Physiological acclimation and oxidative stress of Antarctic sea star *Odontaster validus* to future warming conditions

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Roberta Noon

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Abstract

Climate changes are projected to alter the structure and function of marine ecosystems, and these environmental changes can be amplified in polar regions. Understanding the response of marine species to altered environmental conditions is an expanding field of research as near future scenarios predict changes to an array of key biological factors including temperature, pH and salinity, which will affect species physiology, biochemistry and life history traits. The Antarctic sea star, *Odontaster validus* is a keystone species and opportunistic predator in the nearshore shallow Antarctic marine benthos. Its biology is relatively well understood making it a useful sentinel species to explore the impacts of environmental changes, such responses to Southern Ocean warming and the potential for acclimation.

This thesis uses three measures of physiological fitness over 10 months to experimentally explore the acclimation capacity of *O. validus* to ocean warming. To examine this, adult *O. validus* were acclimated to five temperature treatments (0°C, 1°C, 2°C, 3°C, 4°C). At 3-month intervals, *O. validus* were sampled for respiration, morphometrics and oxidative stress markers.

Of the three measures of physiological fitness, none showed any evidence of acclimation or recovery over time. Temperature significantly increased respiration, antioxidant enzyme activity and levels of oxidative damage markers across almost all treatment. *O. validus* pyloric caecum, a nutrient storage organ, were larger in the 3°C treatment compared to others. The metabolic scaling rate and reproductive gonadosomatic tissue were also not affected by temperature in this study. These responses suggest that whilst *O. validus* is able to metabolically adjust to increased temperatures, physiological acclimation is not achieved (i.e. return to physiological activity over time, that is the same as the ambient states).

The implications for *O. validus* survival in warmer conditions require more investigation, particularly the capacity to maintain key functions such as growth and reproduction in increased temperatures, the sensitivity of early life history stages and the potential pressure of invasive competitors caused by range shifts.

Acknowledgments

This project was envisioned prior to the COVID-19 pandemic. It has been the constant in my life through every lockdown, every announcement, every change large or small, that has occurred over the past few years. At times the continuation of this study part time, whilst working full time, has felt incredibly isolating and endless. The person that started this work feels like a stranger, but that is just because this project has caused, required and evolved alongside an enormous amount of growth. The span of time that it has taken for me to produce this thesis was unprecedented, but the journey has not been one that I would change.

There are a number of people to thank for their contributions to this project over the years and many more for their support, without which this work would not have been possible, and certainly would not have been completed.

Firstly, immense gratitude to my supervisors Miles Lamare and David Burritt for their vision, patience, guidance and feedback throughout the duration of this study. Many thanks to the team at the University of Otago Portobello Marine Laboratory, and the Department of Botany laboratories. I am deeply appreciative of statistical advice from Tim Jowett and Britta Basse from DOC, grateful for their expertise and for tackling my many questions with patience and positivity.

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List of Figures

Figure 1.1 *Odontaster validus* individuals vary in size and colour…… 8

Figure 1.2 Changes in temperatures recorded using hobotemp temperature data logger, for of the four experimental treatments, from November 2018 to November 2019 …….. 16

Figure 1.3 Experimental units for treating sea stars to elevated temperatures. The schematic illustrates a single cabinet with duplicate systems (A) indicating the flow of water from the sump to the header by pumping. Photograph of a single cabinet showing the operational experimental set up (B). Note: The CO_2 and pH controllers were leftover features of the set up from a prior study……...17

Figure 2.1 Example of changes in O_2 concentration in a respiration chamber over a 230 min period. The linear regression slope indicates the rate of change in two concentration per minute……...26

Figure 2.2 Wet weight distribution of *Odontaster validus* individual respiration rates across all temperature treatments…….29

Figure 2.3 Metabolic scaling rates of *Odontaster validus* in five temperatures (top to bottom: -1°C/ / 0°C, 1°C, 2°C, 3°C, 4°C)
……31

Figure 2.4 Average respiration rates of *Odontaster validus* in five water temperatures as sampled in February, August and November of 2019. Error bars show standard deviation...32

Figure 2.5 Radial length and wet weight of *Odontaster validus* sampled for body indices study…….34

Figure 2.6 Average Pyloric Caecum Index of *Odontaster validus* across 5 water temperatures in February, May, August and November of 2019. Error bars show standard deviation…….35

Figure 2.7 Average Gonadosomatic Index of *Odontaster validus* across 5 water temperatures in February, May, August and November of 2019. Error bars show standard deviation…….35

Figure 3.1 Reactive oxygen species (ROS) induce oxidative damage to lipids, proteins, and DNA (From Rezayian et al., 2019)…….48

Figure 3.2 Main cellular antioxidant defences and formation of oxidised lipid products: 6-phospogluconate (6PG), catalase (CAT), cysteine (cys), D-hydroxyacid (DHA), dehydroascorbate reductase (DHAR), DT- diaphorase (DTD), glucose 6-phosphate (G6PDH), !-glutamylcysteine synthetase (GCL), glyoxalase I (Glx- I), glyoxalase II (Glx-II), glutathione peroxidises (GPx), glutathione reductase (GR), glycine (gly), glutamic acid (glu), glutathione synthetase (GS), reduced glutathione (GSH), oxidised glutathione (GSSG), glutathione-S-transferases (GST), GSH conjugated xenobiotic (GS-X), hydroquinone (HQ), "-keto aldehydes ("KA), multidrug-resistance-related protein (MRP), quinone (Q), semiquinone radical (Q•), superoxide dismutase (SOD) and xenobiotic (X). Enzymes and other components of the antioxidant system are shown in orange, blue (glutathione-dependent) or green (detoxification). Oxidised molecules or products are shown in red. Graphic from (2015) as modified from Regoli (2011)……50

Figure 3.3 Average Protein Carbonyl (nmol/ mg protein) for *Odontaster validus* Gonad (upper) and Tube feet (lower) tissue in five temperature treatments from February- November 2019. Error bars show standard deviation. Different colour bars indicate statistical significance between temperatures. Protein carbonyls in gonad and tube feet were statistically different, p <0.0001…… 54

Figure 3.4 Average lipid peroxide (nmol/g FW) for *Odontaster validus* Gonad (upper) and Tube feet (lower) tissue in five temperature treatments from February- November 2019. Error bars show standard deviation. Different colour bars indicate statistical significance between temperatures. Lipid peroxide levels in gonad and tube feet were statistically different, p <0.0003. ……55

Figure 3.5 Average superoxide dismutase (SOD) activity (nmol/ mg protein) for *Odontaster validus* Gonad (upper) and Tube feet (lower) tissue in five temperature treatments from February- November 2019. Error bars show standard deviation. Different colour bars indicate statistical significance between temperatures. SOD activity in gonad and tube feet was statistically different, p<0.0001……. 56

Figure 3.6 Average Catalase (CAT)activity (µmol /min /mg protein) for *Odontaster validus* Gonad (upper) and Tube feet (lower) tissue in five temperature treatments from February-November 2019. Error bars show standard deviation. Different colour bars indicate statistical significance between groups. CAT activity in gonad and tube feet was statistically different, p <0.0001. …… 57

Figure 3.7 Average glutathione peroxidase (GPOX) activity (nmol/ min/ mg protein) for *O. validus* Gonad (upper) and Tube feet (lower) tissue in five temperature treatments from February- November 2019. Error bars show standard deviation. Different colour bars indicate statistical significance between temperatures. GPOX activity in gonad and tube feet was statistically different, p <0.0001…… 58

Figure 3.8 Average glutathione reductase (GR) activity (nmol/ min/ mg protein) for *Odontaster validus* Gonad (left) and Tube feet (right) tissue in five temperature treatments from February- November 2019. Error bars show standard deviation. …… 59

List of Tables

Table 1.1 Target and mean temperatures of experimental fridges during the study

Table 1.2 *Odontaster validus* specimen counts from collection at Mc Murdo Sound, Antarctica and at each of the sampling points in February, May, August and November 2019

Table 2.1 Pairwise slope comparisons of *Odontaster validus* metabolic scaling rates in 1°C, 2°C , 3°C and 4°C relative to the -1°C / 0°C treatment

Table 2.2 Two-way ANOVA for *Odontaster validus* respiration rates at five experimental temperatures and across three sample points in February, August and November 2019. Values with * indicate significance ($p<0.05$)

Table 2.3 ANOVA of *Odontaster validus* Pyloric Index and Gonadosomatic Index from sample points in February, May, August and November in five water temperature treatments

Table 2.4 Summary of average morphometric measurements of *Odontaster validus* in each temperature treatments + standard error of mean

Table 3.1 Mixed effects model output showing significant differences between temperature treatments and oxidative stress markers for *Odontaster validus.*

Chapter 1: General Introduction

1.1 Aims and Hypotheses

The aims of this research are to determine the physiological acclimation potential of the ecologically important Antarctic sea star, *Odontaster validus*, using functional responses to changing temperatures. Considerable research has explored the upper lethal limits of adult *O. validus* (Morley et al. 2012) as well their activity, feeding and metabolic limits (Peck et al. 2008) and showed that they are relatively resilient to the stresses associated with near-future increasing temperatures and decreasing pH compared with sympatric Antarctic benthic invertebrates (Byrne et al. 2016). However, these studies typically last short periods (days to weeks) and are unlikely to capture true acclimation at the physiological level, as these processes occur at slower rates and over longer time periods (Suckling et al. 2015), requiring long-term experimental time scales (months, years). The importance of longer term exposures to combined stressors and the value of such work for understanding species responses through time cannot be overstated. In order to understand the ability of *O. validus* to acclimate, this research uses three indicators of individual fitness: oxygen consumption, morphometrics, and oxidative stress tests to describe the functional responses to increased temperature and the potential of *O. validus* to adapt to climate change.

This study will aim to answer the following four key research questions: (1) how does long term exposure to increased temperatures influence energetic prioritisation of body indices; (2) does respiration of *O. validus* in increased temperatures change over time; (3) does long term exposure to increased temperatures drive oxidative stress; (4) is the antioxidant system of *O. validus* able to acclimate to the physiological demands of increased temperatures over time?

1.2 Climate change in Antarctica

Climate change is driving ecological transformations globally, altering species interactions, causing range shifts and testing the physiological tolerance thresholds of organisms to variation in their environments (Byrne et al., 2016). Global ocean pH is predicted to decrease by 0.3 – 0.4 pH units within this century (Caldeira and Wickett, 2003). Such environmental changes can have significant effects on marine organisms, effecting their physiology, biochemistry and potentially their evolutionary future as well as altering biodiversity and the ecosystem balance (Zhang et al., 2014). In Antarctica, there are both physical and biotic challenges facing marine ecosystems (Agüera et al., 2015a).

Changes to ocean circulation and the delivery of warmer water to the base of ice shelves has caused the warming of Antarctic Continental Shelf Bottom Water (ASBW) (Gille, 2002; Wijk and Rintoul, 2014). ASBW refers to the water occupying the sea floor of the Antarctic Continental Shelf. This water is made up of Circumpolar Deep Water (CDW), Winter Water (WW) and contributing waters from ice shelf melt, sea ice and continental run off (Schmidtko et al., 2014). Temporal variations in the amounts and properties of the contributing waters lead to changes in ASBW. Hydrographic data from the 1980s-2000s shows that ASBW has warmed in most basins and has become fresher and less dense in the Australian Antarctic Basin (Wijk and Rintoul, 2014). Increased ice sheet melting is occurring in the same regions that are experiencing warming ASBW (Schmidtko et al., 2014). The Southern Ocean is predicted to warm between 0.7°C and 1.9°C by 2070 (Rintoul et al., 2018)

The Antarctic peninsula has experienced a 3°C air temperature increase and a 1°C ocean surface temperature increase over the last 50 years (Meredith and King, 2005; Vaughan et al., 2003). As a result of these changes, ectothermal species such as Antarctic marine invertebrates

may be more responsive to changes in their environment compared to endothermic animals who are able to regulate their temperatures (Everatt et al., 2015). Environmental conditions in Antarctica are relatively stenothermal year-round and although the physiology of polar invertebrates has adaptations to withstand the extreme cold, they are not as readily able to cope with relatively high variation or shifts in the thermal regime of their environment (Agüera et al., 2015a). The warming of waters around Antarctic in the past several decades has caused increase rates of ice sheet melt (Gille, 2014). Sea ice losses combined with changes to the thermal dynamics and oceanography of the Antarctic marine environment are predicted to have widespread impacts on the ecosystem from the distribution of salps and krill to the foraging behaviour of mega fauna (Grange and Smith, 2013; Schmidtko et al., 2014).

There are three key ways in which organisms can survive changing environments: acclimatisation, migration and adaptation (Byrne et al., 2016). Adaptation of an entire species takes generations and it is therefore not plausible to study for animals with long generation times (Peck, 2005). For species at lower latitudes, there is the option to shift their distribution poleward in search of conditions that are more suited to their physiological limits, but this option is not available for species that are already in polar waters. Therefore, acclimatisation is the focus of many researchers for polar species, as understanding the phenotypic plasticity of individuals will be important for predicting species winners and losers in the face of climate change (Griffiths et al., 2017).

1.3 Antarctic sea stars (*Odontaster validus*)

Odontaster validus is an abundant cushion sea star with a circumpolar distribution in the Antarctic marine benthos (Kidawa, 2001). The geographic range of *O. validus* is thought to be from South Georgia (58 °S) to McMurdo Sound (78 °S) (Stanwell-Smith and Clarke, 1998).

They occupy a wide depth range from intertidal to 900m but are predominantly found between 15-200m (Peck et al., 2013). *Odontaster validus* range from 2-11cm in size, are coloured bright red dorsally and light pink ventrally, and do not display external sexual dimorphism (Fig 1.1) (McClintock et al., 2008).

Figure 1.1 *Odontaster validus* photographed in McMurdo at 17 m depth. Individuals can vary in size and colour (Photograph: Miles Lamare)

Odontaster validus have a broad omnivorous diet that which includes predation, scavenging and suspension feeding (Kidawa, 2001). Recorded prey types include sponges, molluscs, hydroids, bryozoans and detritus (Dayton et al., 1974). This diverse diet enables them to feed on a variety of sources, combating the challenges of seasonally available prey (Clarke, 1988; McClintock et al., 2008). Low concentrations of predators and employment of such a range of feeding strategies make *O. validus* a keystone predator in the Antarctic marine benthos (Byrne et al., 2016). *O. validus* pyloric caecum (the digestive organ) enables individuals to store energy and therefore survive long periods of starvation (Kidawa et al., 2010). Like most Antarctic invertebrates they are adapted to a stenothermal environment with very cold temperatures,

around -1.8□C and with little seasonal variation (Peck et al., 2014). Individuals have low metabolic rates and slow reproductive cycles with high fecundity and pelagic feeding larvae (Agüera et al., 2015a; Bosch and Pearse, 1990; Gonzalez-Bernat et al., 2013).

O. validus development differs somewhat from other asteroids as it is extremely slow (Pearse, 1969). The reproductive cycle involves the formation of ova over an 18-24 month period, and they employ the same strategy of broadcast spawning and external fertilisation found in most echinoderms. However, gametogenic cycles overlap leading to simultaneous development of oocytes from different generations in the same gonad (Agüera et al., 2015a; Chiantore et al., 2002). The larval stages are long lived, taking up to 165 days to reach juvenile stages, and slow developing with feeding beginning after 4-8 weeks (Bosch and Pearse, 1990; Peck et al., 2009a; Stanwell-Smith et al., 1999). Sexual maturity is thought to be achieved by 3-6 years (Pearse, 1969). The process of reproduction is physiologically demanding and reproductive parameters may be compromised by exposure to environmental stressors as energy is conserved and redirected to physiological defences to improve survival (Lister et al., 2016).

A study exploring the resistance of juveniles to warming in Antarctic marine invertebrates found that juvenile *O. validus* survived to higher temperatures than adults at slow warming rates but performed the same at faster warming rates (Peck et al., 2013). However, this study further emphasises the issue of short term experiments that last only a couple of days compared to long term exposures. In the case of the Antarctic sea urchin (*Sterechinus neumayeri*), reproductive "recovery" was observed during spawning after 17 months acclimation compared with a reduced reproductive output at six months, highlighting both the importance of long term acclimation studies to accurately capture acclimation and the potential for Antarctic invertebrates to adapt to a warmer, more acidic Southern Ocean when provided ecologically relevant time scales to adjust (Suckling et al., 2015).

A significant concern for marine environments on a global scale is ocean acidification, as pH is a function of temperature. *O. validus* have been found to adjust their physiology to changes of 0.3 pH units below optimum (8.1) however this ability is limited and may result in reduced survival (Gonzalez-Bernat et al., 2013). It has been suggested that temperature may override the effects of pH on fertilisation in sympatric species (Byrne et al., 2016). The physiological acclimation potential of *O. validus* across the life stages is important for generating an understanding of how the species will respond to future changes.

