

EFFECTS OF TEMPERATURE ON THE EMBRYONIC CLEAVAGE RATES AND LARVAL  
METABOLISM OF ANTARCTIC INVERTEBRATES

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## Abstract

Global climate change is set to disrupt global ecosystems with changes to both biotic and abiotic environmental factors. The world's oceans are warming at unprecedented rates, with temperature increases of between +1.4 °C and +4.4 °C expected by 2100. These increases in temperature particularly threaten the biota and ecosystems in the high Antarctic regions of Southern Ocean where temperatures have been cold (~ -1.8 °C) and thermally stable for over 30 million years. Warming temperatures can disrupt the biochemical reactions and physiological processes within an individual organism, with potential follow-on effects on populations and ecosystems. The organisms that inhabit the Southern Ocean are adapted to life within a very cold and narrow temperature range and are believed to have limited ability to cope with temperature stress. In this dissertation, I examine how temperature impacts early embryonic cleavage and metabolism, processes that are common to all metazoans. Our study subjects were four species of benthic Antarctic ectotherms, two pycnogonids (*Nymphon australe* and *Ammothea glacialis*), and two dendronotid nudibranchs (*Tritoniella belli* and *Tritonia challengeriana*). I first investigated the effects of temperature on the early cleavage phase. I found that for all four species, embryonic cleavage rate increased rapidly as temperature increased from -1.8 °C (ambient) to +3.5 °C. Consistent with other rate processes that have been measured in Antarctic marine ectotherms, the thermal sensitivity of cleavage rate was high. We also found that overall, thermal sensitivity was very high within the species' natural temperature range (-1.8 to ~0 C), but cleavage rate was less affected by temperature increases above the natural temperature range. This suggests that organisms haven't fully adapted to polar conditions potentially due to the limits of protein synthesis in extremely cold temperatures.

Second, I investigated the effects of temperature on the metabolic rate (as estimated from oxygen consumption) of early, middle, and late larval stages from the same four species. I found that the thermal sensitivity of oxygen consumption was high for the majority of stages, with 8/12 exhibiting  $Q_{10}$  values higher than the 2-4 generally seen for most biological functions. Specific stages of development did not seem to affect thermal sensitivity.

Lastly, I investigated the ability of larvae of one of the four species (*Nymphon australe*) to acclimate to temperature. In most Antarctic ectotherms tested to date, acclimation was completely absent or slow, requiring months to acclimate. I found that after 24 days at +1.0 °C,

larvae *N. australe* had lower overall oxygen consumption rates than those of larvae incubated at -1.8 °C for the same period, a classic sign of thermal acclimation. This is the first time that acclimation has been shown in Antarctic invertebrate larvae. Signs of acclimation occurred in weeks as opposed to months shown by adults of Antarctic invertebrates. Larvae of *N. australe* may be able to optimize their metabolism to match environmental temperature fluctuations and predicted warming on an ecologically relevant time scale.

Together, this work shows that the two essential processes of early cleavage and larval metabolism are thermally sensitive to small increases in temperature. However, if particularly sensitive stages, such as early larval stages, can acclimate to warming waters as shown here, Antarctic ectotherms may be more robust to warming than previously thought.

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## Chapter 1: Background

**“Changing temperatures literally changes the pace of life.”**

**(Zuo et al. 2012)**

### Introduction

Global climate change is set to affect all life forms on our planet across all biomes (Deutsch et al. 2015, Bernatchez et al. 2023). Continuous increases in greenhouse gas production since the industrial revolution are causing our planet to be heated at alarming rates (Schneider 1989, Zandalinas et al. 2021). Current projections by the 2023 Intergovernmental Panel on Climate Change (IPCC) report predict by 2100, the world’s oceans will warm between +1.4 °C and +4.4 °C (Low vs High Emissions). Temperature has been long understood as the strongest driver of the rates of biological functions, dating back over 100 years to the earliest studies on the development of fruit flies, frogs, and planarians (Lillie et al. 1897, Loeb and Northrop 1916, Peck 2016). Through its fundamental effect on the rates of biochemical reactions within an organism (Hochachka & Somero 2002, Delong et al. 2016) temperature impacts processes from the physiology of an organism to distributions and abundances of populations (Thorson 1950, Hoegh-Guldberg and Pearse 1995, Fields and Houseman 2004, Pecorino et al. 2013, Häder and Barnes 2019, Nash et al. 2021). Organisms generally operate within a thermal window, or the maximum and minimum temperatures organisms can successfully tolerate (Flynn and Todgham 2018). Temperatures outside of this thermal window lead to decreases in performance and eventual death (Tsai et al. 2002, Pörtner and Farrell 2008, Miller and Stillman 2012, Ángeles-González et al. 2020). For marine ectotherms, whose body temperatures are dictated by the temperature of their surroundings (Martin and Huey 2008, Zuo et al. 2012), changes in environmental temperature due to climate change may have profound effects (Somero and Devries 1967, Pörtner et al. 1999, Peck 2018). In this chapter, I review the concepts of the thermal environment of the Antarctic, the paradigms of Antarctic biology, and the taxa studied here (Pycnogonida and Nudibranchia).

## Antarctica and the Southern Ocean

About 180 million years ago the continent of Antarctica began to separate from the supercontinent of Gondwana, and by 155 mya the continent had separated from Africa (McLoughlin 2001, Jokat et al. 2003). Fast forward over 100 million years and the Antarctic peninsula has separated from South America, isolating the landmass and allowing the formation of the Antarctic circumpolar current ~30-34 MYA (Livermore et al. 2005). Ice began to form on the Antarctic continent 36-38 MYA (Kennett 1977, Zachos et al. 1992, Lear et al. 2000); the formation of the Antarctic circumpolar current thermally isolated the continent, promoting greater ice growth (Kennett 1977). The fall in atmospheric carbon dioxide ~ 34 MYA created an “icehouse effect” (as opposed to a greenhouse effect seen today), creating a positive feedback loop promoting ice growth and creating the ice-covered Antarctic continent we know today (DeConto and Pollard 2003, Lear and Lunt 2016).

