treatment and the treatments exposed to UV-R. The ratio of the two is a proxy for the cellular stress response efficiency as the amount of glutathione that is available to react and neutralise the potential toxic effects of O_2, O_2^- and HO• (Halliwell & Gutteridge, 2007; Lesser, 2006). GSH is critical for cells as it is needed for the action of GPOX and to maintain an appropriate intercellular redox environment (Halliwell & Gutteridge, 2007). A short UV-R exposure and development within sea water with a pH of 7.6 over a week both deplete the glutathione pool and lower the amount of glutathione (reduced) that is available to maintain a normal cellular redox environment.

Davis et al. (2013) attempted to investigate a similar set of research questions involving ocean acidification and UV-R and temperature on molluscan larvae. Their results showed no significant effect of UV-R on the mortality of *Bembicium nanum* or *Dolabrifera brazieri*. Mortality in their experiment was affected by pH and temperature. Interestingly UV-R slowed the developmental rate in all of their experimental treatments. The slow developmental rate of *S. neumayeri* and short UV exposure does not allow us to make direct comparisons with the response of *B. nanum* or *D. brazier*, but our analysis of the oxidative responses provides important information regarding the impact of these stressors on *S. neumayeri*.

In this study a decrease in the enzymatic antioxidants SOD, CAT, GR and GPOX was seen in the most extreme experimental treatment of full UV/pH 7.6 which caused a significant interaction factor. This decrease in enzyme activity indicates that there is a limit at which *S. neumayeri* is able to respond to oxidative stress caused by environmental stressors. It is at this point where the supply for antioxidants needed to maintain cellular integrity is outpaced by the demand. This drop in the amount of antioxidant enzymes has been observed in the bacteria *Escherichia coli* and has been attributed to ROS inhibiting the function and viability of enzymes and proteins (Imlay, 2013).

A higher proportion of abnormal larvae was seen in the present study than in a previous investigations on the effects of UV-R on *S. neumayeri* Lister et al. (2010) and ocean acidification on *S. neumayeri* by Ericson et al. (2011). There may be two explanations for the higher levels in the present study. Firstly, the level of stress placed on embryos under our full UV treatment and the additional stress caused by a lowered sea water pH was too high for most of the embryos. In many of the samples in the more extreme pH treatments many of the embryos were unable to survive and were dead. These were seen within the preserved sample tubes as cellular debris. Although the UV-R levels that were used in this study were set to a level that is the equivalent of an hour of exposure at the surface of the water with no sea ice. It would be interesting to decrease the UV dose that the embryos received as I believe the dose was too high for embryos that were developing within the pH 7.6 treatments. The other option here would be to extend the number of pH treatments so as to refine the point where *S. neumayeri* is no longer able to produce enough antioxidants to be able to cope with oxidation. It should also be noted that the levels of damage and abnormality in the UV blocked treatments in this experiment were higher than those presented in Chapter 2. This was due to experimental design. The larvae that were sampled in Chapter 2 were sampled straight from the buckets and not exposed to light. The UV

blocked larvae in this experiment were exposed to light with the UV-R components filtered out. This effect could be explained as stress caused by being exposed to light during this experiment. Both the UV exposed and UV blocked treatments were treated the same except for the UV-A and UV-B light being filtered out of the control treatments.

The results from this study are relevant as high latitude regions will be affected by ocean acidification sooner than other areas due to the high solubility of CO2 in cold waters. UV-R will also continue to play a significant role in shaping the lives of organisms in the surface waters of the oceans (Tedetti & Sempere, 2006; Doney et al., 2009; Fabry et al., 2009; Lamare et al., 2011). UV-R is a useful stressor to measure the interaction of ocean acidification on oxidative damage as previous studies have shown that UV-R can directly cause oxidative damage in S. neumayeri and this has been manipulated experimentally and can be compared to the results gathered here (Lesser et al., 2001, 2006; Lister et al., 2010; Lamare et al., 2011).