1.4 Acclimation

Acclimation is a laboratory proxy for acclimatisation to changes in the environment (Peck et al., 2010). Acclimation research seeks to fill the knowledge gap concerning the response of Antarctic fauna to environmental changes, including temperature shifts, increased seasonality, productivity changes and increased ocean acidification (Peck et al., 2010). Current research has highlighted the importance of longer term acclimation studies that measure responses over a year or more and investigation of the potential for transgenerational plasticity within species (Suckling et al., 2015). Short term studies that report no acclimation after four to eight weeks of exposure could be too short to observe full body acclimation, depending on the subject and therefore may not accurately represent the acclimation potential of the organisms studied (Peck et al., 2010). The time taken to fully acclimate to environmental change depends both on the species being manipulated and the conditions being changed (Suckling et al., 2015). Rates of change are also extremely important for determining the outcomes of acclimation experiments and are hard to standardise across literature (Peck et al., 2009a, 2013).

Suckling et al (2015) found that the acclimation time of the Antarctic sea urchin (*Sterechinus neumayeri*) to raised temperature and decreased pH conditions varied from their expected acclimation time of six months and were not fully acclimated until eight months in their

experimental treatments. This was likely due to the added stress of pH change as a factor in the treatments and the combined effects of temperature and pH on the organisms. Throughout the duration of this study, although considered fully acclimated, metabolic rate did not return to the levels measured in the field prior to collection.

Several species of Antarctic fish have been successfully acclimated to water temperatures of 4°C (Peck et al., 2008), whilst the Antarctic clam *Laturnula elliptica* could survive at 12°C but was not capable of key behaviours such as burrowing and feeding at this temperature (Morley et al., 2012). Survival limits and acclimation are different aspects of thermal biology and although both are valuable, they are best considered together and in the appropriate ecological context (Peck et al., 2009). It is important to test behaviour and ecologically important activity such as feeding and reproduction during acclimation studies in order to ascertain the functional mortality threshold of the organism rather than just the upper thermal limit for survival as this will better inform predictions and conclusions about the future of the species in the face of environmental changes (Peck et al., 2009).

1.5 Measuring acclimation

This research will use three physiological measurements of acclimation to determine adult responses to temperature, namely oxidative stress, metabolic rates and energy allocation (morphometrics).

Oxidative stress is a tool that may be used to investigate the effects of temperature on the tissues of *O. validus*. Oxidative stress occurs when the rate of reactive oxygen species (ROS) generation exceeds the scavenging capacity of an organisms antioxidant system (Lister et al.,

2016). In optimum conditions that are not causing stress, the production of ROS is relatively steady and able to be neutralised by the levels of cellular antioxidant defences (Burritt and Lamare, 2016). Changing environmental conditions such as temperature, salinity, pH, pollutants and the presence of photosynthetic organisms can influence redox reactions in aquatic organisms and therefore affect the maintenance of steady state ROS levels (Canesi, 2015). The antioxidant system of marine organisms consists of an interacting network of antioxidant enzymes and low molecular weight scavengers, but identifying oxidative stress can be difficult due to the variation of responses between exposure levels, tissue types and through time (Regoli and Giuliani, 2014). Analysing four tissue types from *O. validus* across five temperature treatments will provide insight into the response of the individuals to stress at a cellular level and across different tissue types to explore any changes that occur in ROS levels through time.

Studies of the temperature sea urchin *Evechinus chloroticus* found that maternal exposure history did not advantageously protect offspring against oxidative DNA damage but did enhance the capacity of embryos to reduce lipid and protein damage as a result of OS (Lister et al., 2017). For the Antarctic sea urchin *Sterechinus neumayeri*, offspring of contaminant exposed mothers were provisioned with greater baseline antioxidant levels which reflected an enhanced capacity to reduce oxidative damage to DNA, lipids and proteins (Lister et al., 2015). Frogs, fish and invertebrates have been documented to experience temperature induced oxidative stress (Lushchak, 2011). Further studies show that additional environmental stressors such as pollutants or pH combined with increased temperatures enhance the likelihood of oxidative damage (Regoli and Giuliani, 2014; Verlecar et al., 2007).

Respiration is a common measurement of physiological responses and fitness and can provide important information about the energy use of an individual and the variation of metabolic

requirements as a result of environmental changes (Marsh and Manahan, 1999; Peck and Prothero-Thomas, 2002). The rate of oxygen use for an individual can be influenced by activity level, ocean acidification and temperature (Zhang et al., 2014). Oxygen consumption is closely related to specimen size (Ruhl et al., 2014). The low resting metabolic rates exhibited by Antarctic marine invertebrates can be 2-10x slower than those of similar tropical or temperate species (Sparks, 2018), and may indicate reduced aerobic scope (Brockington and Peck, 2001). Metabolic rates of *O. validus* have been previously documented in temperatures up to 12°C. Peck et al., (2008) recorded oxygen consumption at -1.5°C as 0.107 mg O_2 g^{-1} dry weight h^{-1} and 0.300 mg g^{-1} dry weight h^{-1} at 12°C. The study found that respiration increased with temperature until 15°C, where it rapidly declined and mortality increased. A separate study of wild populations measured increased oxygen consumption in summer (0.00654 mg O_2 g^{-1} dry $weight^{-1}$ h^{-1}) compared to winter (0.002mg O_2 g^{-1} dry $weight^{-1}$ h^{-1}) (Sparks, 2018). Respiration is regularly used to characterise and measure energy consumption and physiological state of individuals (Stumpp et al., 2019). This technique can be used to measure the metabolic energy requirements of both adult and larval *O. validus.*

Species performance in natural or stressed conditions is determined by the fitness of the individual (Lesser, 2013). Dynamic energy budgets (DEB) describe the use of energy for growth, maintenance, development and reproduction and can be used to model how the allocation of energy may change under different conditions (Agüera et al., 2015; Kooijman and Troost, 2007). For *O. validus*, the DEB predicts that warming will result in increased oxygen consumption, however individual ability to withstand periods of limited food availability decreases as temperature increases (Agüera et al., 2015a). The energy budget of an organism can be impacted by environmental stressors experienced by the individual and have long-term effects on growth, reproduction and population dynamics (Enzor et al., 2017). The energy

allocation of *O. validus* to somatic and reproductive efforts may highlight the extent of physiological acclimation of individuals across temperature treatments.

1.6 Experimental Design

1.6.1 Collection & maintenance

Study subjects were collected from 20 m depth at McMurdo Sound in November 2018 and randomly divided into five environmentally relevant, present day and near future sea temperatures (-1°C, 1°C, 2°C, 3°C, 4°C) for coastal Antarctica. These temperatures were chosen as the average benthic water temperature in Antarctica during winter is around -2°C, and around 1-2°C in summer (Pearse et al., 1991; Peck et al., 2010) and the higher temperatures are similar to those predicted to occur in the near future based on models of ice melt and ocean warming (Schmidtko et al., 2014).

The rate of warming was 0.5°C per week until the temperature was reached. Temperatures were maintained using in five thermostatically controlled fridges (Figure 1.3), with temperature recorded hourly using hobotemp loggers in each fridge (Fig. 1.2). Short term, small temperature spikes occurred in these data every two weeks when water was refreshed and had to be cooled to temperature before being pumped into the tanks again. There was a failure of the thermostat in Fridge #0 (Fig. 2), resulting in a period of fluctuating water temperatures, although no death was recorded in the animals. Due to the thermostat failure, the average temperature in Fridge #0 for the duration of the study was -0.13°C (Table 1:1). This was higher than the target temperature, however it still provides an environmentally appropriate control for the purposes of this work. Seasonal changes were not simulated for this study.

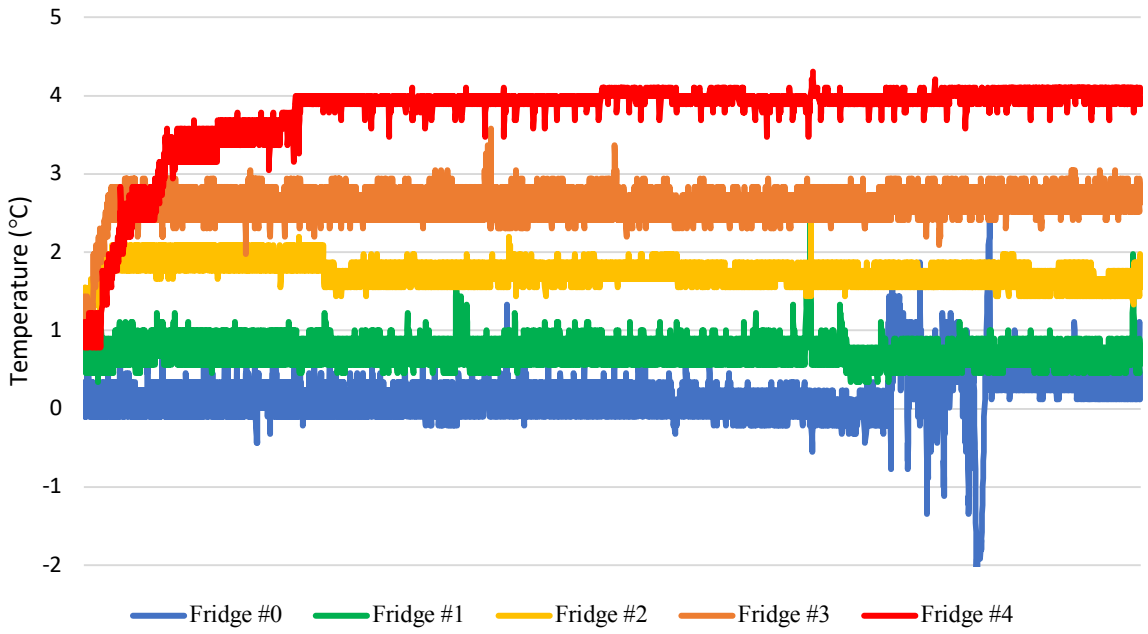

Figure 1.2 Average temperature for each fridge recorded using hobotemp loggers in the experimental tanks from November 2018 to November 2019

The experimental tank set up features 5 separate temperature-controlled fridges with 8 smaller experimental tanks (10 L) in each, supplied with recirculating water via header (40 L) and sumps tanks (80 L). Water circulation was continuous, with water in each experimental take overturned every 3-5 mins. The sumps contain water from Otago Harbour and are cooled to temperature before being pumped to the tanks. Figure 1.3 depicts an overview of the set up. Animals were reared in experimental tanks at densities of ~20 individuals per tank. Feeding took place every two weeks for all animals and the diet was comprised of frozen cod and local cockles.

Table 1.1 Target and mean temperatures (± SE) of experimental fridges during the study

	Fridge #0	Fridge #1	Fridge #2	Fridge #3	Fridge #4
Target Temperature	-1.00°C	1.00°C	2.00°C	3.00°C	4.00°C
Mean Temperature and Standard Error	-0.13 °C ± 0.0042	0.74°C ± 0.0020	1.94°C ± 0.0036	2.87°C ± 0.0025	3.92°C ± 0.0034

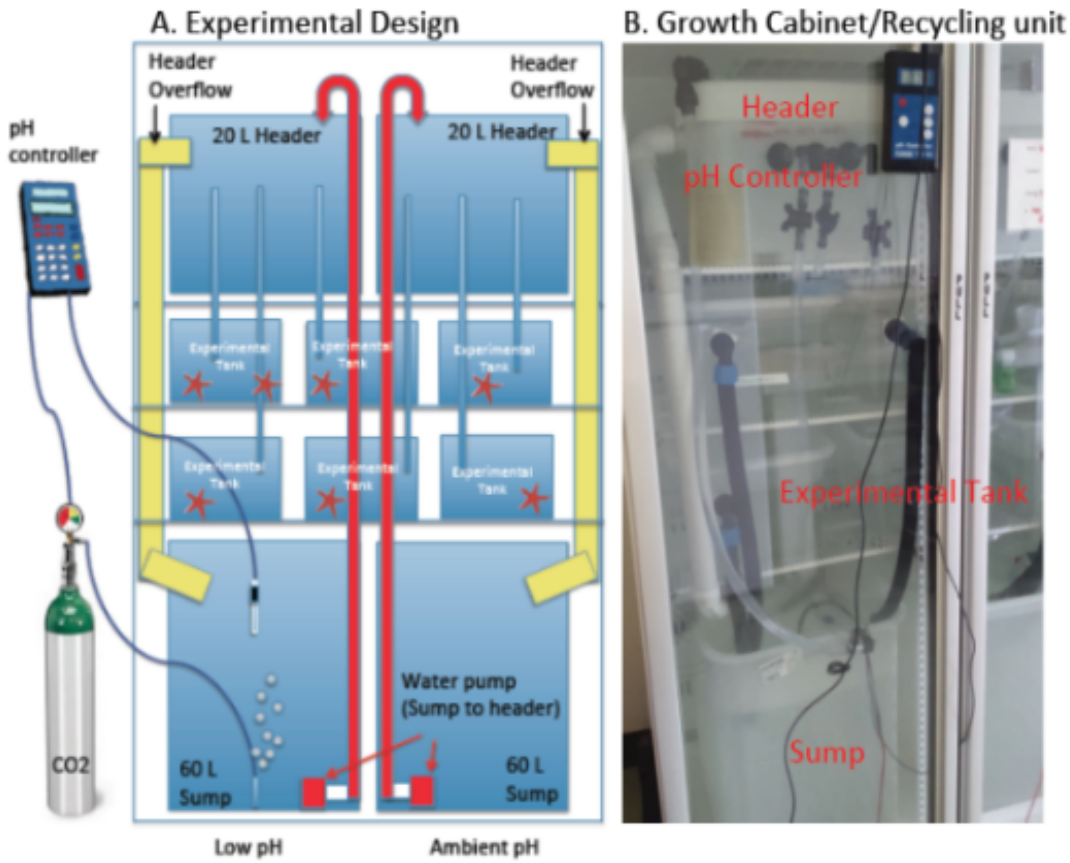

Figure 1.3 Experimental units for treating sea stars to elevated temperatures. The schematic illustrates a single cabinet with duplicate systems (A) indicating the flow of water from the sump to the header by pumping. Photograph of a single cabinet showing the operational experimental set up (B). Note: The CO_2 and pH controllers were leftover features of the set up from a prior study. pH was not manipulated for this study.

1.6.2 Data collection

Dissections and respiration measurements were performed every three months beginning in February 2019 and ending in November 2019. Table 1.1 indicates the total specimen count at each experimental stage. In addition to the 190 animals collected in 2018 there were 12 animals from a previous collection effort in 2016. A total of 160 individuals were used for the studies contained within this thesis, with additional animals stored for alternate purposes.

Table 1.2 *Odontaster validus* specimen counts from collection at McMurdo Sound, Antarctica (T_{ANT}) and at each of the sampling points in February, May, August and November 2019

		Specimen count	*# dissected*	*Mortalities*	*Adjusted specimen count*
T_{ANT}	November 2018	202	0	5	197
T_1	February 2019	197	30	4	163
T_2	May 2019	163	25	1	137
T_3	August 2019	137	25	0	113
T_4	November 2019	113	25	0	88
T_{FINAL}	December 2020	88	0	0	88

Specific laboratory and statistical methodology pertaining to respiration and morphometrics may be found in Section 2.2 and Section 3.2 for oxidative stress.

Chapter 2: Physiological responses of *Odontaster validus* to near future temperatures

2.1 Introduction

Respiration is a well-established measure of metabolic activity and physiological fitness and can provide insight into the energy use of an individual and the variation in metabolic requirements as a result of environmental changes (Marsh & Manahan, 1999; L. Peck & Prothero-Thomas, 2002). Oxygen consumption is closely related to individual size and is known to decrease as body mass increases (Kiørboe et al., 2014; Ruhl et al., 2014). Respiration rates are also influenced by activity levels, nutritional status, temperature as well as environmental stressors (Zhang et al., 2014).

Aerobic scope is the ability of an animal to increase its metabolic rate above maintenance levels and has been described as a key concept which controls the thermal limits of many Antarctic invertebrates (Peck et al., 2010). However, in sea stars, locomotion is primarily facilitated by the water vascular system (Hamilton, 1922) and is therefore not as closely tied to the performance of muscles and oxygen availability. This means that reduced aerobic scope in *O. validus* does not limit activity in the same way that is shown to occur in species of Antarctic fish and invertebrates (Peck et al., 2008). Therefore, as respiration is a direct measurement that can be used to assess the functional health of an individual, it can provide a valuable baseline estimate for metabolic fitness and therefore indicate how *O. validus* responds (and potentially acclimates) to environmental changes such as warming (Suckling et al., 2015).

The Antarctic marine environment is characterised by low (typically sub-zero), but relatively stable sea temperatures, with animals evolving adaptations to withstand the cold temperature (Peck et al., 2014). *O. validus* has an extremely slow metabolism with low somatic

maintenance, around 3 J cm^{-3} d^{-1} at 0°C (Agüera et al., 2015a). The reproductive cycle of *O. validus* is also longer than that of tropical and temperate species; oogenesis takes between 18-24 months, blastulae form around 2 days after fertilisation, gastrulation is initiated after 7 days and the bipinnaria develops after approximately 40 to 55 days (Olson et al., 1987; Sparks, 2018). Previous studies of *O. validus* respiration have documented seasonal and spatial variation in oxygen consumption (Clarke, 1988; Morley et al., 2012). In this respect, *O. validus* have been found to consume significantly more oxygen in summer, up to 44% higher than their winter consumption rate (Souster et al., 2018).