The Southern Ocean surrounding Antarctica is the most biologically productive ocean on earth (Tynan 1998, Mayewski et al. 2008) and contains a diverse and highly endemic fauna (Peck et al. 2014). In addition to its isolating and cooling effects, the deepening and widening of the Antarctic circumpolar current ~31.5 MYA (Egan et al. 2013) increased ocean upwelling and overturning circulation, increasing surface-water nutrient concentrations (Kennett 1977, Egan et al. 2013, Lear and Lunt 2016). Today, numerous species utilize these waters as feeding grounds due to the high biomass of important plankton species, such as the Antarctic krill *Euphausia superba* (Arrigo et al. 1998, Tynan 1998, Cavan et al. 2019). These highly productive waters are not only important for the organisms around Antarctica but are of global importance, as the Southern Ocean plays a major role in the circulation of nutrients around the world’s oceans (Arrigo et al. 1998, Pollard et al. 2005). The Southern Ocean also acts as a climate buffer (Antarctic Climate Change and the Environment Decadal Synopsis (2022 ACCE Decadal)); these waters store a disproportionate amount of heat from anthropogenic warming and act as a carbon sink (Peck et al. 2018, Rintoul 2018, ACCE Decadal), making this region important in climate change scenarios (Peck et al. 2018). Current predictions suggest sea surface temperatures in the Southern Ocean will rise between 0.5 °C and 1.75 °C by 2100 (2022 ACCE Decadal),

threatening the biodiversity and regulatory abilities important to the homeostasis of Earth (2022 ACCE Decadal, Tonelli et al. 2021, Brooks et al. 2022).

### **Paradigms of Antarctic Biology**

The near-freezing waters of the Southern Ocean contain a diverse and highly endemic fauna that has evolved over millions of years in a very cold environment with little spatial or temporal variation in water temperature (Peck et al. 2014, Détrée et al. 2023). As a result, most species, particularly marine ectotherms, are highly cold-adapted, stenothermal, cold-temperature specialists with slow development (Clark and Johnston 1996, Pörtner 2006, Détrée et al. 2023). This specialization allows organisms to survive in some of the coldest waters on earth, but as oceans warm, they are likely to be particularly vulnerable to ocean warming (Peck 2002, Peck et al. 2010, Détrée et al. 2023). Numerous Antarctic invertebrates have been shown to have very long developmental periods from fertilization to hatching or metamorphosis (Peck 2018). For instance, Moran et al. (2019) described the development of the gastropod *Antarctodomus thielei* and found that embryos take 8 years to hatch from their benthic egg masses. While a developmental time of 8 years is on the extreme end of lengthy development in the Antarctic, when compared to tropical or temperate species, the time required to reach a given developmental stage is much greater in a wide diversity of Antarctic ectotherms (reviewed in Peck 2018). We know that with cooling temperatures, development slows in part because increased viscosity and osmolyte concentrations slow protein folding (Peck 2016, Peck et al. 2024). However, the development of Antarctic organisms is slowed beyond what is expected based on temperature alone, suggesting that while the mechanisms underlying development in Antarctic organisms may be cold-compensated to some extent, there is a limit to how much compensation can occur at the coldest limits to biological life (Peck 2016). These early developmental stages in which development occurs over long periods are of particular concern as they have a limited ability to acclimate to temperature stress (Rombough 1997) and are considered to be the most vulnerable points in a life cycle (Bressan et al. 1995, Cowart et al. 2009, Walther et al. 2013).

When organisms experience stress, there are three main responses they can deploy: migration, adaptation, and acclimation (Peck et al. 2010, Habary et al. 2017, Bernatchez et al. 2023, Donelson et al. 2023). While these mechanisms are utilized by many organisms as global warming changes the earth's biomes (Bernatchez et al. 2023), organisms in the Antarctic are limited in their ability to respond. In tropical or temperate regions, organisms can retreat from warmer areas near the equator towards to poles to reach cooler temperatures (Parmesan et al. 1999, Kortsch et al. 2015, Maire et al. 2019, Todgham and Mandic 2020). Migration has limited potential in the Antarctic, however, as movement southward to colder areas in the high Antarctic is not possible, as they already exist in the coldest waters (Todgham and Mandic 2020) and have limited degrees of latitude to migrate along the Antarctic shorelines (Peck 2005). Adaptation through natural selection is also likely to be ineffective due to the long generation time and slow development of most Antarctic ectotherms, which mean that adaptation is unlikely to keep pace with the rate of environmental change (Flynn and Todgham 2017, Peck 2018, Todgham and Mandic 2020). Occurring on shorter time scales, acclimation may be the only mechanism organisms in the Antarctic have to deal with temperature stress (Peck et al. 2010), but its effectiveness is limited and has only been investigated in a few taxa (Pörtner et al. 2007). Of those studies, most concern fish and show they have some ability to acclimate (Franklin et al. 2007, Robinson and Davidson 2008). However, most studies of marine invertebrates suggest that they have very little ability to acclimate to warming temperatures, with those being able to acclimate doing so over extremely long and potentially irrelevant time periods (Peck et al. 2009, Peck et al. 2014, Morley et al. 2019).