Is *Sterechinus neumayeri* being overwhelmed in this multiple stressor environment?

S. neumayeri is relatively robust in response to ocean acidification (Ericson et al., 2011) alone but does not respond well to UV-R (Lesser et al., 2004; Lamare et al., 2007). In a multiple stressor environment that involves warming, ocean acidification and UV-R, it would be expected that this species may not fare well and there would be increased prevalence's of abnormal larvae and blastula (Byrne et al., 2013b; Ericson et al., 2011).

Summary

- An interaction was observed between possible future sea water pH levels and UV-R on the AO response of *S. neumayeri*. This interaction is seen as a decrease in the antioxidant enzyme levels in the most stressful experimental treatment of pH 7.6/full spectrum UV-R exposure. This decrease in enzyme levels is attributed to enzyme inhibition possibly caused by high levels of ROS within the cells of the blastula.
- Exacerbated levels of molecular damage caused by ROS was observed in embryos developing in sea water with a lowered pH and exposed to UV-R, compared to unexposed embryos.

Chapter 4 General Discussion

Summary of findings

This research measured the levels of oxidative damage the antioxidant defences in response to experimentally induced future ocean acidification scenarios in *Sterechinus neumayeri*, *Odontaster validus* and *Patiriella regularis* embryos. To better understand the interactions between multiple stressors, this research also investigated how exposure to future ocean acidification scenarios and UV light interacts. There is a lack of knowledge on the responses of marine animals to ocean acidification and UV-R as single and combined stressors (Pörtner, 2008; Dupont & Pörtner, 2013; Häder et al., 2014). The early embryos of marine invertebrates are a vulnerable life history stage so quantifying how this potential bottleneck responds to ocean acidification and UV-R, and their interactions, can provide insight into how these species will fare into the future (Eckman, 1996; Lamare et al., 2011; Byrne, 2012; Przeslawski et al., 2015).

In this thesis, the following hypotheses were tested: That seawater with a decreased pH caused by increased ρCO_2 will cause oxidative damage during the early embryonic development of *S. neumayeri*, *O. validus* and *P. regularis*. Secondly, the increase in cellular damage caused by ROS in response to seawater with a decreased pH will result in an upregulation of antioxidant enzymes in *S. neumayeri*, *O. validus* and *P. regularis*. Finally the development of *S. neumayeri* embryos in seawater with a decreased pH will be additive with exposure to a second stressor UV-R.

These three hypotheses were tested by artificially lowering the pH of seawater and allowing freshly fertilised embryos to develop until a late blastula stage then examining the embryos morphologically and for oxidative damage to DNA (8-hydroydeoxyguanosine (8-OHdG) formation), proteins (protein carbonyls) and to lipids (lipid hydroperoxides). The levels of damage were then compared to the activities of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPOX), glutathione S-transferase (GST) and the amount of total (GSH + GSSG) and reduced glutathione (GSH).

The results of the present research have shown that IPCC climate change scenarios for oceanic pH in 100 years can cause damage to lipids, proteins and DNA and can elicit an enzymatic response in *S. neumayeri* blastula. Increases in the levels of oxidative damage caused to lipids, proteins and DNA in the 2100 year scenario was similar for *O. validus*. The temperate asteroid, *P. regularis*,

showed little oxidation of lipids and DNA, but did show an increase in protein carbonyls. *S. neumayeri* showed an increase in SOD, CAT, GR, GST and a decrease in the reduced form of glutathione (GSH) in response to these ocean acidification scenarios.

Potential mechanisms causing oxidative stress in sea water with a lowered pH.

The key finding of the experiments presented previously indicate that in acidified conditions for *S. neumayeri*, *O. validus* and *P. regularis*, blastula show damage caused by oxidative stress. Testing the mechanism that is causing oxidative stress in seawater with a reduced pH is beyond the scope of this thesis, however, there are several possible mechanisms that could be causing the internal cellular environment to become pro-oxidant.