Oxygen consumption is used as a proxy for metabolic rate. Metabolism is a fundamental fitness parameter for all organisms and consequently it is crucial to understand factors affecting individual energy requirements (Glazier, 2010). Metabolic scaling is the relationship between metabolic rate and organism mass (Glazier, 2005) according to the power function $R = aM^b$, where a is a scaling coefficient, M is mass and b is the scaling factor (Burgess et al., 2017; Christensen et al., 2022). The scaling factor can be affected by environmental factors including temperature (Gillooly et al., 2001; Verberk et al., 2016), making it an important physiological consideration when exploring species response to climate change.

Morphometrics are a well-established tool used to investigate sea star biology (Sigl et al., 2013). The collection of organ size indices is an important measurement of physiological health and can provide insight into the energy use and allocation of an individual and the variation of metabolic requirements as a result of environmental changes (McClintock et al., 1988). To further understand and predict the response of *O. validus* to climate change, Agüera et al., (2015a) created a Dynamic Energy Budget (DEB). This model type is used extensively in fisheries, aquaculture and conservation to understand species life history traits and to model population dynamics as well as to predict how the energy use of an organism may change under

different conditions (Agüera and Byrne, 2018). In sea star ecology, two measurements are of particular importance: the gonadosomatic index (GI) and pyloric caeca index (PI).

The gonadosomatic index can provide information about sexual maturity, reproductive health and reproductive output. *O. validus* is a broadcast spawner that reproduces during the austral winter and has high fecundity (Bosch and Pearse, 1990; Chiantore et al., 2002). The pelagic feeding larvae take at least 5-6 months to complete development and metamorphose into juveniles, and this means that the latter stages of larval development coincide with the spring algal bloom and therefore the time of maximum planktonic food availability (Agüera et al., 2015a; Bowden et al., 2009). Previous studies have also shown that *O. validus* has a two-year gametogenic cycle and simultaneously develops two cohorts of oocytes as oogenesis takes 18-24 months (Pearse et al., 1991). Measuring GI in this study will provide insight into how acclimated or not individuals are and whether they are physiologically able to invest energy into reproduction. The pyloric caecum is an energy reservoir for *O. validus.* The PI, therefore, reflects the physiological health of sea stars and indicates the nutritional environment that individuals are experiencing. *O. validus* are generalist scavengers and this leads to a seasonal trend in wild populations where PI is greatest during the austral summer (November to February) when productivity is high, food availability peaks, and *O. validus* are very active.

Respiration has been used as a tool to explore the thermal limits of Antarctic marine invertebrates including *O. validus* in a variety of conditions to explore upper lethal limits, geographic and seasonal changes and larval oxygen consumption (Peck and Prothero-Thomas, 2002; Peck et al., 2009a, 2008; Souster et al., 2018). The Dynamic Energy Budget for *O. validus* developed by Agüera et al., (2015a) is a useful tool for comparing the response of *O. validus* to thermal stress.

2.1.1 Research Aims

Two key questions are addressed in this study:

(1) **How does long term exposure to increased temperatures influence energetic prioritisation of body indices in *Odontaster validus*?** To test this, *Odontaster validus* were exposed to five temperatures and dissected at three month intervals. The two key tissues gonad and pyloric caecum were measured for comparison between temperatures through time.

(2) **Does the metabolism of *Odontaster validus* acclimate to increased temperatures over time?** To test this, *Odontaster validus* were exposed to five temperatures and respiration was measured three times across 10 months.

This chapter aims to evaluate the acclimation capacity of *Odontaster validus* during long-term exposure to increased temperatures by measuring the key physiological markers of respiration, metabolism and morphometrics as response indicators. It is expected that both respiration and morphometrics will be affected by increased temperatures.

2.2 Methods

2.2.1 Respiration rates

Respiration was measured using 200 ml sealed chambers and a Presens respirometer (OXY-1 SMA-BT) that were constantly stirred using internal magnetic stir bars. Individual sea stars were chosen at random from each acclimation temperature treatment, and placed in the respiration chambers with water of the same temperature. An empty sealed chamber with 200 mL of seawater was used to control for O_2 changes during measurement. For each temperature treatment, four animals were measured simultaneously. Oxygen consumption was measured prior to fortnightly feeding to ensure individuals were equally starved at the sample point.

Oxygen concentration (% a.s, air saturation) was measured every half hour for 4 hours, or until oxygen concentrations reach 80% of a.s. Following the experiment, individuals were wet weighed before being returned to their experimental tanks. These experiments were repeated every 3 months in February, August and November to determine any changes in the rate of oxygen consumption of individuals across treatments. It was not possible to take measurements in May 2019, due to equipment failure. Individuals were chosen at random at each sample point. The average respiration rate measured at the time of collection (October 2018) in Antarctica were used as a baseline rate. The McMurdo Sound average was 0.0044 mg $O_2\,h^{-1}\,g^{-1}$ and the average across four sites (Mc Murdo Sound, Cape Evans, Arrival Heights and Turtle Rock) in Antarctica was 0.0041 mg $O_2\,h^{-1}\,g^{-1}$

The Presens conversion calculator was used to convert the oxygen % a.s to mg/L (https://www.presens.de›fileadmin›tools_utilities) for ambient air pressure and given chamber water temperature. To determine the rate of oxygen consumption for each animal, oxygen concentration versus time (Fig 2.1), and the rate of oxygen depletion was determined from the

slope of a linear regression. Using this rate and adjusting for chamber water volume and weight of the animal, the oxygen consumption rate as mg O_2 per g (of animal wet weight) per hour (mg O_2 g^{-1} h^{-1}) was calculated. The rate of oxygen consumption was adjusted, accounting for any changes in oxygen associated with the blank control.

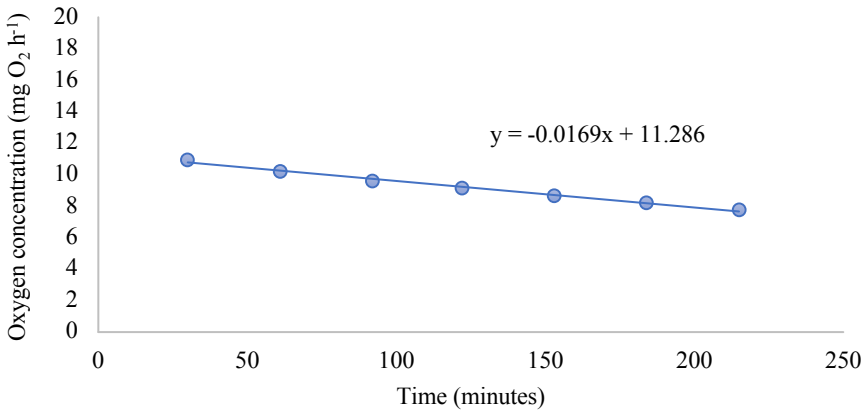

Figure 2.1 An example of changes in O_2 concentration in a respiration chamber over a 230 min period. The linear regression slope indicates the rate of change in concentration per minute.

Q_{10} calculations are a temperature coefficient and describe the rate at which a reaction will increase with a 10°C increase in temperature (Gillooly et al., 2001; Peck, 2016). These were calculated using the equation;

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{\left(\frac{10}{T2-T1}\right)} \qquad \text{Eq. 1}$$

where R = reaction rate and T = temperature. In this case R_1 and R_2 refer to the respiration rate at the baseline temperature (T_1) and the experimental temperature (T_2). This calculation was use to establish Q_{10} coefficients for respiration measurements.

To determine the effects of size on metabolism (metabolic scaling), the oxygen uptake (mg O_2 h^{-1}) and mass (g) were log transformed and plotted for whole animals in each temperature treatment. A linear regression was fit to the data to determine the scaling coefficient, *b* (slope of the line). Pairwise slope comparisons were used to examine differences between the treatments.

2.2.2 Morphometric measurements

For morphometric data, each animal was measured for width and whole wet weight. Animals were then dissected, and three main tissue types wet weighed, namely gonad, pyloric caecum and body wall (including associated structures such as the water vascular system). For each individual, this produces five descriptors: radial length which was measured from the central disc to the tip of the longest arm (mm), wet weight (g), gonad weight (g), pyloric caecum weight (g) and body wall weight (g). The proportionate weight of the pyloric caecum and gonad relative to the pre-dissection wet weight was calculated by dividing the tissue weight by the whole:

Equations for body indices (as published by Agüera et al., 2015a):

Gonadosomatic index (%) = (gonad weight (g)/ total wet weight (g)) * 100

Pyloric index (%) = (pyloric caeca weight (g)/ (total wet weight-gonad)) * 100

Measurements were made at four times of one year (February, May, August and November), with five animals per treatment dissected at each time point.

2.2.3 Statistical analysis

Respiration and morphometric data analysis was performed in R Studio (R Studio Team, 2022). Levene's test for homogeneity of variances was performed on respiration and morphometric data to ensure assumptions were met. Respiration rates were analysed using a two-way ANOVA with oxygen consumption as the response and temperature and month as predictor variables. For respiration analysis, factors were acclimation Temperature (5 levels), Month of measurement (three levels), and Temperature x Month. Tukey's HSD tests were run on significant results. Morphometric data were also analysed using two-way ANOVA for each of the body indices; pyloric caecum index, temperature and month and gonadosomatic index, temperature and month. Factors were acclimation Temperature (5 levels), Month of measurement (four levels) and Temperature x Month. Tukey's HSD tests were run on significant results. Significance level was $p = <0.05$.

2.3 Results

2.3.1 Respiration rate

There was a range in weight of the individuals in which respiration was measured over the course of the study, from 4.71 g to 45.58 g (Figure 2.2). There was also variation in individual respiration rates across the study, with a minimum oxygen consumption of only 0.00138 mg O_2 h^{-1} g^{-1} and a maximum respiration rate of 0.0192 mg O_2 h^{-1} g^{-1} (Figure 2.2).

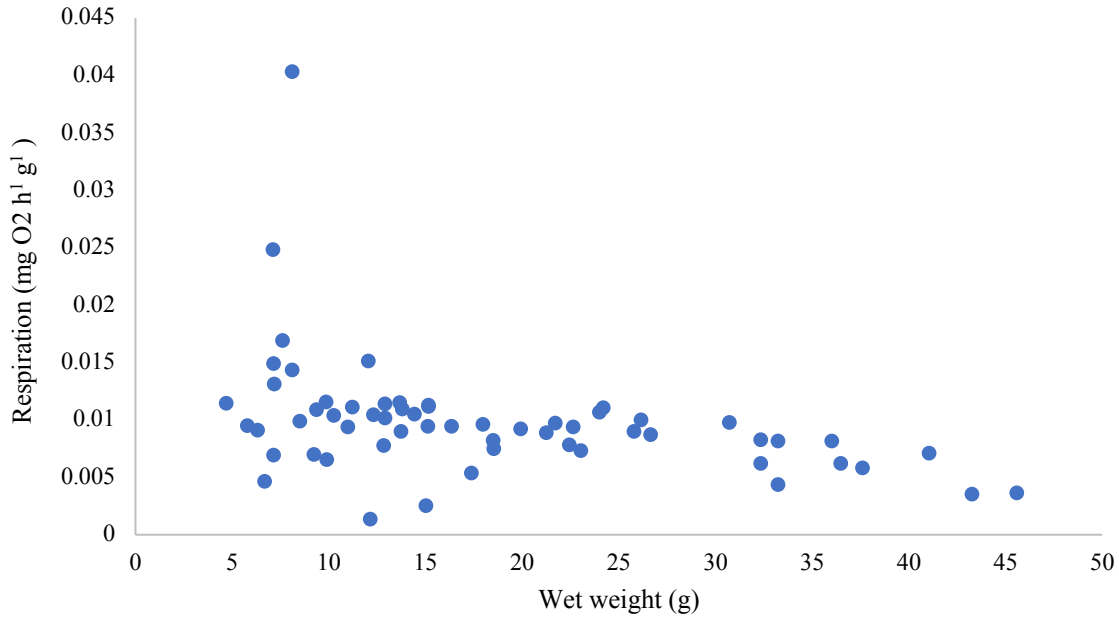

Figure 2.2 Wet weight distribution of *Odontaster validus* versus individual respiration rates across all temperature treatments

Metabolic Scaling

The metabolic scaling exponent *b* is estimated for each temperature by the slope of the linear regression lines of the log transformed data (Figure 2.3). These range from 0.91 in -1°C / 0°C to 0.70 at 4°C. For *O. validus*, oxygen consumption is increased with increasing animal size (Figure 2.3) across the five temperature treatments. In terms of the effects of temperature on

metabolic scaling, there was no significant difference between the metabolic scaling rates at 1°C , 2°C , 3°C or 4°C compared to -1°C / 0°C control (Table 2.1).

Table 2.1 Pairwise slope comparisons of *Odontaster validus* metabolic scaling rates in 1°C, 2°C , 3°C and 4°C relative to the -1°C / 0°C treatment

Slope comparison	***T*-statistic**	**DF**	***P* value**
-1°C – 1°C	0.336	20	0.740
-1°C – 2°C	0.118	20	0.906
-1°C – 3°C	0.432	20	0.669
-1°C – 4°C	0.634	20	0.533

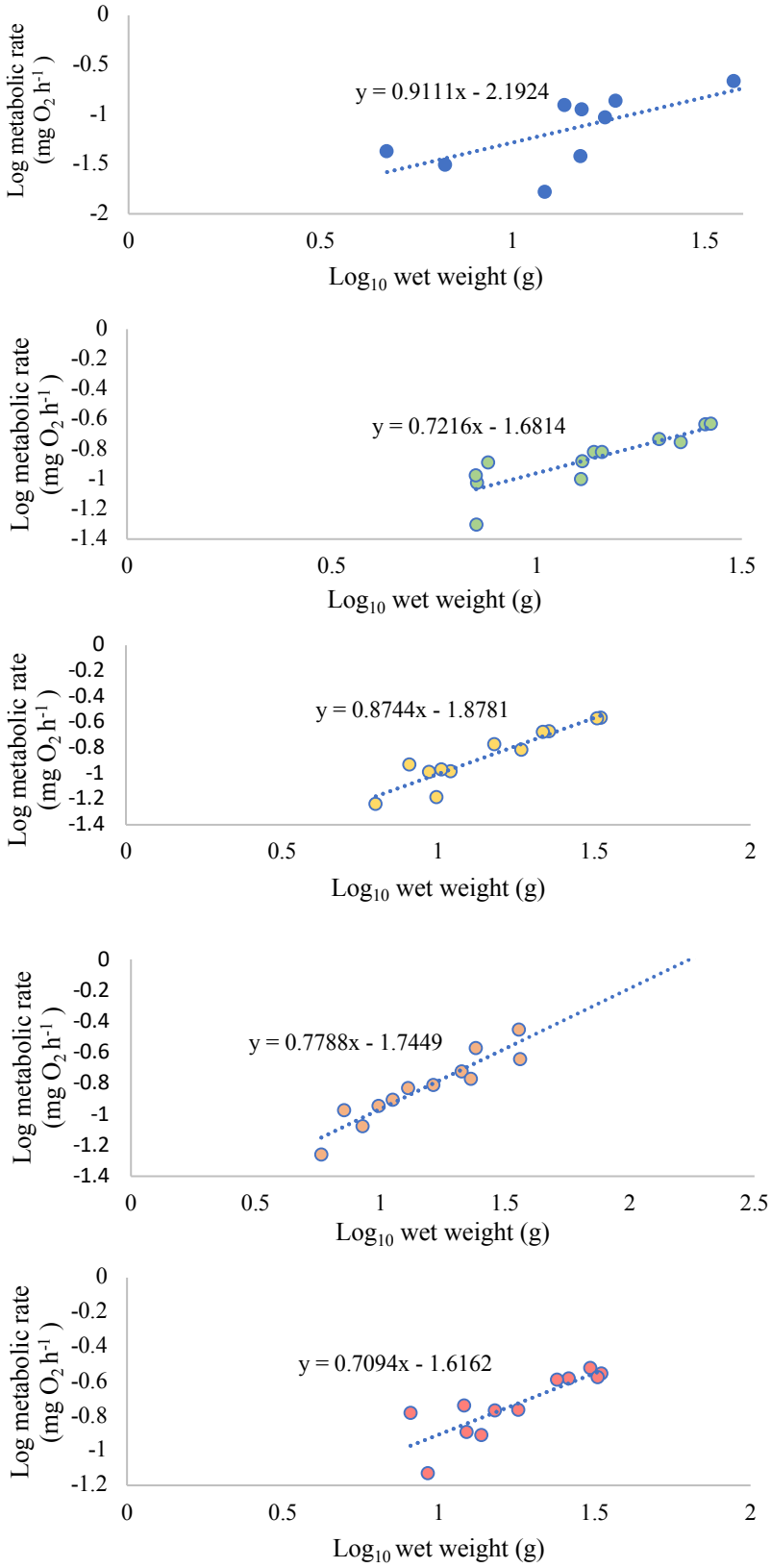

Figure 2.3 Metabolic scaling rates of *Odontaster validus* in five temperatures (top to bottom: -1°C / 0°C, 1°C, 2°C, 3°C, 4°C) in February, August and November of 2019. $N = 4$ per treatment, per month. The slope of the line is an estimate of the metabolic scaling.