## **Pycnogonids**

Two of the focal species in this dissertation are the pycnogonids *Nymphon australe* Hodgson 1902 and *Ammothea glacialis* (Hodgson 1907), both of which are common and widely distributed in the Southern Ocean (*N. australe*: Mahon et al. 2008, Zehnpfennig et al. 2024; *A. glacialis*: Cano-Sánchez and López-González 2009, Cano-Sánchez and López-González 2018). The pycnogonids (phylum Arthropoda, subphylum Chelicerata, class Pycnogonida), more commonly known as sea spiders, are an ancient, and diverse class of marine arthropods (Arnaud and Bamber 1988, Moran et al. 2024, Sharma and Gavish-Regev 2025). The name Pycnogonida

is derived from the Greek words *pykno*, meaning lots of or dense/thick, and *góny*, meaning knees. Pycnogonids are the sister group to the rest of the members of the subphylum Chelicerata, including spiders, scorpions, and horseshoe crabs (Arango 2002, Sharma and Gavish-Regev 2025). They have a worldwide distribution and include almost 1400 described species (Arnaud and Bamber 1988, Zehnpfennig et al. 2024, Sharma and Gavish-Regev 2025). Of these, roughly 21% are found exclusively in the Southern Ocean (Peck 2018). The family Nymphonidae, and more specifically the genus *Nymphon*, is the most common genera of pycnogonid in the Southern Ocean by both the number of species and the estimated population (Mahon et al. 2008, Arango et al. 2011, Collins et al. 2018). Roughly 25% of the 250 species within the genus *Nymphon* are found within the Southern Ocean and many of them are endemic to that region (Arango et al. 2011). The genus *Ammothea* (family Ammotheidae) also shows high diversity and endemism in the Southern Ocean, with roughly 30 of the 41 described species occurring only in Antarctic and Sub-Antarctic waters (Cano-Sánchez and López-González 2014, Cano-Sánchez and López-González 2018).

Sea spiders are typically small and cryptic organisms (Arnaud and Bamber 1988, Brenneis et al. 2017) but in the polar oceans and deep sea they commonly exhibit gigantism and can reach immense sizes with leg spans (leg tip to leg tip) of 70+ cm (Arnaud and Bamber 1988). Many pycnogonids have exclusive male parental care where males brood their young on specialized ovigerous legs through hatching and several larval molts (King 1973, Bain 2003, Cano and López-González 2009, Fornshell 2019, but see Moran et al. 2024 for an exception). Instead of utilizing specialized respiratory structures and pigments, pycnogonids exchange gasses through diffusion across their cuticles and via specialized pores in their cuticles (Woods et al. 2009, Lane et al. 2018, Shishido et al. 2019).

## **Nudibranchs**

The other two focal taxa in this dissertation are the nudibranchs *Tritoniella belli* Eliot 1907 and *Tritonia challengeriana* Bergh 1884 (phylum Mollusca, order Nudibranchia, Family Tritoniidae), both of which are common and widely distributed in the Southern Ocean (*Tritonia*

*challengeriana*: Wägele 1995, Rossi et al. 2021, *Tritoniella belli*: Barnes & Bullough, 1996, McClintock and Baker 1997, Bryan et al. 1998). The family Tritoniidae contains more than 80 described species and are found in all oceans (Moles et al. 2021). Nudibranchs, also known as sea slugs are a diverse order of marine molluscs. whose name is derived from the Latin and Greek words meaning “naked gills.” Unlike many other gastropods, nudibranchs generally lose their shells after metamorphosis from the larval stage and lack shells as adults (Thompson 1959, Faulkner and Ghiselin 1983); perhaps as a consequence, many rely on chemical defenses against predation (Faulkner and Ghiselin 1983, Bryan et al. 1998, Wägele and Klussmann-Kolb 2005). Nudibranchs are found worldwide with over 4700 described species (Dean and Prinsep 2017, Rola et al. 2022). Currently, there are 37 described species within the genus *Tritonia*, only three of which are found in the Southern Ocean (Rossi et al. 2021). Recently, taxonomic reconstruction has placed *Tritoniella challengeriana* in a new genus *Myrella* along with the subantarctic species *Myrella dantarti* (De Vasconcelos Silva et al. 2023). The genus *Tritoniella* is represented by six species all of which are found in Antarctic and sub-Antarctic waters (Schächinger et al. 2022, De Vasconcelos Silva et al. 2023).

Most nudibranchs are simultaneous hermaphrodites as adults (Sekizawa et al. 2013, Cheney and Wilson 2018). When mating, nudibranchs alternate roles between donating and receiving sperm (Sekizawa et al. 2013, Heller 2015). After copulation nudibranchs can store donated sperm allowing for the possibility of post-copulatory female choice of fertilization (Sekizawa et al. 2013). After eggs are fertilized, animals produce egg masses containing embryos embedded in a gelatinous matrix that, for most taxa, are deposited on the substrate (Carroll et al. 1990, Page 2007, Moran and Woods 2007). *Tritoniella belli* and *Tritonia challengeriana* produce benthic, gelatinous egg masses that contain hundreds to thousands of fertilized zygotes (McClintock and Baker 1997, Bryan et al. 1998, Moran and Woods 2007). Based on our observations and those of Woods and Moran (2008), both species hatch from the gelatinous egg mass as swimming-crawling larvae close to metamorphosis.

## Approach

In Chapter One of this dissertation I address how temperature impacts the early embryonic cleavage rates of two pycnogonids and two dendronotid nudibranchs from McMurdo Sound, Antarctica. The early cleavage phase is highly conserved and is one of the most important stages of the metazoan life cycle (Stitzel and Seydoux 2007, Liu et al. 2014). Early cleavage phases are regulated by the maternal complement (Tardos et al. 2003, Baroux et al. 2008) and are believed to have limited mechanisms such as acclimation to compensate for increases in temperature (Rombough 1997, Flynn and Todgham 2017). Here I test the effect of temperatures ranging ~4 °C from -1.8 °C to +2.3 °C on the rate of early embryonic cleavage. In Chapter II, I address how temperature impacts the metabolism of early life history stages of the same four species.