Oxidative stress caused by lowered seawater pH and or increased pCO_2 could alter the intra or extracellular pH of cell. Tomanek et al. (2011) have suggested several hypotheses why ocean acidification may cause an upregulation of antioxidant proteins in *Crassostrea virginica*. The first hypothesis is that the CO_2 is reacting with peroxynitrate ($ONOO^-$), a very reactive nitrogen species that is formed through the interaction between superoxide anions and nitric oxide (Dean, 2010). The second is that high CO_2 and/or lower seawater pH is affecting the function of the mitochondria as well as increasing the production of radical species (Tomanek et al., 2011). *C. virginica* and most molluscs have a limited ability to regulate inter and extracellular pH, this may be detrimental for the production of some antioxidants as the functioning of many proteins and enzymes can be pH specific (Tomanek et al., 2011). As well as potentially altering pH-specific enzyme functions, the mitochondrial election transport chain (ETC) may become impaired under low pH/high pCO_2 conditions by increasing electron escape in the ROS generating mitochondrial complexes I and III and/or by partially inhibiting the flow through the downstream ETC complexes which would then, in turn release more ROS (Murphy, 2009; Starkov, 2006). Kaniewska et al. (2012) recognised this effect in a study on the coral *Acropora millepora* in which there was a down regulation of genes associated with the triocarboxylic acid (TCA cycle) and the ETC in coral exposed for 28 days to sea water with similar pH values to this study. This indicates a reduced capacity for the cells to produce ATP or NADPH, while they also noticed an upregulation of genes associated with oxioreducatase activity. Kaniewska et al. (2012) suggests that this impairment to the mitochondria and or the photosynthetic symbiont would both result in an increase in the levels of ROS within the cells. This study has observed an increase in the amount of damage caused by ROS to proteins, lipids and DNA when the organism does not have high baseline levels

of antioxidants to begin with. It should be noted that although the species investigated in this thesis do not have photosynthetic symbionts, any disruption to mitochondria that are adapted to cold environments may result in higher ROS levels outside of the mitochondria (Lesser, 2006). A third hypothesis put forward by Tomanek et al. (2011) is that a decrease in intracellular pH may cause a release of chelated transition metals like those of Fe^{2+} from intracellular stores. This is of importance because excesses of intracellular iron can induce oxidative stress by inducing Fenton reactions and generating hydroxyl radicals (Stohs & Bagchi, 1995; Halliwell & Gutteridge, 2007). An increased Fenton reactions are suspected to increase DNA strand breaks when the levels of these transition metals become high enough (Imlay & Linn, 1987). In both mammalian cells and in the marine snail, *Littorina littorea*, stress responses that involve intracellular acidosis can increase the amount of oxidative damage that has been observed to be prevented by iron chelators and the release of the iron-chelating protein ferritin (English & Storey, 2003; Bronk & Gores, 1991). In the experiments presented here, increases in lipid hydroperoxides, which can be caused by the reaction between polyunsaturated fatty acids and hydroxyl radicals was observed (Lesser, 2006). This could be a significant cause of oxidative stress for Antarctic species due to the increased proportions of polyunsaturated fatty acids that help the cellular membranes of ectothermic animals that live in high latitude waters remain fluid in cool temperatures (Regoli et al., 2011).

In a bacterial system, high pCO_2 exacerbated the effects of oxygen toxicity (Ezraty et al., 2011). If this is to be the case in marine invertebrate systems, it is of concern because the oxidation of amino acids and arsenic (III) that occurs in the Fenton reaction is dependent on the presence of the bicarbonate ions, which will increase under future climate change scenarios (Stadtman & Berlett, 1991; Berlett et al., 1990; Hug & Leupin, 2003; Doney et al., 2009). CO_2 may also interact with ROS such as hydroxyl radicals (HO) and hydrogen peroxides (H_2O_2) and CO_2 appears to increase H_2O_2 induce oxidative damage in a dose-dependent manner, i.e. 8-oxo-guanine levels and the carbonyl levels in bacteria (Ezraty et al., 2011). This could help explain the increases in oxidative damage that has been observed in this study on *S. neumayeri* and *O. validus* embryos and, perhaps to a lesser degree, the damage caused to protein carbonyls in *P. regularis* embryos. The results presented within this thesis are consistent with the fact that Antarctic species are prone to both an increased threat of oxidative damage due to their high oxidative environment and the increased saturation of CO_2 in their waters due to the cold temperatures compared to the tropics and temperate waters (Lesser, 2006; Fabry et al., 2009).