The average respiration of animals tested from the same temperature through time showed that the highest rate of oxygen consumption was found in animals living in 4°C in November, with an average respiration rate of 0.01501 mg O_2 h^{-1} g^{-1}. The lowest average respiration rate (excluding the McMurdo Sound baseline) was 0.00465 mg O_2 h^{-1} g^{-1} and was recorded for individuals in -1°C/ / 0°C in August (Figure 2.4). Oxygen consumption in *O. validus* was significantly different among temperature treatments (p = 0.03, Table 2.2).

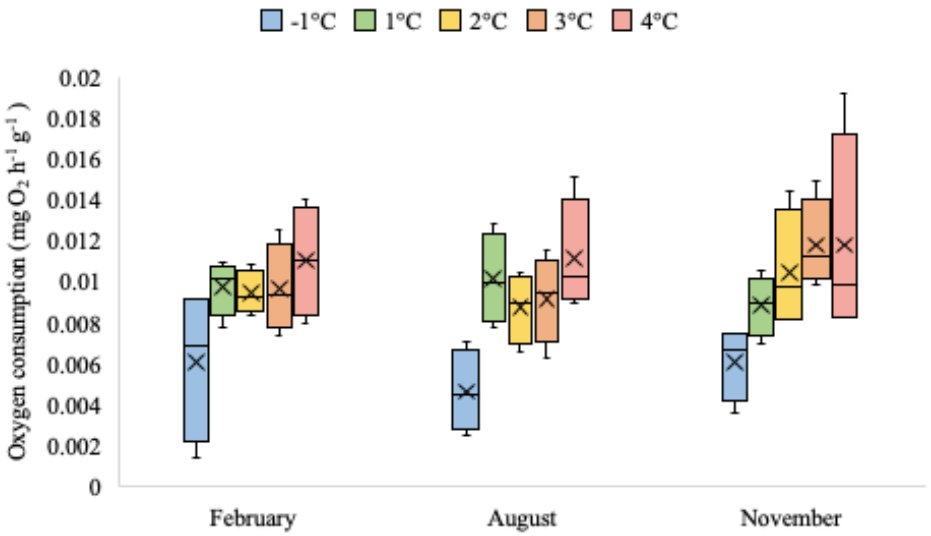

Figure 2.4 Average respiration rates of *Odontaster validus* in five water temperatures as sampled in February, August and November of 2019. Error bars show standard deviation.

Baseline respiration rates from -1.7°C in McMurdo Sound, Antarctica were 0.0044 mg O_2 h^{-1} g^{-1}. Throughout the duration of the study, respiration rates of individuals in the experimental temperatures did not return to the measured baseline. The Q_{10} for oxygen consumption in the 4°C treatment compared to the McMurdo Sound (-1.7°C) was 4.7.

Table 2.2 Two-way ANOVA for *Odontaster validus* respiration rates at five experimental temperatures and across three sample points in February, August and November 2019. Values with * indicate significance ($p<0.05$)

Source	DF	F	Sum of sq.	P
Temperature	4	3.72	0.00009	0.026*
Month	2	0.45	0.00000057	0.838
Temperature x Month	8	0.81	0.0013032	0.631

Post hoc Tukey's HSD tests showed that each month, the significant difference was between the -1°C / 0°C and 4°C temperatures with more oxygen consumed by individuals in the warmer 4°C treatment, $p = 0.049$. There was no significant interaction between month and temperature. Month was not significantly affecting oxygen consumption ($p= 0.73$, Table 2.2). Warmer temperatures had greater variation in respiration rates (Figure 2.4).

2.3.2 Body morphometrics

Individuals analysed for the body indices analysis had radial lengths of between 26.1 mm and 60.3 mm (Figure 2.5), and wet weights were between 4.0 g and 41.8 g. These ranges led to high variation in both body indices measured.

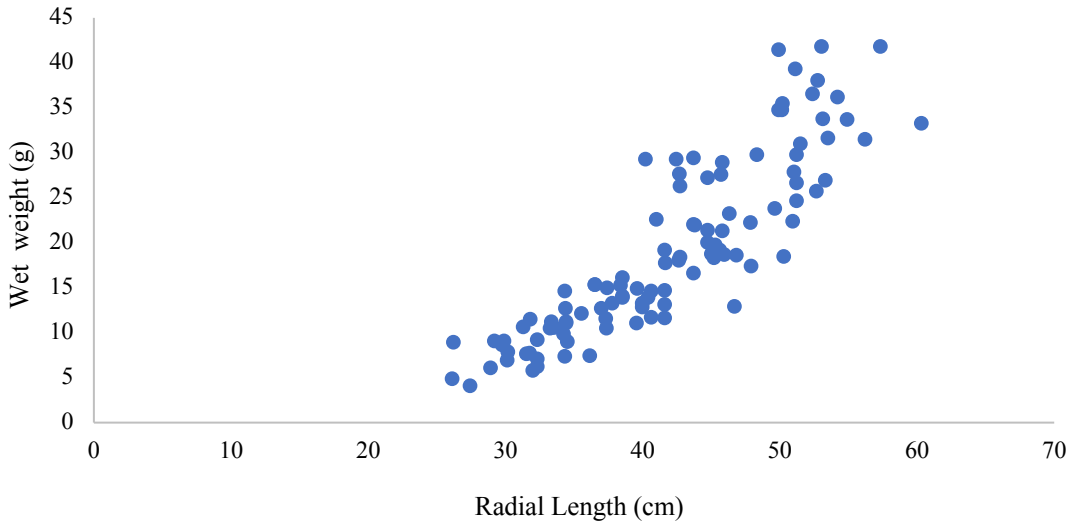

Figure 2.5 Radial length (measured from the centre of the disc to the tip of the longest arm) and wet weight of *Odontaster validus* sampled for body indices study

The maximum pyloric caecum index measured across the study was 20.2%, whilst the minimum was only 9.8% (Fig. 2.6). The minimum gonad index was recorded at 3.7% and maximum was 10.1% (Figure 2.7). Mean GI and PI across all treatments and months were 5.8% and 14.1%, respectively.

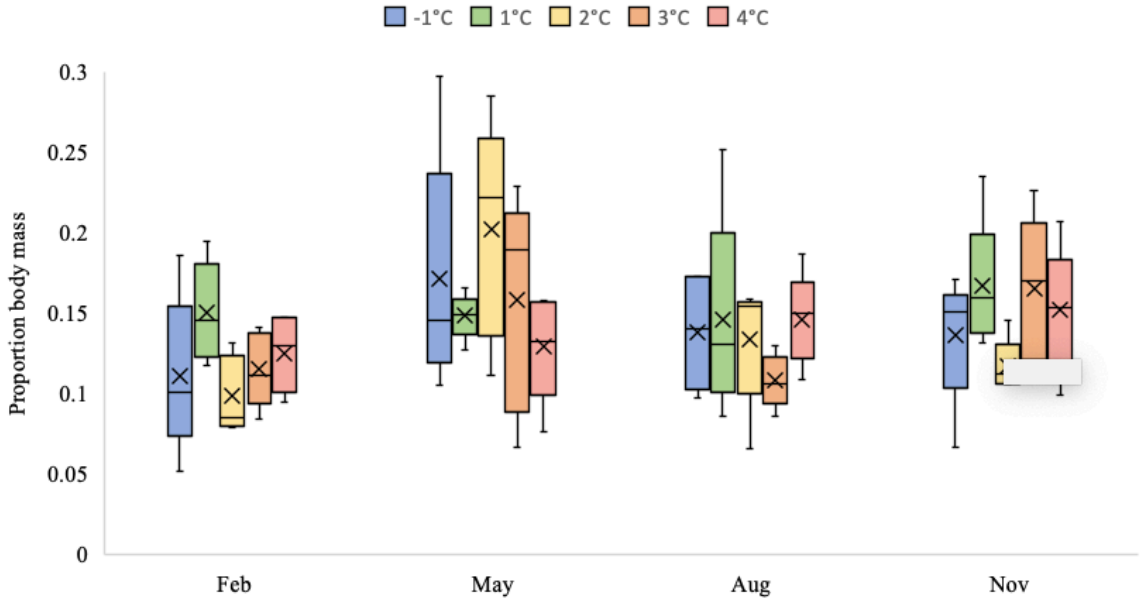

Figure 2.6 Average Pyloric Caecum Index of *Odontaster validus* across 5 water temperatures in February, May, August and November of 2019. Error bars show standard deviation.

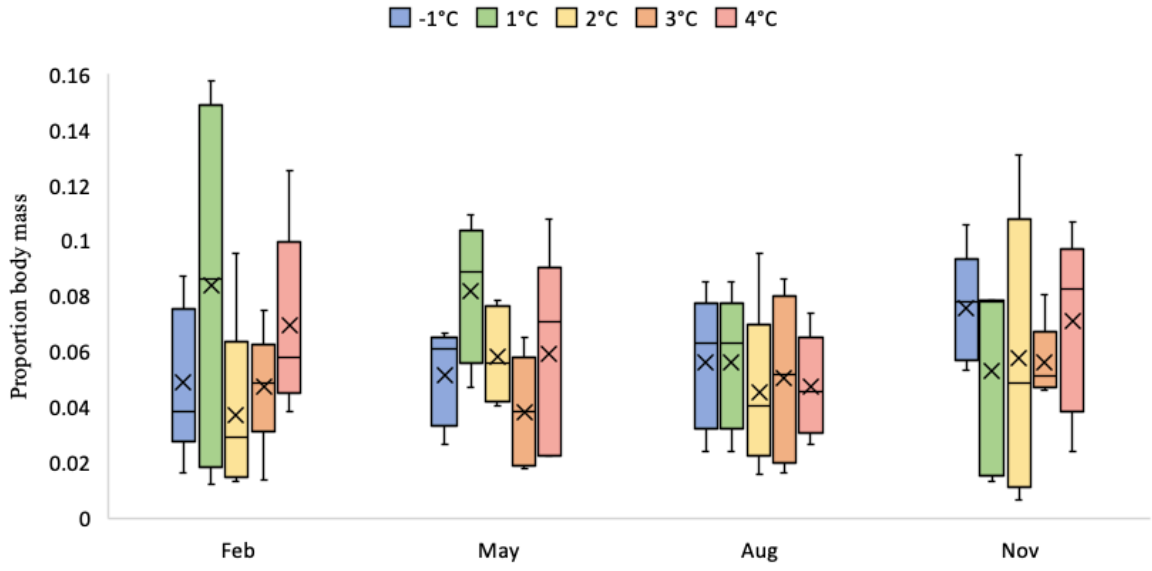

Figure 2.7 Average Gonadosomatic Index of *Odontaster validus* across 5 water temperatures in February, May, August and November of 2019. Error bars show standard deviation.

There were no significant differences in the pyloric index of *O. validus* among temperature treatments (p=0.86, Table 2.3) or month (p=0.09, Table 2.3). Gonadosomatic index was significantly different among temperature treatments (p=0.03), a post-hoc Tukey's HSD test

showed that the significant difference was between gonad indices from -1°C / 0°C and 3°C treatments (p=0.02) with greater GI measured in individuals in the colder treatment. Month was not significantly influencing gonadosomatic indices (p=0.92, Table 2.3).

Table 2.3 ANOVA of *O. validus* Pyloric Index and Gonadosomatic Index from sample points in February, May, August and November in 5 water temperature treatments

Source	DF	F	Sum of sq.	P
A. Gonadosomatic Index				
Temperature	4	3.693	0.00272	0.035*
Month	3	0.158	0.00008	0.922
Temperature x Month	12	0.759	0.00221	0.690
B. Pyloric Caecum Index				
Temperature	4	0.314	0.00069	0.863
Month	3	2.620	0.00435	0.098
Temperature x Month	12	1.145	0.00664	0.336

2.4 Discussion

In this study the acclimation potential of *O. validus* to increased temperatures was investigated using physiological measures of fitness. Acclimation would be demonstrated by observing metabolic rates in the treatments that are adjusted overtime to become comparable with those measured in the field (baseline). In this respect, the respiration rates recorded do not show evidence of acclimation, with respiration rates of individuals in all treatments consistently greater than rates recorded in the field, even after 10 months. Respiration has been used as a measurement of acclimation in several species (Pörtner, 2010). This is possible as oxygen consumption is an indicator of metabolic stability, and thus, provides insight into the phenotypic plasticity of species and their ability to respond to environmental changes (Peck et al., 2010; Somero, 2010).

The extremely cold conditions that characterise the Antarctic marine environment have led to a number of studies exploring the potential for Antarctic fish and invertebrates to respond to environmental change. *Sterechinus neumayeri,* the Antarctic sea urchin has been documented to survive in increased temperatures similar to the ones used in this study, however full body acclimation was not achieved (Peck et al., 2010). In the same study, Peck et al. (2010) investigated acute thermal limits of six Antarctic invertebrates and found that only one, the Antarctic mollusc *Marseniopsis mollis,* was capable of physiological acclimation after 60 days at 3°C. In contrast, *S. neumayeri* oxygen consumption was not significantly different between measurements taken immediately after the temperature increase and after 60 days. This implies that *S. neumayeri* was not able to adjust metabolically to the increased temperature, and suggests that if acclimation is possible, the time required exceeds 60 days (Peck et al., 2010).

Peck et al., (2009b) found that the Antarctic brittle star *Ophionotus victoriae* is not capable of acclimating to increased temperatures. At 3°C, mortality occurred first at 19 days and all

individuals in the study had died by 32 days. They also found high mortality rates at 2°C with an average survival rate of 42 days and mortality first occurring at 24 days. This observation suggests that warming may be detrimental to *O. victoriae* populations as 2°C is not far from summer peak temperatures in some parts of Antarctica, which can be between 0.5°C to 1.5°C (Morley et al., 2012). In contrast, Antarctic notothenioid fish, *Pagothenia borchgrevinki* was found to acclimate to 4°C (Robinson and Davison, 2008). There was no significant difference between metabolic rate of fish in 4°C treatment compared with fish in 0°C after 1 month exposure (Robinson and Davison, 2008). This highlights that acclimation capacity in the Antarctic marine environment may be highly variable and species-specific (Peck et al., 2010).

Recording the greatest respiration rate in the highest experimental temperature (4°C, November), and the lowest oxygen consumption in the coldest temperature (-1°C, August) is expected due to the increased metabolic demand of warmer water with biological reactions occuring at faster rates as temperature increases (Agüera et al., 2015). Calculating a Q_{10} of 4.7 indicates that the increase in respiration rate with a temperature rise of 10°C is greater than the rate for normal biological reactions, which have Q_{10} values of 2-3 (Peck and Prothero-Thomas, 2002). The Q_{10} for this reaction is similar to those calculated for *O. validus* larval oxygen consumption and development in increased temperatures. Peck and Prothero-Thomas (2002) found that respiration of gastrula at 2°C compared with -0.5°C had a Q_{10} temperature effect of 4.4 and the difference in days to reach the bipinnarial stage had a Q_{10} of 4.5.

The lowest respiration rate (0.0046 mg O_2 h^{-1} g^{-1}) was only slightly higher than the baseline measurement in McMurdo Sound although this measurement is much lower than all other respiration rates recorded for this study. The average rates of between 0.009-0.015 mg O_2 h^{-1} g^{-1} (4°C), 0.008-0.010 mg O_2 h^{-1} g^{-1} (2°C) and 0.005-0.007 mg O_2 h^{-1} g^{-1} (0°C) suggest that while the sea stars have adapted to their current conditions, oxygen consumption has not

returned to background levels measured on collection at McMurdo Sound which would be expected under complete acclimation (Suckling et al., 2015).

Respiration in *O. validus* has been documented varying seasonally in wild populations, with 44% higher oxygen consumption in summer (Souster et al., 2018) but this trend was not observed across the entirety of this study (Figure 2.4). In this study, such a trend would present as a pattern of oxygen consumption that was lower in August, peaking in February and November however respiration rate did not significantly differ across the 10-month study. Respiration of Antarctic sea urchin *S. neumayeri* has also been found to vary seasonally, however only 15-20% of this increase is thought to be temperature related, with the rest from feeding, growth and spawning (Brockington and Clarke, 2001).

Food availability during the Antarctic winter is low and *O. validus* are able to withstand long periods of starvation (McClintock, 1994). This leads to seasonal variation in the activity levels and metabolism of wild populations of *O. validus* and other sympatric invertebrates, with the majority of feeding, growth and reproductive activity occurring during the austral spring and summer (November – February) which is a period of high productivity and warmer sea temperatures in Antarctica (Clarke, 1988; Morley et al., 2012). In this laboratory study, *O. validus* were fed regularly every two weeks, with near constant light and temperatures and it is possible that this obscured or reduced seasonal trends in metabolic rates seen in the natural environment and attributed to food availability in the Antarctic marine benthos (Brockington and Clarke, 2001).