Metabolism or the sum of all the biochemical processes that provide an organism with energy, is another important process common to all metazoans (Clarke and Fraser 2004, de Nava and Raja 2022). Temperature is known to increase metabolic rate (Hochachka and Somero 2002, Schute 2015) but if temperature exceeds the functional range of an organism, energy may be allocated away from growth and reproduction and towards cellular stress responses (Sokolova et al. 2012, Todgham and Mandic 2020). While organisms can tolerate temporary increases in energetic demand, long term exposure can be energetically costly and eventually detrimental if energetic equilibrium cannot be maintained (Sokolova et al. 2012, Sokolova 2013, Wingfield 2013). Here I show the effect of temperature on three stages of development for each of the four species and assess the thermal sensitivity of metabolism in early life history stages. Chapter III focuses on acclimation (in the lab) and acclimatization (in situ); mechanisms organisms can utilize in order to compensate for increases in temperature stress. Here I show the acclimatory ability of one larval stage of the pycnogonid *Nymphon australe* to temperatures of +1.0 °C and test for signs of seasonal acclimatization between the austral summer and austral winter.

These data are among the first Antarctic studies to examine the impact of temperature on the highly conserved early cleavage phase and the acclimatory ability of larvae of Antarctic invertebrates. There is an overall limited amount of experimental data showing the effects of temperature on vital processes such as early embryonic cleavage and larval metabolism of Antarctic invertebrates. Success of these early life history stages is vital to the success of

vulnerable populations in the Antarctic. Along with a lack of knowledge pertaining to the effects of temperature on these vital processes, a lack of understanding on whether larvae and embryos can acclimate limits our understanding of how organisms may compensate for increasing temperature. These data allow us to better understand how these organisms may be impacted by climate change and may give insights into what role early life history stages play in the resilience or vulnerability of Antarctic taxa.

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## CHAPTER 2: EFFECTS OF TEMPERATURE ON THE EARLY EMBRYONIC CLEAVAGE OF ANTARCTIC INVERTEBRATES

### Abstract

The Southern Ocean surrounding Antarctica is the coldest ocean on Earth and many of the ectotherms that live there are highly sensitive to temperature. A fundamental and essential process in the development of all metazoans is early embryonic cleavage, which transforms the zygote into an embryo. The maternal complement of RNA and protein regulates the first few stages of embryonic development and is highly conserved across metazoans. These early developmental stages in an organism's life are vulnerable to stressors such as temperature as they have poor acclimatory abilities when compared to adults. We measured the thermal sensitivity of early cleavage of four Antarctic ectotherms, two pycnogonids (*Nymphon australe* and *Ammothea glacialis*) and two dendronotid nudibranchs (*Tritoniella belli* and *Tritonia challengeriana*). For Antarctic fauna, few studies have investigated the effect of increasing temperature on early developmental stages; these are often more vulnerable to environmental stressors than later stages. Uncleaved zygotes were placed in an aluminum thermal gradient block and maintained at a range of 12 temperatures between -1.8 °C (ambient) and 3.5 °C until embryos at each temperature reached at least the 32-cell stage. Embryos reached the 32-cell stage in approximately half the time at the highest temperature compared to the lowest (ex. 7 days vs. 18 days). Q10 values for developmental rate across the whole temperature range were high (>2-3) indicating high thermal sensitivity of early cleavage. Breakpoints were present in the thermal range indicating a change in thermal sensitivity over the ~5.0 °C range. Additionally, development appeared normal even at the highest temperature, but abnormalities, developmental arrest, and embryo death were more frequent at higher temperatures. These data show that early cleavage rates are highly sensitive to increases in temperature, and that there is considerable variation in embryos' ability to develop normally at temperatures > 4 °C degrees above ambient.

## Introduction

Temperature is a major environmental factor impacting the physiology, distribution, and abundance of organisms (Thorson 1950, Hoegh-Guldberg and Pearse 1995, Fields and Houseman 2004, Pecorino et al. 2013) because of its fundamental effects on the rates of biochemical reactions within organisms (Hochachka & Somero 2002, DeLong et al. 2016). Climate change is causing warming of the world's oceans, leading to great concern about how many marine organisms will respond to these changes (Hoegh-Guldberg and Pearse 1995, Hofmann and Todgham 2010, Bylenga et al. 2015). The Southern Ocean which surrounds Antarctica is an area of particular concern, because it stores a disproportionate amount of the world's heat and is projected to warm anywhere from 0.5 °C to 1.75 °C (low vs high emissions scenario) over the next 100 years (ACCE 2022 Decadal Report). The frigid waters of the Southern Ocean contain a diverse and highly endemic fauna that has evolved for millions of years in a very cold environment with little temporal or spatial variation in water temperature (Peck et al. 2014).

The evolutionary history of cold adaptation in Antarctic marine organisms has led to a paradigm of stenothermality and vulnerability to climate and ocean warming, which has received considerable experimental support (reviewed in Peck 2018). One open question about the Southern Ocean marine fauna is how early life history stages, which are widely considered to be one of the most vulnerable points in the life cycle (Bressen et al. 1995, Cowart et al. 2009, Walther et al. 2013), will be affected by warming. This question has rarely been addressed in Antarctic organisms, but a large body of work from warmer regions has found that increases in temperature during early development speed up both metabolism and developmental rate (reviewed in Hoegh-Guldberg and Pearse 1995). Early developmental stages often have poor acclimatory abilities compared to adults, leaving early stages unable to compensate their physiological processes for increases in temperature (Rombough 1997, Flynn and Todgham 2017). Increases in temperatures can negatively affect organisms by causing a metabolic imbalance, such that energy supply cannot keep pace with energy demand (Todgham and Mandic 2020). Early developmental stages can be strongly affected by such metabolic imbalances because proper energy maintenance is vital to ensure organisms have enough energy to fuel them through ontogeny (Hoegh-Guldberg and Emlet 1997). Also, by decreasing the

energy required for biological reactions to proceed, rising temperature increases an organism's developmental rate (within functional limits) (Hochachka and Somero 2002). Rapid cellular divisions during development may increase the incidence and severity of developmental abnormalities (Reitzel et al. 2004, Byrne and Prezeslawski 2013). While the effects of temperature on early development are well studied in warmer regions (Briere et al. 1999, Gillooly et al. 2002, Lawrence and Soame 2004), where organisms generally experience considerable temperature fluctuation, little is known about the effects of temperature on development for Antarctic organisms, or how the effects of temperature will change with progressive warming of the world's oceans.