How the oxidative stress response will be affected in a multiple stressor environment.

Ocean acidification will be a source of environmental stress in *S. neumayeri*, *O. validus* and *P. regularis* and it is important to consider how these species will react to other stressors when exposed to a more acidic environment. These multiple stressor combinations can be highly varied and complex (Pörtner, 2008; Byrne, 2012; Kroeker et al., 2013; Häder et al., 2014).

It has been suggested that increased temperatures through ocean warming may offset some of the negative effects of ocean acidification, especially, those associated with delays in development as a result or reduced pH (Byrne, 2012). However a meta-analysis of the results from studies involving both ocean acidification and ocean warming is unclear, although generalised patterns suggest that warmer conditions decrease the sensitivity of organisms to ocean acidification. With this pattern is a recognition of a high degree of variation between groups of animals and population bottlenecks will be species and life history stage dependant (Kroeker et al., 2013). Physiologically, the negative effects of ocean acidification may be offset by slight increases in temperature. This may increase the metabolic rate but may, on the other hand, decrease the thermal tolerance windows of the organisms in question (Pörtner, 2008). For animals that are close to their thermal limits already, a decrease in seawater pH could reduce this tolerance. For example the mortality of *P. regularis* blastulae increased with exposure to pH 7.6 and a 4 °C increase in temperature above the ambient (Byrne et al., 2013a). A 2 °C increase in temperature increased larval size but did not ameliorate the negative effects of ocean acidification for this species (Byrne et al., 2013b). The effects of increased temperature and decreased seawater pH are hard to predict on ROS and enzyme responses. The increased temperature could increase the activity of some repair enzymes (e.g. Photolyase, Lamare et al. (2006)) but could increase stress through an increased conductance of O_2 and increase the viscosity of mitochondrial membrane increasing ROS leakage (Lesser, 2006). The ability of animals to acclimatise to changes in temperature and maybe to a lesser extent decreases in pH should also be noted with decreases in the stress response to heat shock seen in fish and the echinoderm larvae *Strongylocentrotus purpuratus* (Abele & Puntarulo, 2004; Lesser, 2006; Kelly et al., 2013).

UV-R in future climate change scenarios will continue to shape ecosystem processes (Häder et al., 2014). The size of the spring ozone hole is thought to have stabilised over the Arctic and the Antarctic and is expected to significantly decrease in size over the next century (IPCC, 2013).

Despite this, it is likely that the amount of oxidative stress within the oceans caused by UV-R will increase with increasing sea surface temperatures. Increased sea surface temperatures may decrease the depth of the upper mixing layer and increase the amount of time planktonic organisms such as larvae are exposed to UV-R and decrease the amount of time in which organisms can repair when they are passively transported deeper in the water column (Häder et al., 2014). This will increase the amount of ROS formed by exposure to UV-R at the surface and will be of concern for mid latitude species like *P. regularis* and especially for high latitudes species that have their planktonic developmental stages during the Antarctic spring and summer, such as *S. neumayeri* (Lister et al., 2010). It should be noted however that the interactions between these stressors is not well understood and the interactions may be species and life history phase dependant because different species and stages have different susceptibilities to UV-R due to sunscreens, DNA repair rates and the ability to self-shade or relocate (Lamare et al., 2011). For example, increased temperature appears to ameliorate the effects of UV-R and decreased seawater pH for the embryos of two intertidal mollusc species (Davis et al., 2013).