Temperature increased the oxygen consumption of *O. validus,* but metabolism was not significantly different among sampling points in February, August and November. Echinoderm metabolic scaling rates are predicted to be between 0.6 and 0.8 (Lawrence and Lane, 1982),

which fall within the range of the present study. For many ectotherms, the metabolic scaling rate decreases as temperature increases (Glazier, 2010). For *Ophionereis schayeri* the metabolic scaling exponent *b* was greater at warmer temperatures: b = 0.7 at 18.5°C and b = 0.635 at 15.5°C (Christensen et al., 2022), a similar difference as between *O. validus* at -1°C and 4°C (Figure 2.4). The metabolic scope of *O. validus* across the five temperature treatments was not significantly different, indicating that for the experimental animals in this study the relationship between size and oxygen consumption was not different between temperatures. In contrast, some species of molluscs (Clark et al., 2013) and crustaceans (Strong and Daborn, 1980) show increased sensitivity to temperature as size increases which may indicate a greater risk of climate change and drive decreases in body size (Peck et al., 2009a).

Morphometric measurements of *O. validus* were collected as a measurement of fitness and to explore allocation of energy across the temperature treatments. Average pyloric caecum index and gonadosomatic index were both lower in all treatments compared to the McMurdo Sound baseline (Table 2.4). Unexpectedly, there was no difference over time in the PI or GI indices.

Table 2.4 Summary of average morphometric measurements of *Odontaster validus* in each temperature treatments + standard error of mean

Temperature (°C)	**Average Radial Length (mm)**	**Average Weight (g)**	**Average GI (proportion body mass)**	**Average PI (proportion body mass)**
-1.8°C	44.435 ± 1.85	69.224 ± 2.310	0.193 ± 0.006	0.194 ± 0.006
-1°C	44.086 ± 1.855	24.344 ± 2.458	0.057 ± 0.006	0.143 ± 0.012
1°C	41.294 ± 1.767	19.635 ± 2.018	0.068 ± 0.010	0.164 ± 0.009
2°C	39.249 ± 1.563	15.610 ± 1.931	0.049 ± 0.008	0.146 ± 0.014

3°C	41.187 ± 1.963	17.059 ± 2.137	0.048 ± 0.005	0.144 ± 0.011
4°C	42.695 ± 1.740	17.751 ± 1.755	0.062 ± 0.007	0.148 ± 0.008

The pyloric caecum is an important energy reserve for sea stars (Kidawa et al., 2010). Typically, the pyloric index would be high for most of the year, apart from in the immediate lead up to the reproductive season where it supports gametogenesis (Sparks, 2018). Gonad index would be expected to peak in June before reproduction occurs (Agüera et al., 2015a). The absence of these trends in the data may indicate that temperature is altering the normal cycles of energy allocation in the gonad and pyloric caecum tissues. *O. validus* are generalist scavengers with the ability to store nutrients in the pyloric caecum (Agüera et al., 2015a). The metabolic cost of increased temperatures may lead to a greater risk of starvation for *O. validus* during seasonal low availability of food sources (Christensen et al., 2022).

A Dynamic Energy Budget (DEB) Model for *O. validus* (Agüera et al., 2015b) estimates that Pyloric Index (PI) comprises around 30% of adult body mass. This prediction holds true for several individuals sampled for this study however, there is considerable variation in PI across and within the treatments. The minimum PI in this study is 5%, while the maximum was 32% with an average of 14.8%. Agüera et al., (2015b) documented an average PI of 26% for individuals at McMurdo Sound. The variation measured may be a result of regular feeding in the lab compared to in natural conditions where food availability is highly variable both spatially and temporally (Agüera et al., 2015b). Pyloric caecum mass did not vary significantly throughout the duration of this study. This is likely due to the fortnightly feeding regime preventing the usual cycle of near starvation during the austral winter and intensive feeding during summer experienced by benthic invertebrates in Antarctica (Brockington and Clarke,

2001). It may also indicate that the animals did not get the environmental cues for reproduction so the PI stayed high, while the GI stayed low and did not vary.

O. validus gonadosomatic index for this study varied from 1.0 to 15.7%. This is in line with the findings of McClintock et al., (1988) for individuals collected from depths of 20 – 30m. Seasonal variation in gonadosomatic index was found by McClintock et al. (1988), not seen in the animals in this study. McClintock et al. (1988) found a clear trend of both location and depth effecting gonadosomatic index with larger indices measured in specimens collected from shallower depths and larger gonadosomatic indices at McMurdo Sound compared with East Cape Armitage. The highest GI in the McClintock et al. (1988) study was 50% of body mass, much higher than any individuals measured in this study, however the DEB model by Agüera et al. (2015b) predicts that gonadosomatic index should make up between 8-10% of total body mass.

The absence of seasonal trends in this study may be attributed to several limitations of the sample including high variation of individual body mass within the temperature treatments, lack of environmental cues, nutritional issues and low sample sizes at each dissection period (n=5 per temperature, per dissection period). The trends in GI may have been further limited by the unusual reproductive cycle of *O. validus* which includes a longer oogenesis between 18-24 months and the simultaneous production of two sets of oocytes (Agüera et al., 2015b). There was an effort made after the final sampling period in November to spawn larvae from the remaining individuals for additional research, however the output was too low to produce sufficient samples across all treatments suggesting the animals had not undergone gametogenesis (or had already spawned unobserved). In a separate study, McClintock (1989) showed variation between males and female *O. validus* energy allocation with females

dedicating 50% of their resources to PC. For this thesis, specimens were not sexed so there is no potential to explore variation in energy allocation between the sexes.

Studies of tropical brittle star *Ophionereis schayeri* found that exposure to increased temperatures from a simulated winter heat wave led to increased rates of respiration and limb regeneration but also increased mortality (Christensen et al., 2022). The heat wave treatment found that respiration rates did not immediately return to ambient levels and remained increased for 4 weeks. Cross generational effects of temperature on tropical sea urchin *Echinometra sp. A* were explored with both physiological and behavioural indicators (Uthicke et al., 2021). After 9 months, offspring with parents from ambient conditions had greater respiration rates and increased growth compared to offspring with parents from warmer experimental conditions, although neither of these differences were found at 14 months. At both 9 and 14 months, righting times were significantly affected by parental treatment.

This ten-month time series study found that respiration and gonadosomatic index were both significantly influenced by increased temperatures. Month was not a significant predictor of body indices or oxygen consumption. It is likely that the seasonal trends expected in the pyloric caecum and gonadosomatic index are not present due to the constant temperature and regular feeding in the laboratory as this does not reflect the seasonal variation in temperature and food availability found in natural conditions. Oxygen consumption is known to increase with temperatures as biological reactions occur faster in warmer conditions and this requires a greater metabolic rate, however after 10 months, respiration of individuals in the -1°C treatment did not match the McMurdo Sound baseline suggesting that full body acclimation has not taken place. These measures of acclimation are indicators of overall health and vary

considerably between individuals. Both respiration and body indices studies were limited by small sample sizes which may have masked some trends.

The lack of variation in body indices, resource allocation and respiration with time highlights the effects of temperature on key life processes; metabolism and reproduction. Increased temperatures caused greater oxygen consumption to sustain metabolism and interfered with the expected pattern of energy allocation between tissues. Although individuals in this study did not spawn, the cost of life in higher temperatures may affect future generations by potentially reducing reproductive fitness as more energy is required to sustain other physiological functions.

Chapter 3: Oxidative stress response of *Odontaster validus* in near future temperatures

3.1 Introduction

Oxidative stress is an important physiological response to environmental stressors (Costantini et al., 2010; Lushchak, 2011). Marine organisms may experience oxidative stress as a result of thermal shifts, UV radiation, and exposure to pollutants (Lister et al., 2010). Antarctic marine ectotherms experience increased pro-oxidant pressure and metabolic costs associated with antioxidant defences as a result of the high solubility of O_2 in the cold stenothermal environment (Lesser, 2006). The recorded and predicted changes in the Antarctic marine environment are likely to impact benthic organisms such as *O. validus* physiologically at the cellular level (Benedetti et al., 2016) including, potentially, oxidative stress.

Reactive oxygen species (ROS) occur as a product of aerobic metabolism and there are a range of enzymatic and non-enzymatic defences that are employed to scavenge ROS under standard environmental conditions (Sies, 1993). ROS encompass oxygen free radicals, such as superoxide anion radical ($O_2^{\cdot -}$) and hydroxyl radical ($^{\cdot}OH$), and nonradical oxidants, such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (Valavanidis et al., 2006). In times of stress, the production of ROS exceeds the rate of removal by antioxidants (Ayala et al., 2014), and this imbalance can result in oxidative damage to lipids, proteins and DNA (Figure 3.1). Oxidative stress is a term used to define a state during which the levels of ROS exceed the level associated with normal cellular function (Zorov et al., 2014).

Oxidative damage can occur to a range of cellular structures, including lipids, proteins and DNA. Lipid peroxidation occurs when ROS attack lipids and a self- propagating chain reaction of oxidation occurs resulting in the formation of toxic lipid peroxyl radicals and

hydroperoxides (Ayala et al., 2014). Polyunsaturated fatty acids (PUFAs) are vulnerable to lipid peroxidation, particularly by hydroxyl HO• at the C–H bonds on hydrocarbon side chains (Lister, 2015). PUFAs are important for maintaining membrane fluidity in cold temperatures and are therefore found in high concentrations in the membranes of aquatic animals (Monserrat et al., 2007). Protein carbonylation is a major biomarker of oxidative stress (Davies et al., 1999). Carbonyl (CO) groups are produced on protein side chains, introducing ketones and aldehydes when proteins are oxidised (Dalle-Donne et al., 2003). This is an irreversible effect of oxidative stress as carbonated proteins cannot be repaired (Song et al., 2020). ROS can also damage DNA by causing mutations, deletions and other lethal genetic effects including base degradation, cross linking to proteins and strand breakage (Lesser, 2006).

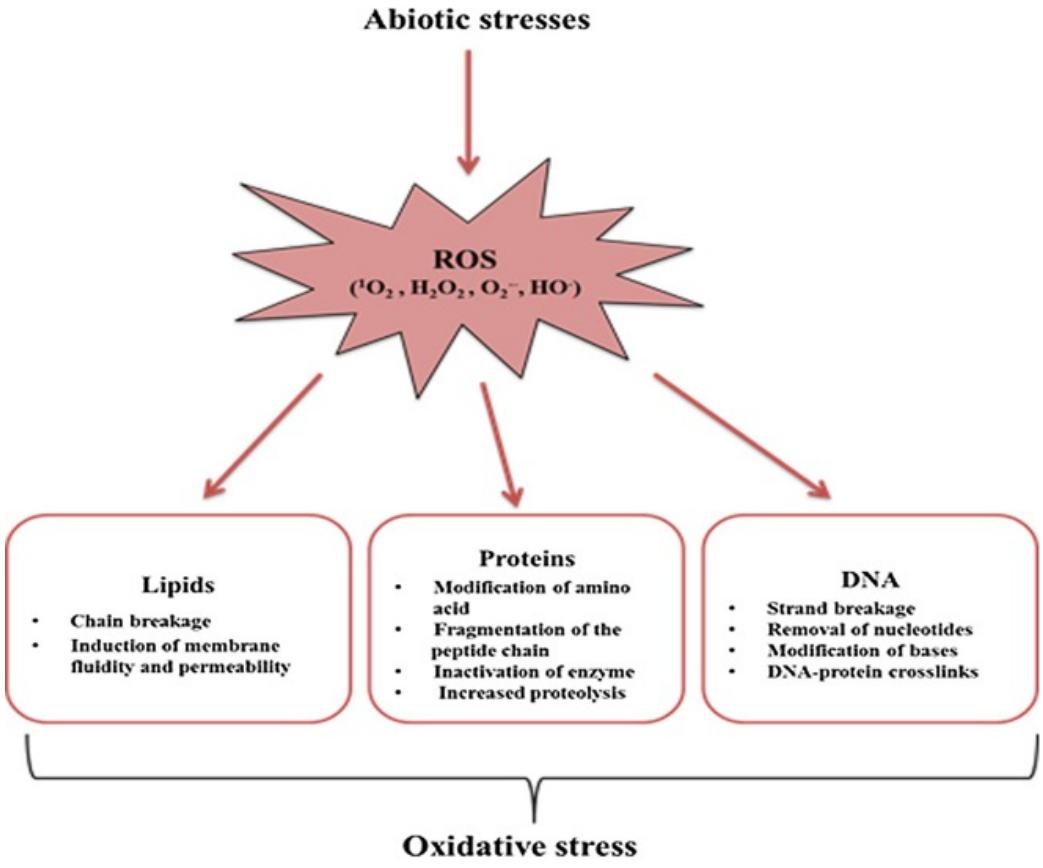

Figure 3.1 Reactive oxygen species (ROS) induce oxidative damage to lipids, proteins, and DNA (From Rezayian et al., 2019)

Antioxidant defences can protect marine organisms against the toxic effects of free radicals and overcome oxidative damage by scavenging ROS and converting them into less toxic molecules (Buttemer et al., 2010; Rezayian et al., 2019). The antioxidant system is composed of enzymatic and non-enzymatic defences (Figure 3.2). Low molecular weight compounds such as vitamins A, C and E, uric acid, glutathione, β-carotene, carotenoids, and phenolic compounds make up the non-enzymatic defences (Birben et al., 2012). The most efficient and effective enzymatic antioxidants that protect cells from oxygen species, radicals and thereby oxidative stress are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPOX) and glutathione reductase (GR) (Figure 3.2). Mechanisms employed by antioxidant enzymes to prevent oxidative damage include removing free radicals to prevent lipid peroxidation, interrupting oxidation chain sequences, or removing peroxidases to prevent further ROS generation (Castell et al., 1997).

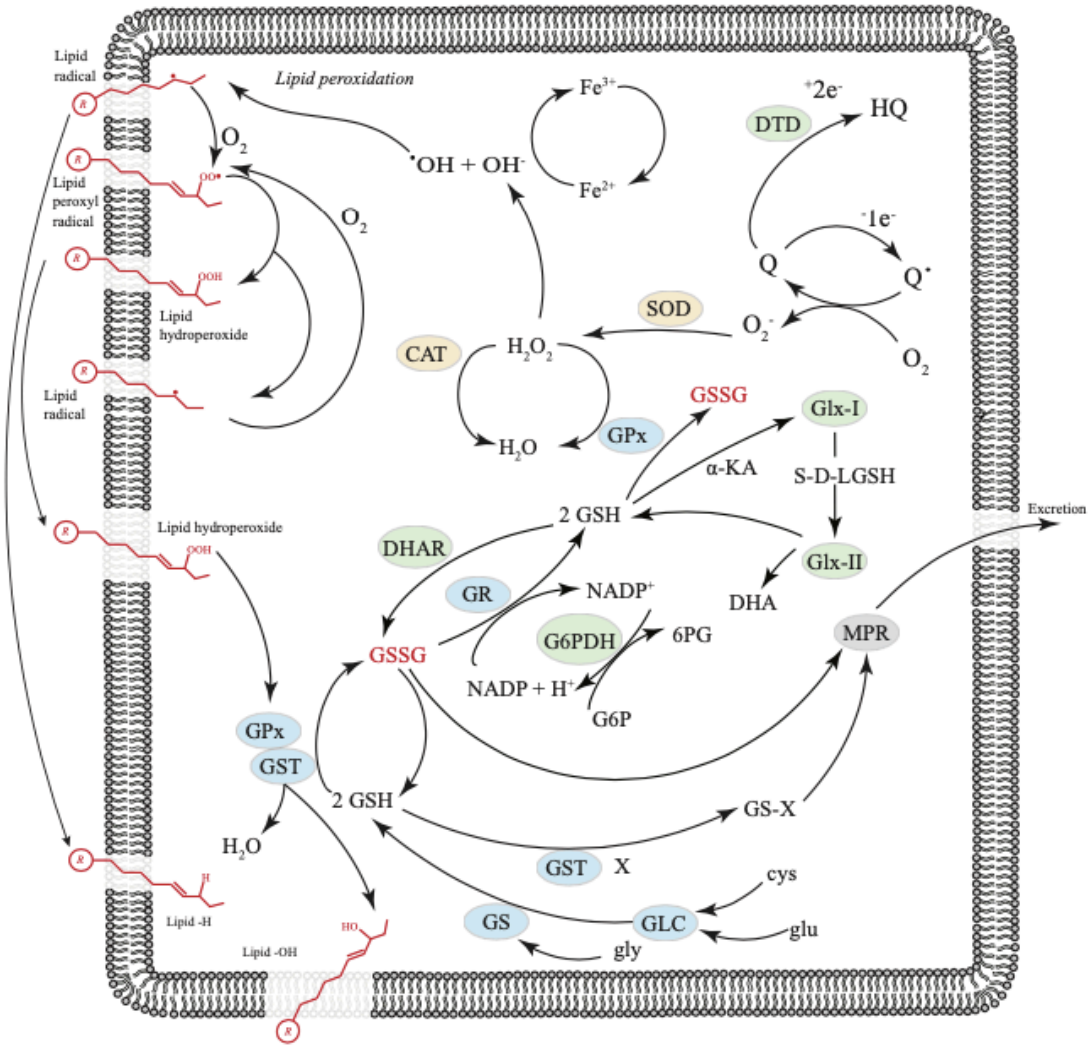

Figure 3.2 Main cellular antioxidant defences and formation of oxidised lipid products: 6-phospogluconate (6PG), catalase (CAT), cysteine (cys), D-hydroxyacid (DHA), dehydroascorbate reductase (DHAR), DT- diaphorase (DTD), glucose 6-phosphate (G6PDH), !-glutamylcysteine synthetase (GCL), glyoxalase I (Glx- I), glyoxalase II (Glx-II), glutathione peroxidises (GPx), glutathione reductase (GR), glycine (gly), glutamic acid (glu), glutathione synthetase (GS), reduced glutathione (GSH), oxidised glutathione (GSSG), glutathione-S-transferases (GST), GSH conjugated xenobiotic (GS-X), hydroquinone (HQ), "-keto aldehydes ("KA), multidrug-resistance-related protein (MRP), quinone (Q), semiquinone radical (Q•), superoxide dismutase (SOD) and xenobiotic (X). Enzymes and other components of the antioxidant system are shown in orange, blue (glutathione-dependent) or green (detoxification). Oxidised molecules or products are shown in red. Graphic from (2015) as modified from Regoli (2011).