One of the most conserved and important stages of the metazoan life cycle is the cleavage phase, when the zygote is transforming through cleavage into an embryo. All metazoans undergo a cleavage phase, and during this time the maternal complement of RNA and protein in zygotic cytoplasm contain all the necessary machinery to drive the first stages of embryonic development (Stitzel and Seydoux 2007, Liu et al. 2014). Maternal control of development lasts until roughly the mid blastula stage, when maternal products are degraded and embryonic control is activated (Tardos et al. 2003, Baroux et al. 2008). Across metazoans, the cleavage phase generally speeds up with temperature within the functional range of an organism; above that range development fails due to exhausted aerobic capacity, increased anaerobic metabolism and eventually the denaturation of proteins (Pörtner and Farrell 2008, Ángeles-González et al. 2020). Temperatures below the functional range of an organism slow development due to intracellular increases in viscosity and osmolyte concentrations which may also impact protein folding and stability (Peck 2016, Peck et al. 2024). Elements of the maternal complement that regulate cell division are highly conserved across the major branches of the metazoan tree of life (Liu et al. 2014), suggesting a potentially powerful set of mechanisms for identifying convergent evolution of thermal adaptation traits.

Important but largely unexplored questions for cold-adapted Antarctic marine organisms are (1) Will predicted levels of warming cause embryonic development to fail, or to cleave abnormally? (2) If the mechanics of early cleavage are robust across a given range of warming, will there be ecologically- or physiologically- relevant changes in developmental rate? Warming-driven

increases in developmental rate may lead to a mismatch between hatching times and environmental conditions, such that animals enter the juvenile environment during an inopportune time (Cushing 1975, Schmalenbach and Franke 2010). There is considerable concern about the potential collapse of the Antarctic ecosystem due to climate change (ACCE 2022 Decadal Report), but the role of thermal sensitivity of embryonic and larval development has received little attention.

Here we examine the impacts of temperature on the cleavage rate and early development of four Antarctic invertebrate species from two different phyla. Using Arrhenius plots, we test for the effect of temperatures ranging from ambient (-1.8 °C) to +4.0 degrees, encompassing predictions for 2100 (Tonelli et al. 2021) on the rate of cleavage of the early embryo, as well as the incidence of developmental abnormalities. These data are among the first Antarctic studies to examine the impact of temperature on the earliest and potentially most vulnerable life history stage.

## **Methods**

### **Study Organisms**

Four Antarctic species were used in this study, including two sea slugs, *Tritoniella belli* Eliot 1907 and *Tritonia challengeriana* Bergh 1884 (phylum Mollusca, class Gastropoda, order Nudibranchia), and two sea spiders *Ammothea glacialis* (Hodgson 1907) and *Nymphon australe* Hodgson 1902 (phylum Arthropoda, class Pycnogonida, order Pantopoda). *Tritoniella belli* and *Tritonia challengeriana* both produce benthic, gelatinous egg masses that contain numerous eggs (>1000) (McClintock and Baker 1997, Bryan et al. 1998, Moran and Woods 2007). Both species of sea spider carry their young on the ovigerous appendages (Cano and López-González 2009, Fornshell 2019), where offspring develop through hatching and several larval molts. All four species are widely distributed throughout the Antarctic (*N. australe*: Mahon et al. 2008, Zehnpfennig et al. 2024, *A. glacialis*: Cano and López González 2009, *Tritonia challengeriana*: Wägele 1995, Rossi et al. 2021, *Tritoniella belli*: Barnes & Bullough, 1996, McClintock and Baker 1997, Bryan et al. 1998) and were chosen for the relative ease of collection of newly fertilized embryos. All four species were collected by divers on SCUBA from McMurdo Sound

during the 2019-2022 field seasons and were stored in flow-through sea tables with temperatures between  $-1.57\text{ }^{\circ}\text{C}$  and  $+0.56\text{ }^{\circ}\text{C}$  (average:  $-1.07\text{ }^{\circ}\text{C} \pm 0.36\text{ (SE)}\text{ }^{\circ}\text{C}$ ). Egg masses of *N. australe* were collected entirely from the field from mating pairs of adults, whereas egg masses of *A. glacialis*, *Tritoniella belli*, and *Tritonia challengeriana*, were collected from an adult that was depositing an egg mass in the field or in some cases, in the laboratory. Exact time of fertilization (and therefore time spent at the zygote (1-cell) stage) was unknown for any mass. A total of 10 *Tritoniella belli*, 2 *Tritonia challengeriana*, 4 *N. australe*, and 1 *A. glacialis* egg masses were assessed. Each set of embryos for each of the egg masses were from the same spawn, and hence were at least half-siblings. Embryos of *Tritoniella belli* and *Tritonia challengeriana* were removed from the egg masses by gently tearing the gel sleeve surrounding the embryos with fine forceps until embryos fell freely out of the egg mass (see Moran and Woods 2007). For *N. australe* and *A. glacialis* embryos were gently removed from the oviger and then separated using fine forceps. Embryos were kept in  $0.2\mu\text{m}$  – freshly filtered seawater chilled at  $-1.8\text{ }^{\circ}\text{C}$  during handling using a digital chilling plate (IC20, Torrey Pines, Carlsbad, US).