Latitudinal and ecological consequences of oxidative stress, OA and UV-R.

For marine invertebrates the amount of oxidative stress inflicted upon them will likely vary between species and habitats (Byrne, 2012). It is for this reason that it is important to identify those key environmental stressors that exist within ecosystems across latitudes and those population processes that must be understood in order to understand how climate change will to affect differing areas and influential animals (Byrne, 2012; Lamare et al., 2011). For the species tested here small decreases in the fitness and delays in life history steps from spawning, through to settlement may have large implications for the populations to maintain their size (Eckman, 1996). For all species, an increase in oxidative damage during the early development will divert energy resources that would have been used for development into protective and repair mechanisms. For the Antarctic species, delays in the early development may lead to these species missing the critical timings of food availability that are strongly linked with the breakup of the sea ice and phytoplankton blooms (Pearse, 1969; Pearse & Bosch, 1986; Bosch et al., 1987). On top of this, increases in development time will lead to an increase in the likelihood of predation which will have further impacts upon wider population dynamics into the future (Lamare & Barker, 1999). For the intertidal *P. regularis*, ocean acidification may not be as much of a threat as increased temperature. Intertidal organisms appear to be fairly robust to fluctuations in environmental conditions as they have adaptions to deal with regular environmental fluctuations (Byrne et al., 2013a; Davis et al., 2013; Kroeker et al., 2013).

UV-R will be a significant threat to *S. neumayeri* (Lister et al., 2010) and is likely to be a threat to *P. regularis*. The winter spawning *O. validus* will not be affected by UV-R during early development and appears to be somewhat robust to ocean acidification although it did show that it was not entirely unaffected. Temperate and tropical species appear to have the ability to tolerate small levels of stress in short term experiments but will need to acclimate to the conditions presented with climate change and then have the genetic ability to adapt to decreasing pH, increasing temperatures and multiple other stressors, there is some hope with this given the short time period to sexual maturity (Fabry et al., 2009; Byrne, 2012; Kelly et al., 2013). The slowly growing and long lived Antarctic species will however only be two to three generations away from the experimental conditions presented in this thesis so their ability to tolerate environmental stress is of critical importance (Pearse & Bosch, 1986; Bosch et al., 1987; Eckman, 1996). Transgenerational effects may however, provide a much more shorter term and immediate response as shown by *S. neumayeri* embryos that were spawned from urchins from a pollution contaminated site and compared to embryos that were spawned from urchins from a pristine un-polluted site. The embryos from pollutant exposed mothers had higher baseline levels of antioxidants and had a higher capacity to minimise oxidative damage caused by experimental stress from pollution (Lister et al., 2015). This is an example of the ability of mothers to increase the resilience of offspring to stress however this increase in maternal reproductive investment must come at a cost.

Future directions

Using oxidative stress biomarkers on a wider range of species to further help understand both the physiological and ecological effects of climate change is important because it has the ability to detect small fluctuations in stress levels in developing organisms that may not be detected through morphological measurements alone (Halliwell & Gutteridge, 2007). It is also important for this reason that the molecular mechanisms that are causing oxidative stress in response to ocean acidification are better understood and used experimentally alongside proteomic studies. This will allow us to better understand the relationship between gene responses and the levels of stress presented to the developing larvae.

It will also be of importance for future studies to continue to combine multiple stressors together so that the complex interactions that occur within the oceans for keystone species are understood and teased apart. It is for this reason that larval stages are crucial to the study of climate change biology as these life history stages can form a bottleneck for the ability for

populations to survive. It should also be noted that although the ozone hole will close in the future that this will not stop UV-R being a significant stressor that can still cause significant damage to organisms even when the protective ozone layer is present (Lamare et al., 2011; Häder et al., 2014).

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Appendix

Ocean acidification enhances oxidative damage in *Sterechinus neumayeri* embryos when exposed to UV radiation.