Superoxide dismutase (SOD) is a critical enzyme for antioxidant defences as it converts superoxide radicals to the relatively less toxic hydrogen peroxide and water, therefore preventing the formation of lipid peroxides (Ighodaro and Akinloye, 2018; Rezayian et al.,

2019). SOD activity increases are associated with increased organism resistance to environmental stressors, and are shown to result in increased oxidative stress tolerance in plants (Sharma et al., 2012).

Catalase enzymes (CAT) are present in most living tissue that use oxygen and are usually located in the peroxisomes (Ighodaro and Akinloye, 2018), they help to modulate oxidative stress at the cellular level (Sies et al., 2022). Catalase is highly efficient and converts hydrogen peroxide (H_2O_2) to water and molecular oxygen (Glippa et al., 2018; Wang et al., 2018).

Glutathione peroxidase (GPOX) is an essential intracellular enzyme that uses glutathione to reduce hydrogen peroxides and lipid peroxides to water or the corresponding alcohols and oxygen (Ayala et al., 2014). GPOX is a selenoprotein and its activity is dependent on the micronutrient cofactor known as selenium. Hydrogen peroxide has the highest lifetime in sea water and a high steady state concentration, and can readily pass through membranes (Lesser, 2006). The critical inhibition of lipid peroxidation makes GPOX an extremely important enzyme for protection against oxidative stress (Ighodaro and Akinloye, 2018).

Glutathione reductase (GR) has an important role in the ascorbate-glutathione cycle and maintains the reduced state of glutathione (GSH), making it a key part of oxidative stress defenses (Rezayian et al., 2019). GR activity has been documented to increase in conditions of environmental stress (Sharma et al., 2012).

Antioxidant defenses play important roles in protecting marine organisms from oxidative stress (Carney Almroth et al., 2015; Wang et al., 2018). When the scavenging capacity of antioxidant defences is overcome, an imbalance occurs which can cause tissue damage, and ultimately

results in the impairment of vital physiological processes (Ansaldo et al., 2005). As a result of their importance in organism defence mechanisms, the activities and levels of enzymatic antioxidants are commonly used as biomarkers of oxidative stress (Valavanidis et al., 2006).

3.1.1 Research Aims

Two key questions are addressed in this study:

(1) **Does long term exposure to increased temperatures lead to oxidative stress in *Odontaster validus?*** To test this, *Odontaster validus* were exposed to five temperatures, four key antioxidant enzymes and two oxidative stress markers were measured.

(2) Is the antioxidant system of *Odontaster validus* able to acclimate to the physiological demands of increased temperatures over time? To test this, *Odontaster validus* were exposed to five temperatures for ten months and sample groups were dissected at three month intervals.

For *O. validus*, measures of oxidative damage; lipid peroxide, protein carbonyls, and antioxidant enzymes; SOD, CAT, GPOX, and GR are used to explore the sensitivity of *O. validus* to long term exposure to increased, environmentally relevant temperatures ranging between -1°C / 0°C and 4°C.

3.2 Methodology

3.2.1 Treatments and dissections

Odontaster validus were held in 10L tanks with continuous water circulation in five thermostatically controlled fridges (Figure 1.3, Chapter 1). Treatments were -1°C, 1°C, 2°C, 3°C and 4°C. Feeding occurred every two weeks. Tissue samples ~100 mg of gonad, body wall, tube feet and pyloric caecum were dissected, placed in cryovials, snap frozen in liquid nitrogen and stored at -80°C. The activities of antioxidant enzymes and oxidative damage products were assessed in each of the four tissue types of *O. validus* using established methods (Lister et al., 2010).

3.2.2 Extractions

To each sample, 900 µl of extraction buffer (pH 7.0) was added to the 2mL cryovial. The extraction buffer contained 0.1 mM Na_2 EDTA, 1% PVP-44, 1 mM PMSF and 0.5% *v*/v Triton X-100) at a 1:9 ratio (*w*/*v*). The sample was disrupted using a BioSpec Mini-BeadBeater machine and 2.3 mm Zirconia beads for 42 seconds. Cryovial contents were transferred into 2mL centrifuge tubes and centrifuged at 4°C for 20 minutes at 14,000 RMP. The supernatant was semi purified using 500 µl Amicon ultrafilter units centrifuged at 4°C for 20 minutes at 10,000 RMP. After centrifugation, 400 µl phosphate buffer (50 mM pH 7.0) was used to reconstitute the protein. The protein sample was then transferred into a 1.5 mL Eppendorf® tube and aliquoted into four separate tubes and stored at −80 °C for later biochemical analysis. Lipids were extracted by adding 600 µl of methanol:chloroform to each sample and leaving the sample to stand for 1 min before adding 400 µl of Chloroform and mixed for 30 seconds. Deionised water 400 µl was added and the sample mixed for 30 seconds. Once the phases separated, the chloroform phase was collected, transferred into Eppendorf® tubes and stored at −80 °C for lipid analysis.

3.2.3 Assays

Pyloric caecum and body wall tissues were found to be too pigmented to produce samples suitable for assay, so assays were only run on gonad and tube feet tissue. All assays were carried out using a PerkinElmer (Wallac) 1420 multilabel counter (Perkin Elmer, San Jose, California, USA), controlled by a PC, and fitted with a temperature control cell and an auto-dispenser. Data were acquired and processed using the WorkOut 2.0 software package (Perkin Elmer, San Jose, California, USA). Glass microplates were used for the lipid peroxide analysis. All assays were completed by Dr David Burritt.

3.2.4 Oxidative damage to proteins and lipids

Protein carbonyl levels were determined in the semi-purified protein extracts via reaction with 2,4-dinitrophenylhydrazine (DNPH) as described by Reznicj and Packer (1994). The protein contents were determined as per Fryer et al. (1986) with minor modifications. Lipid hydroperoxide levels were determined using the ferric thiocyanate method described by Mihaljević et al. (1996).

3.2.5 Antioxidant enzyme assays

Superoxide dismutase (SOD; EC 1.15.1.1) was assayed using a Cayman/Sapphire bioscience kit (605-70602) as per the manufacturer's instructions. Catalase (CAT; EC 1.11.1.6) was assayed using the chemiluminescent method of Maral et al. (1977). Glutathione peroxidase (GPx; EC 1.11.1.9) activity was measured according to the spectrophotometric method described by Paglia and Valentine (1967). Glutathione reductase (GR; EC 1.8.1.7) was assayed using the method of Cribb et al. (1989) with minor modifications. Enzyme assays were validated using heat denatured protein extracts as controls and by running assays in the absence of substrates/cofactors as appropriate.

3.2.6 Statistical analyses

To assess the relationship between increased temperatures and oxidative stress markers, a linear mixed effects model was constructed allowing for repeated measures using R package nlme (Pinheiro et al., 2021) and emmeans (Lenth, 2022). The model included a random effect (called "SpecimenID") to control for the induced correlation across 12 measurements take from each specimen across two tissue types and six oxidative stress markers. Model selection involved finding the model with the lowest Akaike Information Criterion (AIC) value across a range of candidate models with different fixed and random effect structures. Non-significant 2-way and 3-way interactions were removed. The fixed effect equation for the final model is given as: **OS(value) ~ OS(type)+ Temp + Month + Tissue + Variable:Temp + Variable:Tissue**

Residual diagnostics were used to check the validity of the model assumptions which were found to be acceptable. Statements of significant differences were based on accepting $p < 0.05$. All analyses were conducted using RStudio (R Studio Team, 2022).

3.3 Results

3.3.1 Oxidative damage

The levels of protein carbonyl (Figure 3.3) and lipid peroxide (Figure 3.4) in *Odontaster validus* tissues were significantly different among temperature treatments throughout the 10-month study. Sample month was not a significant predictor of oxidative damage markers or antioxidant activity. Table 3.1 shows the significance levels of differences in antioxidant enzyme activities and oxidative damage markers between temperature treatments.

Table 3.1 Mixed effects model output showing significant differences between temperature treatments and oxidative stress markers for *Odontaster validus.*

	Protein Carbonyls	**Lipid Peroxides**	**SOD**	**CAT**	**GPOX**	**GR**
Temperatures	<0.0001*	0.0003*	<.0001*	<.0001*	<.0001*	<.0001*
0°C -1°C	<0.0001*	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*
0°C -2°C	0.0268*	<.0001*	0.0268*	<.0001*	<.0001*	<.0001*
0°C -3°C	<0.0001*	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*
0°C -4°C	<0.0001*	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*
1°C -2°C	0.7034	1.0	0.7034	0.9988	0.9956	0.9790
1°C -3°C	<0.0001*	<.0001*	<.0001*	<.0001*	0.0024*	0.0261*
1°C -4°C	<0.0001*	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*
2°C -3°C	<0.0001*	<.0001*	<.0001*	<.0001*	0.0077*	0.0916
2°C -4°C	<0.0001*	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*
3°C -4°C	0.1086	0.0929	0.1086	0.0580	0.0033*	0.0011*

Average protein carbonyl levels in gonad tissue ranged from 1.61 – 2.96 nmol/mg protein. Tube feet protein carbonyl activity averaged from 2.16 – 3.64 nmol/mg protein. Protein carbonyl levels were highest in the warmer temperatures (p=0.0011, Table 3.1). Somatic cells from *O. validus* tube feet show a pronounced increase in protein carbonyl levels between the 3°C and 4°C temperatures across each of the 4 sample months. Protein carbonyl levels were significantly higher in tube feet compared to gonad tissue (p<0.0001, Figure 3.3).

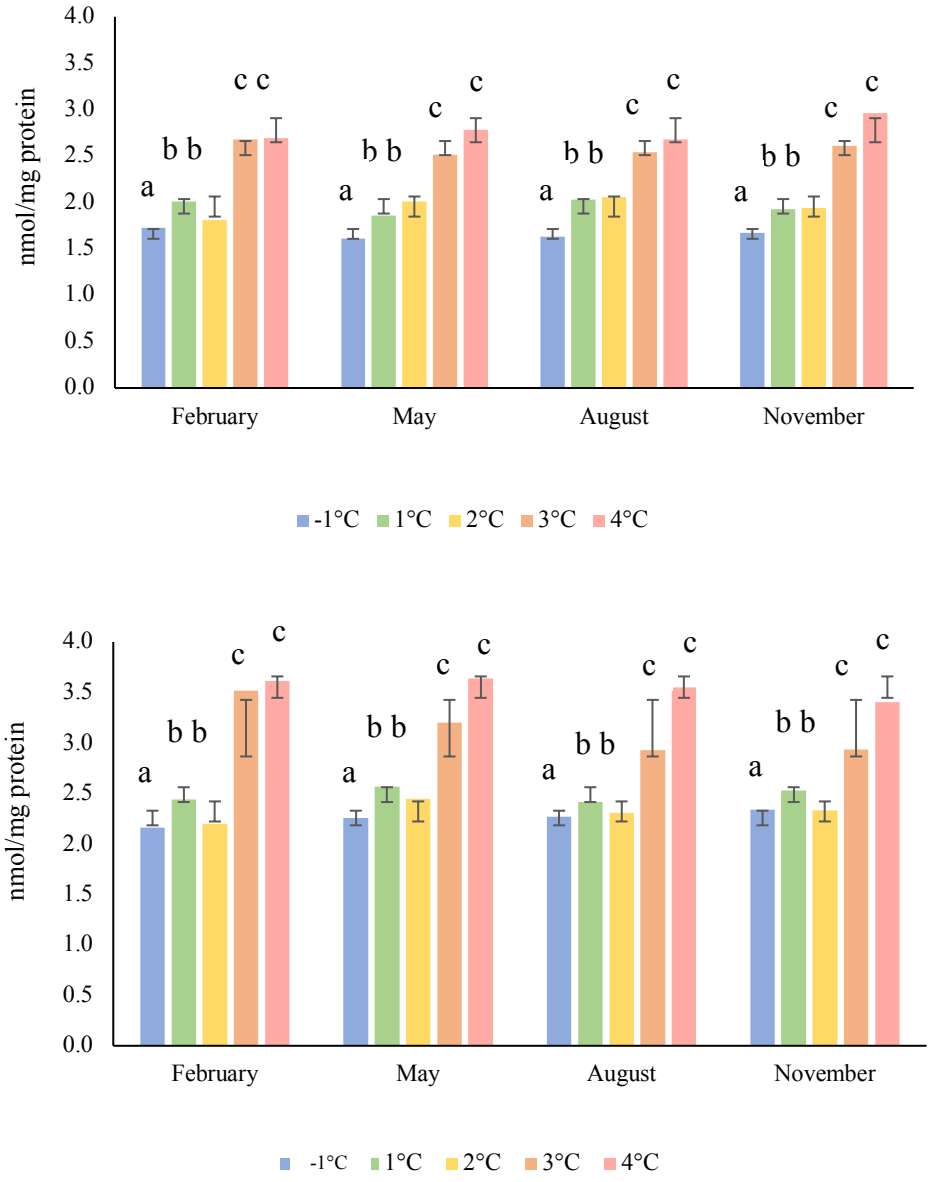

Figure 3.3 Average Protein Carbonyl (nmol/ mg protein) for *O. validus* Gonad (upper) and Tube feet (lower) tissue in five temperature treatments from February- November 2019. Error bars show standard deviation. Different letters indicate statistical significance between temperatures. Protein carbonyls in gonad and tube feet were statistically different, p = <.0001.

Lipid peroxide levels show a clear temperature trend but no effect of month in either gonad or tube feet tissues (Figure 3.4). The average concentrations are similar between the tissue types with average lipid peroxide levels in gonad tissue ranging from 3.84 – 8.24 nmol/g FW and from 4.29 – 9.39 nmol/ g FW in the somatic tube feet tissue. The mixed effects model showed

that there was a significant difference between lipid peroxide levels in gonad tissue and tube feet tissue (p=0.0003), as well as between different temperatures through time (Table 3.1).