### **Experimental Temperature Exposures**

To test the effects of temperature on cleavage rate, we maintained embryos of all four species at a range of temperatures in a thermal gradient block up to the 32-cell stage. We did so separately for one or more sets of embryos from different parents ( $n= 10$  for *Tritoniella belli*,  $n= 2$  for *Tritonia challengeriana*,  $n= 4$  for *N. australe*,  $n= 1$  for *A. glacialis*), and each set of embryos was at the 1- or more rarely 2-cell stage when the experiment was initiated. Two slightly different protocols were followed due to the availability of different thermal gradient blocks in 2019 vs. 2021 (Table 1). In both setups, the thermal gradient was established by two continuously running pumps (RTE-211, Neslab/Thermo Fisher Scientific, Waltham, US) sending chilled glycol at two temperatures through the two ends of an insulated aluminum block drilled to hold small glass incubation chambers for embryos. In the first setup, the aluminum block measured 54 cm x 6 cm and had ten rows of three wells drilled to hold small tubes. We set the water baths such that the ten rows of wells contained a thermal gradient from  $-1.8\text{ }^{\circ}\text{C}$  to  $+3.3\text{ }^{\circ}\text{C}$ , with three wells at each of the ten temperatures. Each well in the block held a hand-blown glass microrespiration chamber (as used in Woods and Moran 2008) with a diameter of 10.7 mm or roughly the size of

a 1.5 mL Eppendorf tube. The second setup used a larger block (47.3 cm x 34.5 cm) with a 12 x 9 setup of holes drilled accommodate standard-sized 20-ml glass scintillation vials (diameter 27.2 mm). This block was set to create a thermal gradient of -2.2 °C to +2.2 °C. These temperature gradients were chosen to encompass predicted temperature changes in both high (increase of 1.75 °C) and low (increase of 0.5 °C) emission predictions (Tonelli et al. 2021, ACCE 2022 Decadal Report) and modern-day seasonal variation in McMurdo Sound (in prep.).

Experiments were performed the same way in both gradient blocks. Embryos were first removed from masses and rinsed in filtered sea water at -1.8 C. Rinsed embryos were then haphazardly assigned to groups of either 7-13 (nudibranchs) or 3 (pyncogonids), determined by embryo size and number of embryos available from that particular batch. Groups of embryos were placed into glass vials containing either 700 uL (1<sup>st</sup> setup) or 18 mL (2<sup>nd</sup> setup) of 0.2µm - filtered seawater. Vials were then maintained in the thermal block, with three replicate vials at each of the 12 temperatures within the gradient for a total of 36 vials per set.

To assess developmental rate, embryos were removed from the block once per day and the developmental stage of embryos in each vial was determined under a stereomicroscope (Wild M5A, Wetzlar, DE) and recorded (1 = uncleaved zygote (1 cell), 2 = first cleavage (2 cells), 3 = second cleavage (4 cells) , 4 = third cleavage (8 cells), 5 = fourth cleavage (16 cells), and 6 = fifth cleavage (32 cells)). Development was mostly synchronous within vials, but if embryos in a particular vial were at different stages (e.g. a mix of 2- and 4-cell), the most common stage (> 50%) was assigned as the stage of that vial. Each group of embryos were also photographed with a microscope camera (Gryphax Subra, Jenoptik, Jena, DE). During handling, embryos were maintained at -1.8 °C using a digital chilling plate (IC20, Torrey Pines, Carlsbad, US). Half the volume of seawater in each vial was replaced daily with freshly filtered seawater at temperature.

Each set of embryos was staged daily until the 32-cell stage (fifth cleavage) was reached, after which blastomere number became difficult to determine accurately. We also noted any embryo that appeared to be developing abnormally, for example if cell divisions were chaotic or irregular, or there were signs of loss of cellular integrity (i.e. external cell fragments or burst egg envelope).

## Cleavage rate and Arrhenius Transformations

To assess the relationship between temperature and cell division, for each set of embryos, we first estimated the cleavage rate (cleavages  $\text{h}^{-1}$ ) of each individual vial as the slope of a linear regression between cleavage stage (one of the following: first cleavage (2 cells), second cleavage (4 cells), third cleavage (8 cells), fourth cleavage (16 cells), or fifth cleavage (32 cells) and the number of hours of incubation. We then generated an Arrhenius plot from these data by taking the natural log of the cleavage rates (cleavages  $\text{h}^{-1}$ ) for each of the vials and plotting them against the inverse of their incubation temperature in Kelvin ( $1/T$ ). In order to compare with other studies and to calculate metrics such as activation energy and  $Q_{10}$ , the data was plotted with the x axis transformed as  $1/(Tx10^{-3})$  (Hoegh-Guldberg and Pearse 1995, Peck et al. 2024).

To assess the temperature dependence of developmental rate for each set of embryos, we first used the Boltzmann-Arrhenius equation, Boltzmann gas constant ( $R$ ), and the slope from the Arrhenius regressions to calculate activation energy ( $E_a$ ) as  $E_a = -R/\text{Slope}$ . We then estimated  $Q_{10}$ , the change in any rate process standardized to a 10 °C change in temperature (Hirche 1984, Hochachka 1991), using the activation energy ( $Q_{10} = e^{(E_a/R)(10/T_1T_2)}$ ) (see Hoegh-Guldberg and Pearse 1995, and Manogaran et al. 2019 for additional details).

To explore whether thermal sensitivity changed across the temperature range, which can indicate transitions between rate-limiting processes (Hoegh-Guldberg and Pearse 1995, Clarke 2017, Crapse et al. 2021) or deviations in the activation energy of a process (Hochachka and Somero 2002, Clarke 2017), we then fit a broken-stick regression to each Arrhenius plot to look for breakpoints in the data followed by a Davies test to determine the significance of the breakpoint (Davies 1987, Robinson and Williams 1993, Manogaran et al. 2019). If two or more regression lines were a better fit than a single one, we then calculated the Arrhenius breakpoint temperature (ABT), the temperature(s) at which the best-fit regression lines cross (Davies 2002, Robinson and Williams 1993, Manogaran et al. 2019). All modeling and computations were performed in the R Studio statistical package (Version 2024.04.1+748, Posit Software, PBC).