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Background

The response of marine organisms to multiple stressors caused by anthropogenic climate change is relatively understudied and the synergetic effects are largely unknown. Two biologically important stressors for animals in the Antarctic marine environment are; increases in UVB (290-320nm) radiation caused by ozone depletion (Lamare et al. 2011), and changes in the carbonate chemistry of sea water resulting in reduced seawater pH causing ocean acidification (OA) (Fabry et al. 2009).

We have used measures of oxidative damage through reactive oxygen species and the resulting upregulation of antioxidant enzymes as a measure of how the embryos of the Antarctic sea urchin, *S. neumayeri*, respond to these two stressors. Forms of oxidative damge that we have measured are DNA damage through oxidation of guanine and damage to lipids through the formations of lipid hydroperoxides. Oxidative stress has not been directly measured in response to OA, the response is well known for UVR in *S. neumayeri*.

Our Aims

1. To explore whether future ocean acidification causes oxidative stress in the embryos of the Antarctic sea urchin, *Sterechinus neumayeri*.
2. To investigate if ocean acidification decreases the ability of embryos to deal with the additional physiological stressors of UVR exposure.

Conclusions

- Ocean acidification does cause oxidative stress in *S. neumayeri* embryos.
- The effects of UVR on oxidative damage is increased when embryos are reared in future ocean acidification scenarios.
- This suggests there are synergistic effects with UV-R at low pHs. Blastula appear not to be able to upregulate antioxidant enzyme activity in response to the increased oxidative damage obtained through a dose of UVR when reared in low pH conditions.
- The cumulative effects of ocean acidification and UV radiation at this early life history stage of *S. neumayeri* may have serious implications for population dynamics if future climate change scenarios are to be true.

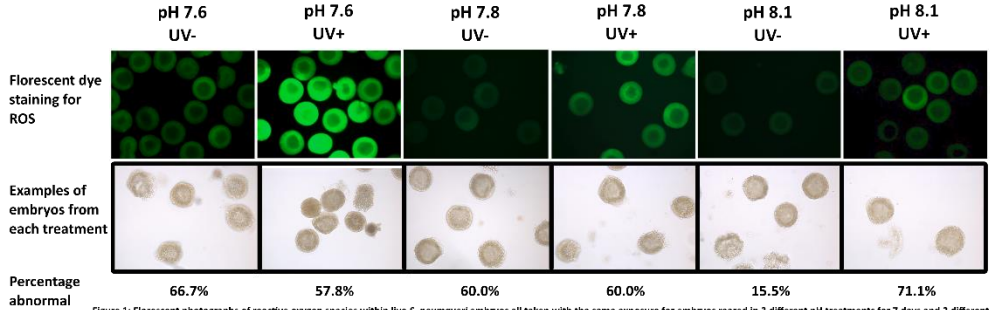

Figure 1: Florescent photographs of reactive oxygen species within live *S. neumayeri* embryos all taken with the same exposure for embryos reared in 3 different pH treatments for 7 days and 2 different light treatments for 1 hour doses. Below these photos are embryos preserved in buffered formalin for abnormality counts and below that the percentages of abnormal embryos based on the classification as per Lister et al. (2010). pH 8.1 is the ambient seawater. Both received 1hr doses of light the UV+ spectrum treatment received light > 290nm. The UV- treatment received light >400nm with UVB and UVA blocked.