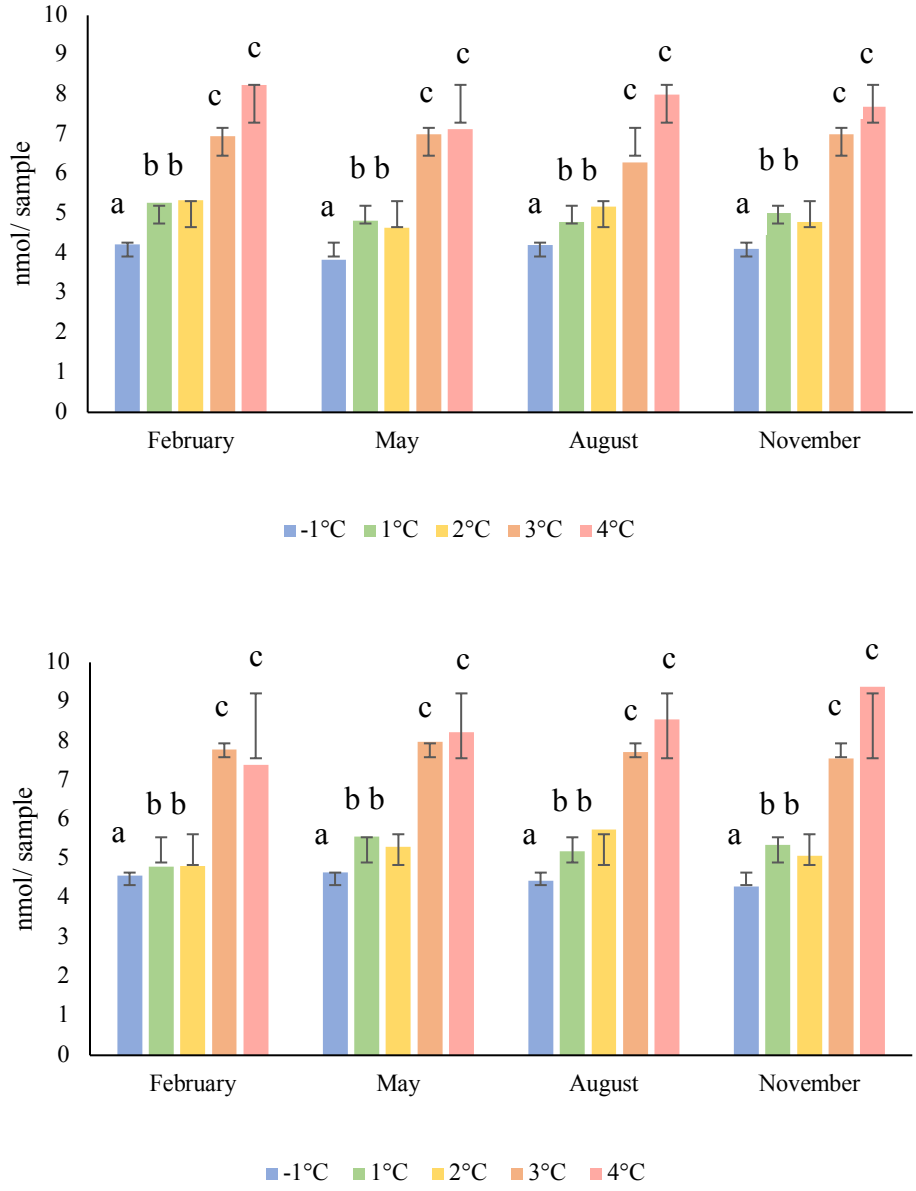

Figure 3.4 Average lipid peroxide (nmol/g FW) for *O. validus* Gonad (upper) and Tube feet (lower) tissue in five temperature treatments from February- November 2019. Error bars show standard deviation. Different letters indicate statistical significance between temperatures. Lipid peroxide levels in gonad and tube feet were statistically different, p = <.0003.

3.3.2 Antioxidant responses

The responses of antioxidant enzymes in *O. validus* were significantly different between temperatures and tissue types, but not month. Activity of superoxide dismutase (SOD), was significantly different between gonad and tube feet tissue ($p<0.0001$). Average SOD activity in gonad tissues was between 11.2 – 23.54 units / mg protein and between 10.12-19.13 units / mg protein in the tube feet tissue (Figure 3.5).

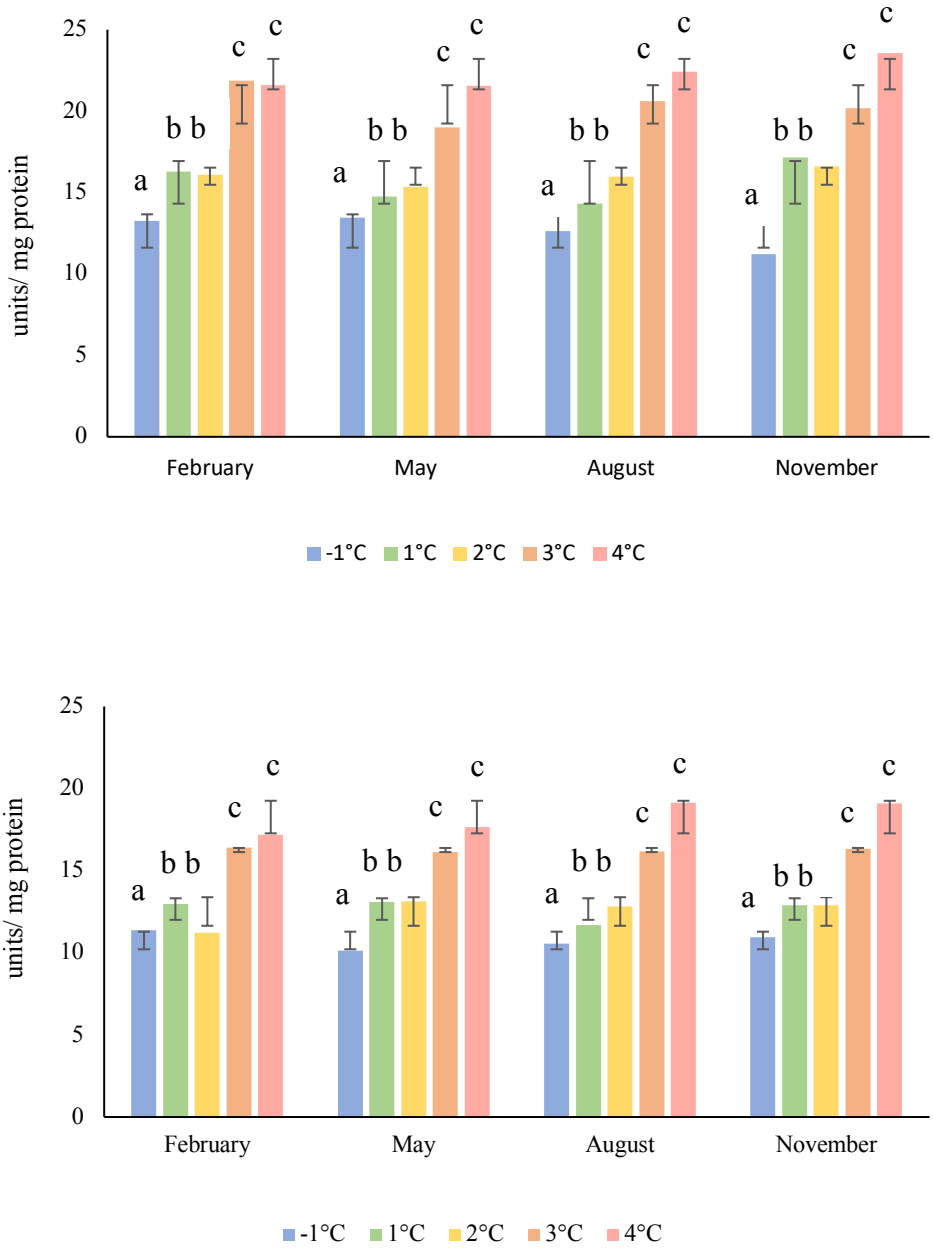

Figure 3.5 Average superoxide dismutase (SOD) activity (nmol/ mg protein) for *O. validus* Gonad (upper) and Tube feet (lower) tissue in five temperature treatments from February-

November 2019. Error bars show standard deviation. Different letters indicate statistical significance between temperatures. SOD activity in gonad and tube feet was statistically different, p = <.0001.

Catalase (CAT) activity was greater in gonad tissue than in tube feet tissue (p= <0.0001). Average activity ranged from 6.68-13.51 µmol /min/mg protein and 5.74-11.55 µmol /min/mg protein in gonad and tube feet respectively (Figure 3.6). The trend is similar to the activity of SOD as above.

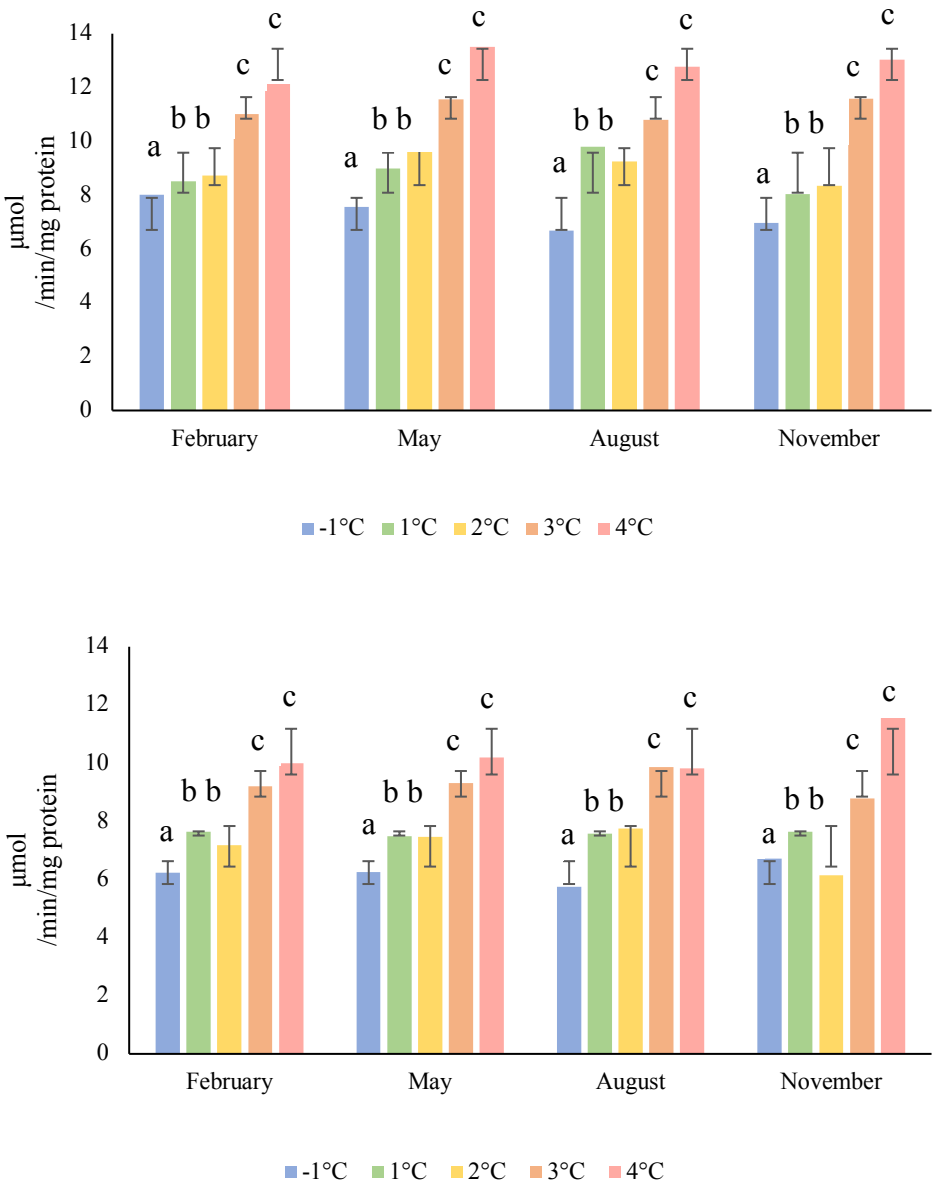

Figure 3.6 Average Catalase (CAT)activity (µmol /min /mg protein) for *O. validus* Gonad (upper) and Tube feet (lower) tissue in five temperature treatments from February- November 2019. Error bars show standard deviation. Different letters indicate statistical significance between groups. CAT activity in gonad and tube feet was statistically different, p = <.0001.

Average activity of glutathione peroxidase (GPOX) in gonad tissue was variable and measured between 52.05 – 113.44 nmol/ min/ mg protein. In tube feet, GPOX activity was between 38.41 – 80.91 nmol/ min/ mg protein. There was a significant difference in the activity of GPOX in the tissue types ($p<0.0001$). GPOX activity was also affected by temperature with increased activity in higher temperatures and between treatments (Figure 3.7, Table 3.1). Month was not a significant predictor of GPOX activity.

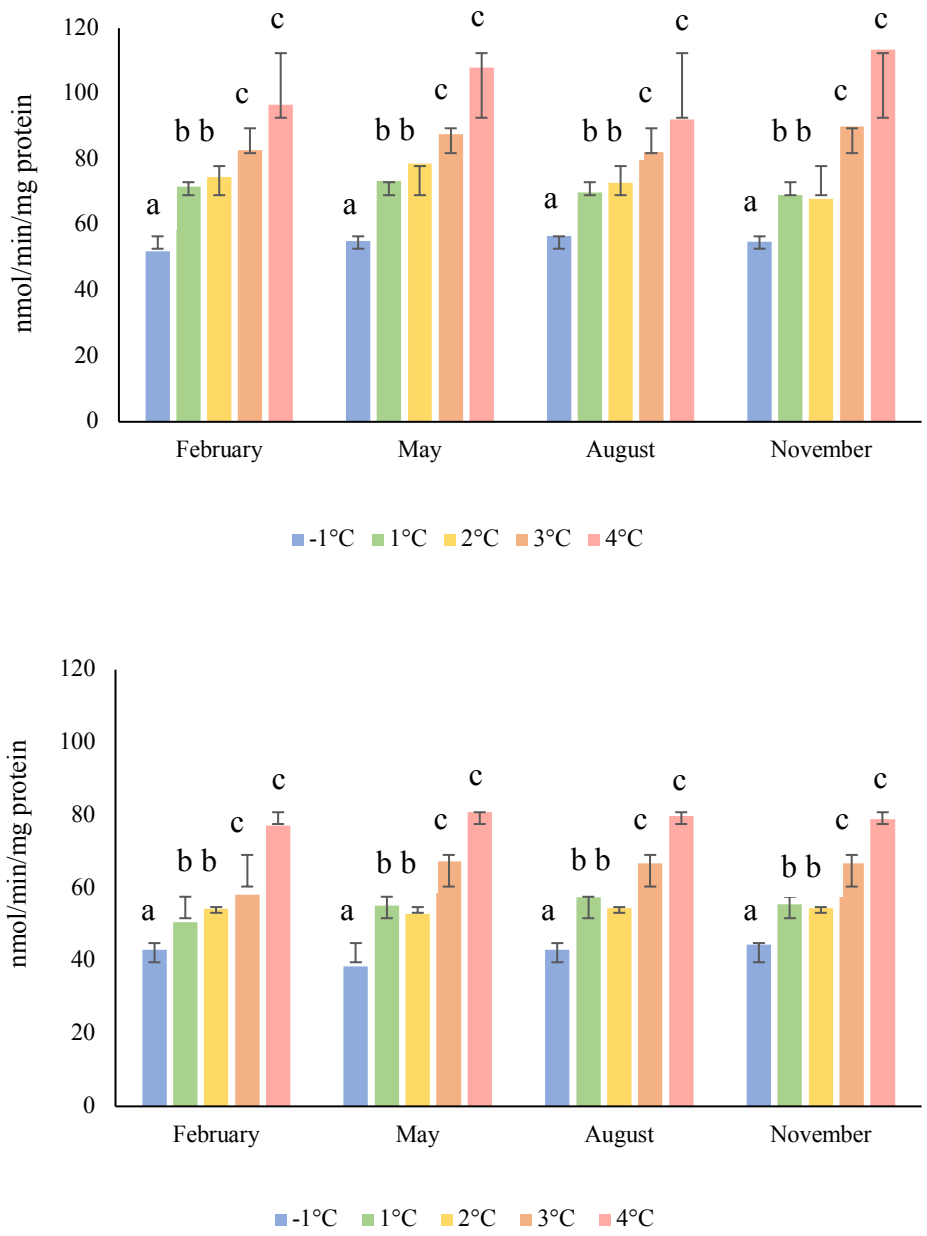

Figure 3.7 Average glutathione peroxidase (GPOX) activity (nmol/ min/ mg protein) for *O. validus* Gonad (upper) and Tube feet (lower) tissue in five temperature treatments from February- November 2019. Error bars show standard deviation. Different letters indicate statistical significance between temperatures. GPOX activity in gonad and tube feet was statistically different, p = <.0001.

Glutathione reductase (GR) activity was significantly different between gonad and tube feet tissue ($p = <0.001$). Average GR activity in gonad tissue throughout the duration of the study was between 27.34 – 48.06 nmol/ min/ mg protein. Activity in tube feet tissue was between 23.69 – 43.64 nmol/ min/ mg protein.