## Results

### Cleavage Rate and Developmental Success

The number of hours to reach the 32-cell stage for all sets of embryos at each experimental temperature are shown in Table 2. The slowest cleavage was seen in the single brood of *Ammothea glacialis*; embryos of *A. glacialis* took 821 h to reach the 16-cell stage at -1.8 °C, and none had reached the 32-cell stage by the time the experiment was terminated at 888 hours (37 d). The four sets of *Nymphon australe* took on average  $652 \pm 9$  (SE) h to reach the 32-cell stage. For the nudibranchs, *Tritonia challengeriana* took  $494 \pm 49$  h to reach the 32-cell stage and *Tritoniella belli* took  $454 \pm 20$  hours at -1.77 °C.

Abnormal development was rare outside of the three sets of embryos in which development was disrupted at higher temperatures (Tb 3, Tc 1, Tc 2; Table 2). If abnormal development occurred in the sets of embryos in which development was not disrupted, it generally happened at the upper end of the temperature treatments ( $>+1.6$  °C) with less than 10% of each vial being affected (i.e. 1 of 13 embryos developing abnormally). Only three sets of embryos failed to develop normally across the whole temperature range. For one set of *Tritoniella belli* (Tb 3), embryos at the highest temperatures (+2.6 °C and +3.3 °C; Table 2) took longer to develop to the two-cell stage than embryos at -1.8 °C and did not advance past the two-cell stage. For the two egg masses of *Tritonia challengeriana* (Tc 1\* & Tc 2\*; Table 2), embryos developed normally up to +1.27 °C but embryos kept at +1.65, +1.94, and +2.26 °C did not cleave, remaining at the 1-cell stage for the entirety of the experiment.

### Thermal Sensitivity

ABTs,  $Q_{10}$ s,  $E_{as}$ , slopes and intercepts for all sets of embryos are shown in Table 3. Arrhenius plots for each set of embryos are shown in Figures 1 and 2.

For the species with the most replication, *Tritoniella belli* (10 sets of embryos), the Arrhenius plots of three of the ten were best described by a single regression; these three masses had an

average  $Q_{10}$  of  $7.5 \pm 0.8$  (SE). For the other seven, significant breakpoints were detected. The average ABT for these seven masses was  $-0.65 \text{ }^\circ\text{C} \pm 0.2$ .  $Q_{10}$  values calculated from the slope below the ABT ranged from 128.4 to 12.5 (average  $36.6 \pm 16.1$ ) while above the ABT  $Q_{10}$ s were universally lower and ranged from 3.6 to 6.9 (average  $5.1 \pm 0.6$ ).

For the two masses of *Tritonia challengeriana*, no breakpoint was observed at the lower end of the temperature range, but there were clear shifts in thermal sensitivity at the higher end of the temperature range because development ceased at temperatures  $1.65 \text{ }^\circ\text{C}$  and above. To visually show the breakpoints (dashed line) as no development was observed (so therefore no data), artificial data points (X's) were added to Figure 2 to show stoppage in development. Across the thermal range where embryos appeared to be developing normally,  $Q_{10}$  values for *T. challengeriana* were 66.4 and 9.0 (average  $37.7 \pm 28.7$ ) for the two sets of embryos.

Two of the four sets of embryos of *N. australe* had Arrhenius plots that were best described by a single linear regression line; these had  $Q_{10}$  values of 10.6 to 8.3 (average  $9.5 \pm 1.2$ ) across the entire temperature range. The other two sets of embryos had significant ABTs at  $-0.85 \text{ }^\circ\text{C}$  and  $-1.66 \text{ }^\circ\text{C}$  with an average ABT of  $-1.26 \text{ }^\circ\text{C} \pm 0.41$ . Before the breakpoint  $Q_{10}$  values were 26.3 and 13.4 (average  $19.9 \pm 6.5$ ), while after the breakpoint they dropped to 6.3 and 6.5 (average  $6.4 \pm 0.1$ ). Lastly, the single egg mass of *A. glacialis* had an Arrhenius breakpoint at  $-0.75 \text{ }^\circ\text{C}$ , with  $Q_{10}$  values of 83.0 before the breakpoint and 3.8 after.

## Discussion

As the waters around the Antarctic continue to store a disproportionate amount of heat from global climate change, much concern has been raised on how the already vulnerable fauna inhabiting the Southern Ocean will be impacted by increased temperatures (Peck 2018, Todgham and Mandic 2020, ACCE 2022 Decadal Report). Embryonic and larval stages are among the most vulnerable points in the life cycle (Bressen et al. 1995, Cowart et al. 2009, Walther et al. 2013). Early embryonic cleavage is of particular concern because embryos have a limited ability

to acclimate (Flynn and Todgham 2017) and rapid anthropogenic change may overwhelm mechanisms designed to buffer embryos from stressors (Hamdoun and Epel 2007). Elevated temperatures during embryonic development can disrupt gene expression and essential biochemical processes, resulting in developmental abnormalities and decreases survival rates (Hasan et al. 2023). Here we show the effect of temperature on the early embryonic cleavage for four species from two different phyla in the Antarctic. Overall, cleavage rate was slow across all four species (relative to temperate species), with embryos taking days to undergo the first five cleavages even at the highest temperature. Temperature had a large impact on cleavage rate, with high  $Q_{10}$  values seen in cleaving embryos of all four species. We also found evidence that for the majority of sets of embryos, the underlying kinematics of the cleavage phase changed over the experimental temperature range as indicated by significant Arrhenius breakpoints in 10 of 17 experiments.