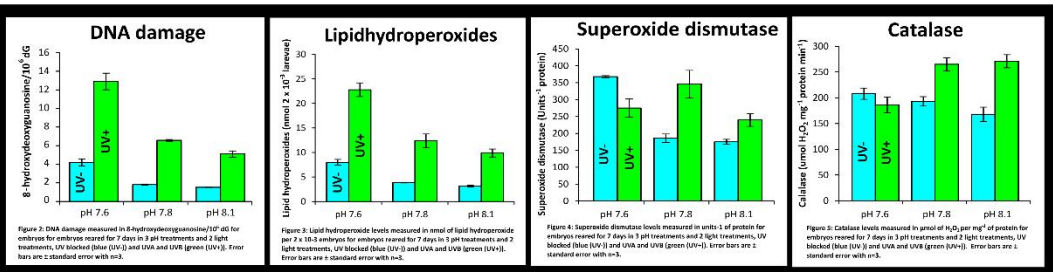

Figure 2: DNA damage measured in 8-hydroxydeoxyguanosine/10^6 dG for embryos for embryos reared for 7 days in 3 pH treatments and 2 light treatments, UV blocked (blue (UV-)) and UVA and UVB (green (UV+)). Error bars are ± standard error with n=3.

Figure 3: Lipid hydroperoxide levels measured in nmol of lipid hydroperoxide per 2 x 10-3 embryos for embryos reared for 7 days in 3 pH treatments and 2 light treatments, UV blocked (blue (UV-)) and UVA and UVB (green (UV+)). Error bars are ± standard error with n=3.

Figure 4: Superoxide dismutase levels measured in units-1 of protein for embryos reared for 7 days in 3 pH treatments and 2 light treatments, UV blocked (blue (UV-)) and UVA and UVB (green (UV+)). Error bars are ± standard error with n=3.

Figure 5: Catalase levels measured in μmol of H_2O_2 per mg^{-1} of protein for embryos reared for 7 days in 3 pH treatments and 2 light treatments, UV blocked (blue (UV-)) and UVA and UVB (green (UV+)). Error bars are ± standard error with n=3.

Methods

S. neumayeri were collected from Cape Evans, Antarctica. Sea water acidified by bubbling CO_2 through filtered sea water until a pH of pH7.8 and pH7.6 was achieved. There were three replicates for each treatment. Urchins were spawned via intracoelomic injections of 0.5M KCl. Eggs from 13 females were fertilized using dilute drops of sperm from 3 males. Fertilisation was close to 100% after 20mins. Embryos were split into pH treatments and left to develop for 7 days. Embryos were then spilt into 2 light treatments and placed underneath UV lamps for a 1 hour exposure. Embryos were placed underneath filters, one blocking wavelengths smaller than 290nm exposing embryos to UVB, UVA and PAR (UV+ treatment), the second filter only exposing the embryos to PAR (UV- treatment). Embryos were photographed live after addition of 2', 7'-dichlorodihydrofluorescein diacetate ROS probe directly after light exposure. embryos were preserved in buffered formalin and photographed for abnormality analysis as per Lister et al. (2010). Lots of 20,000 embryos were frozen in liquid nitrogen and stored at -80°C for oxidative analysis. DNA extraction as per instructions with DNeasy blood and tissue kit. Lipidhydroperoxides assayed as per Mihaljevic et al. (1996). Superoxide dismutase assayed as per Banowetz et al., (2004). Catalase assayed as per Maral et al., (1977), as described by Janssens et al., (2000).



Results

- pH and UV both significantly increased the levels abnormality (p=0.039 and p<0.001 respectively) and there was a significant interaction between the 2 treatments (p=0.002). Tukey post hoc significance testing shows that the embryos in ambient seawater (pH 8.1) that received no UVR were different at the 95% significance level. There were no differences between the other treatments.
- pH significantly increased the levels of DNA damage (p=0.000). Exposure to the full UV spectrum treatment also significant increased the amount on DNA damage (p<0.001), the interaction of pH and UV treatment was close to being significant (p=0.053).
- Lipidhyroperoxides levels significantly increased in the pH and light treatments with p<0.001and p=0.000 respectively. There was no significant interaction between pH and UV (p=0.329).
- Superoxide dismutase levels varied significantly with pH (p<0.001) and UV (p<0.001). There is a significant interaction between pH and UV on superoxide dismutase levels (p<0.001).
- Catalase levels were significantly different between the light treatments (p<0.001). pH did not have a statistically significant effect on for catalase expression (p=0.301) although with decreasing pH the mean catalase level has increased.

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