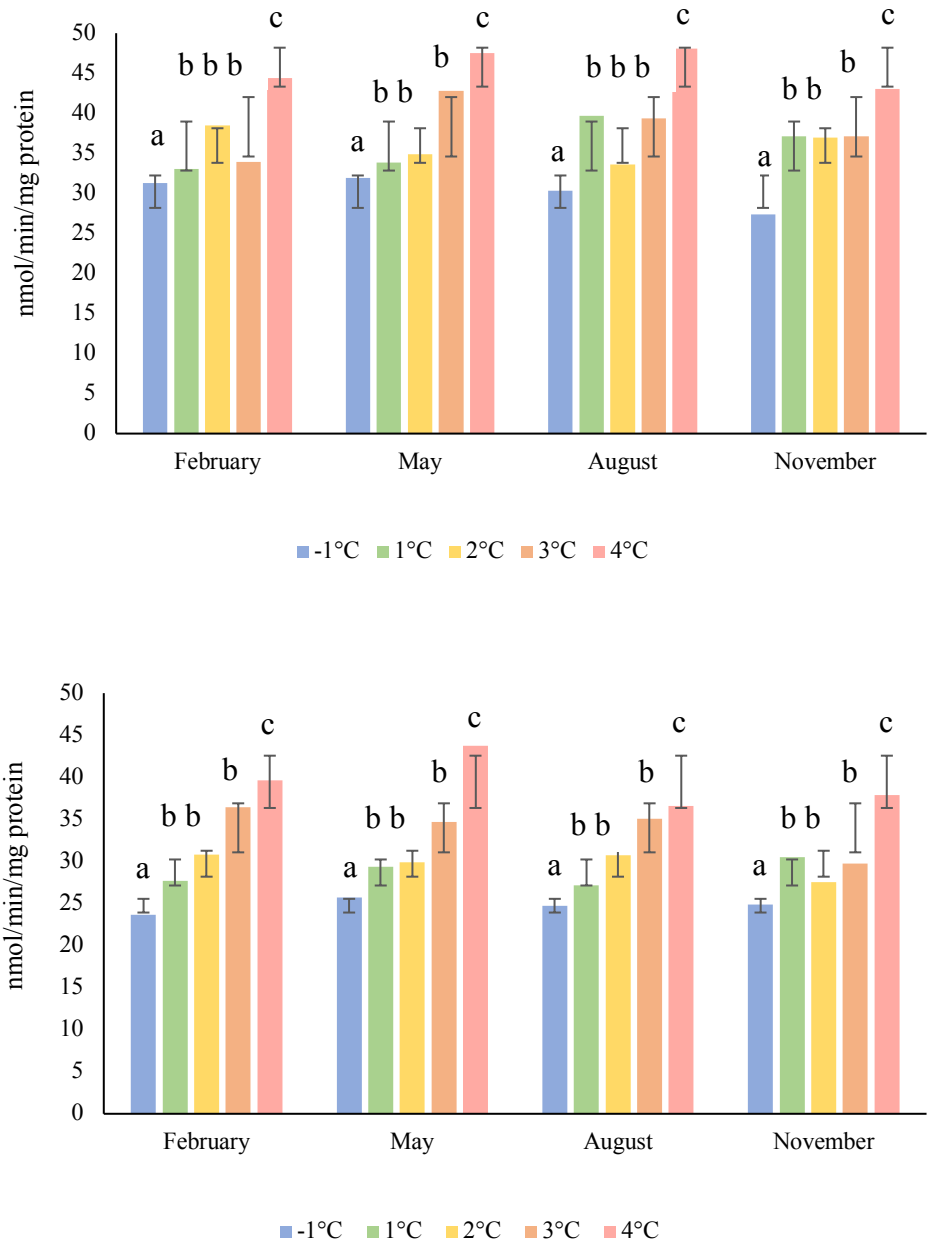

Figure 3-8 Average glutathione reductase (GR) activity (nmol/ min/ mg protein) for *O. validus* Gonad (left) and Tube feet (right) tissue in five temperature treatments from February- November 2019. Different letters indicate statistical significance between temperatures. Error bars show standard deviation.

Antioxidant enzymes and oxidative damage markers responded differently to the temperature treatments and this response was variable between somatic tube feet tissue and reproductive gonad tissue. The data shows a significant difference between -1°C / 0°C and 4°C across the study whilst the 1°C and 2°C treatments are not significantly different regardless of the variable measured (Table 3.1). Enzyme activity in the 3°C and 4°C treatments tends to be similar and noticeably greater than in the -1°C / 0°C, 1°C and 2°C treatments. This trend is most pronounced for protein carbonyls (Figure 3.3), lipid peroxides (Figure 3.4) and SOD (Figure 3.5). There were significant differences in all oxidative damage markers and antioxidant responses between the tissue types. Gonad tissue had higher activities of GR, GPOX, SOD and CAT, whilst tube feet had higher levels of protein carbonyl and lipid peroxide. The smallest difference between tissue types was in the lipid peroxide levels.

3.4 Discussion

This study investigated the sensitivity of *O. validus* to oxidative stress and the response of the antioxidant system to increased temperatures. This was also assessed to see if long term exposure to increased temperatures altered oxidative stress responses. Among the key characteristics of polar marine invertebrates are low rates of metabolism and reactive oxygen species (ROS) formation associated with lower antioxidant enzyme activities (Abele and Puntarulo, 2004; Buttemer et al., 2010; Lister, 2015; Pörtner, 2002). *O. validus* in this study had similar levels of antioxidant activity for SOD and CAT throughout this 10-month study compared with reported measures of a sympatric species Antarctic limpet *Nacella concinna* (Ansaldo et al., 2005). Activity levels of SOD and CAT were much lower than measures reported for Antarctic sea urchin *Sterechinus neumayeri* and Antarctic clam *Laturnula elliptica* collected from three locations in Antarctica (Lister, 2015), but activity of GPOX and GR were greater in *O. validus* than those found in either *S. neumayeri* or *L. elliptica*.

There is no evidence of *O. validus* acclimation through time, with no effect of sample month on antioxidant enzyme activities or oxidative damage markers in any of the temperature treatments. The increase in levels of protein carbonyls, lipid peroxides and antioxidant enzymes are consistent with the increase in metabolism required at warmer temperatures (Lushchak, 2011). However, the stress and metabolic demands of living at increased temperatures may lead to greater vulnerability of *O. validus* and other echinoderms to other environmental stressors including pollution, pathogens and ocean acidification (Uthicke et al., 2021).

Oxidative stress in marine invertebrates and fish

The use of oxidative stress analysis as a tool in ecological studies concerning climate change and abiotic and anthropogenic factors is increasing (Glippa et al., 2018). Elevated temperatures are documented to cause increased metabolism and molecular oxygen (Wang et al., 2018). For Antarctic bivalve *Yoldia eightsi,* prolonged exposure to 2°C water temperatures resulted in an imbalance of ROS production and antioxidant removal (Abele et al., 2001). Atlantic oysters exposed to the combined conditions of elevated temperature and cadmium found that evidence of oxidative stress increased as temperature increased (Lannig et al., 2006). However, some studies have found that acute thermal stress caused significant upregulation of antioxidant enzymes in bony ice fish (Carney Almroth et al., 2015) whilst longer term (3 weeks) did not show this. Oxidative damage was found after three weeks that was not detectable after the acute 12 hour exposure, suggesting that the antioxidant defence system returned to baseline during the long term exposure and defences were not sufficient to prevent damage (Carney Almroth et al., 2015).

O. validus antioxidant enzyme activity was not found to increase or decrease with time. The antioxidant enzyme defenses have been found to activate due to warming, both in cladocerans, mussels and crabs (Glippa et al., 2018). Antarctic scallop *Adamussium colbecki* were found to have specific antioxidant responses to increased temperatures and cadmium between the digestive and gill tissues (Benedetti et al., 2016). In polar, oxygen rich marine environments antioxidant enzymes compensate for exposure to chronically cold seawater temperatures and under standard conditions are present in similar levels to those found in temperate marine invertebrates (Lesser, 2006; Viarengo et al., 1998). Reactive oxygen species (ROS) are a by-product of cellular respiration and therefore the low respiration rates of species adapted to the polar marine environment result in less cellular ROS (Abele et al., 2002). The increased base

levels of antioxidant activity in polar species relative to metabolism are thought to be an important strategy to cope with prooxidant pressures in extreme marine environments like Antarctica which is characterised by high levels of dissolved oxygen, highly seasonal productivity and food availability and low relatively consistent temperatures (Abele and Puntarulo, 2004; Regoli et al., 2011).

Physiological acclimation of *Odontaster validus*

It is possible that the temperature treatments in this study, although environmentally relevant, are not sufficient to trigger an change in oxidative damage markers and antioxidant enzymes in *O. validus* during long term exposure. Morley et al., (2012) demonstrated that the acute upper lethal limit for *O. validus* was around 12-16°C, but individuals also survived in temperatures of 9-12°C in short term studies (Peck et al., 2008). Heat shock response, a defence mechanism employed by many marine species as a protection against thermal stress, is not often present in Antarctic marine invertebrates (Clark et al., 2017) and this is attributed to millions of years of adaptation to the cold. *O. validus* is among the species that does not exhibit a heat shock response (Clark et al., 2008; González et al., 2016).

Examination of multiple tissues is important as it is indicative of whole animal responses to environmental changes and stressors (Clark et al., 2017). The upregulation of antioxidant enzymes CAT, GPOX, and GR in the gonad tissue compared to the tube feet may suggest that *O. validus* is able to selectively protect its reproductive organs from oxidative damage. Tube feet are part of the water vascular system and facilitate movement in *O. validus* but are less complicated structurally and physiologically than the gonad or pyloric caecum tissues. Studies of pollution response in eels found that measures of oxidative damage and antioxidant enzymes varied between tissue types (Regoli et al., 2011). Antioxidant levels of Antarctic scallop *A.*

colbecki and Mediterranean scallop *P. jacobaeus* differed between the gills and digestive tissues (Viarengo et al., 1995), echoing what this study has found with *O. validus*. *A. colbecki* digestive glad has higher levels of both free radical scavengers and enzymes compared to the antioxidant defences of the gills. For *O. validus* SOD and CAT activity levels were both greater in the gonad tissue compared to the tube feet and these enzymes are usually found to be correlated (Carney Almroth et al., 2015). SOD has been shown to respond to temperature stress in temperate and polar marine invertebrates (Abele et al., 2001) and forms the first line of defense against oxidative stress as it catalyses the removal of ROS. *O. validus* also had greater levels of GR and GPOX in the gonad compared to the tube feet. This disparity between the defences of different tissues may be due to the increased uptake of ROS-generating natural and anthropogenic xenobiotics, as well as other pollutants by the tube feet compared to the gonad tissue (Regoli et al., 1997; Viarengo et al., 1995).

Greater variation in the higher temperature treatments may indicate a range of responses to increased temperatures. Body size and age can be controlling factors on the responses of individuals to thermal stress and therefore their resilience to environmental changes (Gillooly et al., 2001). The oxidative stress theory of aging suggests that individuals that live longer have less cumulative oxidative damage and may have structural characteristics that make them less susceptible to oxidative damage (Buttemer et al., 2010). This is an important consideration for research exploring oxidative stress mechanisms as the inverse relationship between maximum life span potential (MLSP) and antioxidant levels has implications for the future of species in the face of climate change and increasing ecosystems changes. *O. validus* are long-lived and reach sexual maturity between 3-6 years or older (Pearse, 1969). If exposed to conditions that result in oxidative stress, the associated reduction in MLSP has important implications for this

keystone species as changes to the population density would impact ecosystem function, balance and biodiversity (Buttemer et al., 2010; Clark et al., 2017).

Limitations of the study

The mixed effects model found that month was not a significant predictor of oxidative damage markers, nor antioxidant enzyme activity in either tissue type. Warmer temperatures are known to cause increased metabolism (Abele et al., 2001; Gillooly et al., 2001). In this study, prolonged exposure to increased temperatures did not result in oxidative stress but led to increases in the levels and activity of all indicators as a result of the metabolic demands of increased temperatures.

A key element that is absent from this study is the ability to measure the lab effect. This would have required taking samples of *O. validus* at McMurdo Sound when they were collected for comparison to the values later measured. Animal sex and age data were also not collected during the sampling process and this factor would have been an interesting addition to the study. Sex data would have enabled comparisons between male and female individuals and allowed exploration any differences in the antioxidant enzyme activity or oxidative damage and therefore acclimation potential between the sexes. Age data were also not collected and this can be important for assessing gonad development and sexual maturity as there are differences in biochemical composition of animals at each life stage, including levels of lipid peroxidation and antioxidants (Glippa et al., 2018). Differences in the age, sex and maturation of individuals may account for some of the variation within the treatments.

Chapter 4: General Discussion

4.1 Summary of findings

The objectives of this study were to examine the physiological acclimation potential of *O. validus* to increased temperatures similar to those anticipated in the near future as a result of climate change. This was measured using three key indicators of individual fitness; metabolic rate, morphometric analysis of the energy reserve organ pyloric caecum and reproductive tissue, and the measure of oxidative stress markers and antioxidant defences.

O. validus were found to consume more oxygen in higher temperatures, and both antioxidant enzyme activity and levels of oxidative stress markers increased in warmer treatments. The gonadosomatic index but not the pyloric caecum index was also affected by temperature, with lower GI found in animals in 3°C compared to -1°C. This suggests *O. validus* are able to physiologically adjust to increased temperatures by increasing their metabolic rate and increasing antioxidant enzyme activity to protect against oxidative damage. However, this adjustment is not acclimation. The elevated respiration rates and Q_{10} suggests that *O. validus* were experiencing physiological stress. Across the ten-month study, there was no effect of time in any of the fitness parameters studied. Evidence of acclimation in these physiological measures would tend to present in an initial response to temperature, followed by a gradual return to pre-exposure or near pre-exposure levels.

Maintenance of the body at increased temperatures comes with energetic trade-offs and reduces the energy available for other life processes (Sokolova, 2021). Individuals in warmer temperatures had lower GI indices compared to individuals in colder temperatures, but all gonadosomatic indices were much lower than the McMurdo Sound average. A high energetic

investment is required to produce oocytes and sperm, particularly as female *O. validus* develop two cohorts of oocytes simultaneously. Lab effects and continuous food supply likely masked any variation in PI across the study.

Increased temperatures are not the only condition predicted to challenge Antarctic benthic invertebrates. Changes to the Southern Ocean including pollutants, sea ice melt, ocean acidification and ecosystem shifts will all place further stress on species at the poles (Chown et al., 2015; Griffiths et al., 2017; Somero, 2010). The cumulative nature of these stressors and documented importance of integrative studies highlight the need for future work in this field to involve multiple stressors and explore their interactive effects.

The predicted effects of climate change on animal physiology is a topical and growing area of research. The known principles and constraints of temperature on biology are well established, governing many life processes and shaping ecosystems globally (Bruno et al., 2015; Sokolova, 2021). For ectothermic organisms, including marine invertebrates, temperature affects metabolism, growth and reproduction (Christensen et al., 2022; Somero, 2012). This study suggests that ocean warming as a result of climate change will lead to *O. validus* adjusting their metabolism. However, this adjustment to meet increased energetic demands will reduce the resources available to support growth and reproduction, and which may impair the survival of the species in future climate change scenarios (Christensen et al., 2022).

This study did not explore the cumulative effects of environmental stressors on oxidative stress markers. Antarctic marine organisms including *O. validus* are predicted to face a range of anthropogenic and ecological challenges in the near future including pollution, invasive species, and shifts in the oceanographic environment simultaneously (Aronson et al., 2007; Crain et al., 2008; Somero, 2010). Although *O. validus* is an opportunistic scavenger and is

thought to be relatively robust to temperature stressors (Agüera et al., 2015; Kidawa et al., 2010; Peck, 2016), the lack of acclimation over this ten-month study highlights that whilst *O. validus* are able to survive and function long term at increased temperatures by increasing oxygen consumption and activity of antioxidant defences, there will be a metabolic cost associated with increased temperatures that their physiology may not be able to reconcile through acclimation.

Key findings

- Temperature significantly increased *Odontaster validus* respiration rates at the highest temperature (4°C) compared to the lowest (1°C / 0°C), and antioxidant enzyme activity and levels of oxidative damage markers were higher at all other temperatures compared to at -1°C / 0°C.
- Pyloric caecum but not gonadosomatic index was increased in the 3°C treatment compared with -1°C. The metabolic scaling rate was not increased with temperature.
- *Odontaster validus* are able to increase their metabolism in warmer temperatures but this is likely to result in energetic trade-offs for vital processes such as growth and reproduction.

4.2 Future directions

This study was limited in two key ways: small sample sizes and lack of individual sex data. The small sample sizes led to individual variation across all measures of respiration, morphometrics and oxidative stress. Destructive sampling of Antarctic benthic invertebrates is logistically challenging but a larger number of study subjects may have helped reduce the variation across the physiological parameters measured for this study. If a larger number of individuals were available they could also have been grouped by size, leading to more

confidence in the results found. Metabolism, morphometrics and oxidative stress can all vary with age, activity, nutritional status, size, sex and reproductive status (Monserrat et al., 2011). In addition, the absence of oxidative stress data from Mc Murdo Sound means there is no baseline data with which to compare the findings of this study, and assess for lab effects.

The larval stages of *O. validus* may be the most vulnerable to environmental stressors, given that the adults have a higher lethal temperature limit compared to sympatric species (Morley et al., 2012; Peck et al., 2010, 2008). For this reason, the analysis of multi-generational effects would be beneficial to the understanding of *O. validus* survival under future climate change scenarios (Lamare et al., in review). Non-genetic inheritance, often referred to as transgenerational plasticity, whereby offspring capacity to resist pollutants and environmental stressors, has been found to reflect parental exposure history (Lister et al., 2017). Maternal effects can influence the phenotype of offspring (Marshall, 2008), and therefore maternal provisioning has a direct impact on development and future survival (Uthicke et al., 2021). Lister (2015a, 2015b) found that offspring of Antarctic sea urchin benefitted from maternal antioxidant provisioning when mothers had been exposed to environmental contaminants. However, Uthicke et al, (2021) measured negative carryover effects in both physiological and behavioural traits from parents exposed to thermal stress. This highlights the species-specific nature of stress responses and transgenerational plasticity in the marine environment, and the need for long term multi stressor, multi-generational studies.

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