### **Upper thermal limits to cleavage**

At and above their thermal limits, organisms can no longer keep up with basic biological processes as oxygen delivery systems fail and proteins begin to denature, ultimately leading to death of the organism (Pörtner and Farrell 2008, Miller and Stillman 2012, Ángeles-González et al. 2020). The highly endemic fauna that live in the Southern Ocean inhabit a narrow range of very cold sea water temperatures (Peck et al. 2014, Todgham and Mandic 2020). Evidence suggests that the vast majority of Antarctic marine ectotherms have poor capacities to survive at elevated temperatures (Peck 2018). Species such as the bivalve *Limopsis marionensis*, brachiopod *Liothyrella uva*, and arthropod *Paraceradocus gibber* were unable to survive extended periods with temperatures between +2.0 and +4.5 °C (Peck 1989, Pörtner et al. 1999, Peck et al. 2010). However of the 17 sets of embryos, only three showed clear upper thermal limits to cleavage within our temperature range, with the rest appearing to developing normally throughout the range. All three sets with clear upper limits were nudibranchs; one was *Tritoniella belli* and, both of the sets of embryos from *Tritonia challengeriana*. Embryos from the *Tritoniella belli* egg mass (Tb3) developed up to +1.62 °C, above which development became slower with rising temperature and then stopped after ~90 hours. For *Tritonia challengeriana* (Tc1 & Tc2) development failed at and above +1.65 °C; at these temperatures, embryos remained

at the one-cell stage for the duration of the experiment. Failed development at and above +1.62 °C suggests an upper thermal limit for these species, and that their embryos may be highly susceptible to increasing water temperatures, particularly *Tritonia challengeriana*. These temperatures are only 2-3°C above summer maximum temperatures in McMurdo Sound (Hunt et al. 2003, Cheng and Detrich 2007) and less than 1 °C warmer than sea surface temperatures on the Antarctic peninsula in summer (Barnes et al. 2006, Cárdenas et al. 2018) where these organisms are also found (Wägele 1995, Barnes & Bullough, 1996, McClintock and Baker 1997, Bryan et al. 1998, Cano and López González 2009). Thus, our data suggest that both species may be vulnerable to warming on scales that some models predict will happen by the end of the century (Tonelli et al. 2021, ACCE 2022 Decadal Report)

### **Slow Development in Antarctic Invertebrates**

Temperature affects a wide variety of organismal processes, from metabolic rate (Newell 1969, Moran and Woods 2007) to developmental rate (Pearse 1969, Peck 2016). In the frigid waters surrounding McMurdo Sound, which hover around the freezing point of seawater (-1.8 °C) for most of the year (Hunt et al. 2003, Cheng and Detrich 2007), numerous Antarctic ectotherms have been shown to have a very long developmental periods from fertilization to hatching or metamorphosis (Peck 2018), including the two phyla in this study, arthropods (Bahrndorff et al. 2021, Moran et al. 2024) and molluscs (Hain and Arnaud 1992, Moran et al. 2019). At the extreme, development of one species of gastropod took more than 8 years to hatch from their egg casing (Moran et al. 2019). The two nudibranchs in this study, *Tritonia challengeriana* and *Tritoniella belli*, both hatch from egg masses as swimming/crawling larvae after > 1 year (Bryan et al. 1998, Woods and Moran 2008). Direct comparisons between the rates of Antarctic development and rates of warmer locations are difficult to interpret because developmental rates of Antarctic species were slowed beyond what would be predicted from the amount of cooling alone (Peck 2016, Peck 2018). This suggests that while the mechanisms of development of Antarctic organisms may be cold-compensated to some extent, there may be a limit to how much compensation is possible at the lowest end of the temperature range (Peck 2016).

Compared to the length of larval development, much less is known about the length of the cleavage phase in most invertebrates, particularly Antarctic taxa. To our knowledge, no previous studies have measured the rate of the early cleavages of Antarctic nudibranchs or sea spiders. Comparisons of early cleavage timing between Antarctic and warmer-water taxa are further complicated by the fact that for most non-Antarctic species, when embryos are cooled to Antarctic temperatures they cease development altogether (Lawrence and Soame 2004), supporting some degree of adaptation to cold temperatures by Antarctic taxa. The cleavage phase is controlled by the maternal complement, not the embryonic or larval genome (Baroux et al. 2008, Liu et al. 2014), and so may be operating under different sets of rules and constraints than later stages. However, if we assume that the cleavage phase is affected in the same way by temperature as larval development, then comparative studies can shed some light. Peck (2016, Figure 1) showed that the natural log (of the developmental rates of the Antarctic gastropods ranged from -2.0 to -2.6 (y axis on Arrhenius plot); these numbers were lower than the predicted values for Antarctic temperatures from a regression of natural log developmental rate vs. natural log temperature for a wide range of urchins from different latitudes, suggesting cold-driven rate limitation. When we transformed the cleavage rates of embryos from our four species, all of them, regardless of the experimental temperature, had even slower rates (natural log Developmental rate < 3.4, see Figure 1&2) than the Antarctic species in Peck's data set; around -1.8 C, rates were even lower. Thus, early cleavage of our Antarctic taxa appears to be very slow indeed, but further study is needed to determine how temperature stress in early development persists through adulthood.

### **Temperature Sensitivity of Cleavage Rate**

As warming of the Southern Ocean continues, understanding the effect of temperature on the essential and ubiquitous cleavage phase will shed light on how early life history stages of Antarctic organisms will likely be affected by global climate change. In our experiments, thermal sensitivity of cleavage rate was overall very high. Our data showed  $Q_{10}$ s and activation energies that were generally well above (and sometimes an order of magnitude higher than) the range of 2-4 ( $Q_{10}$ ) and activation energy (57.9-67.5 kJ/mol) exhibited by most biological processes (Hochachka & Somero 2002, Delong et al. 2016, Flynn and Todgham 2017). Some

studies suggest that even narrower ranges of  $Q_{10}$  values of 1-2 are common for organisms that are adapted to their environmental thermal regimes (Johansen et al. 2024), further highlighting the high overall thermal sensitivity of our Antarctic taxa. In addition to high overall thermal sensitivity, we also found that thermal sensitivity changed across the temperature range for 12/17 sets of embryos. For three sets of embryos (Tb 3, Tc 1, Tc 2), as described above, embryos either failed to cleave (leading to a developmental rate of zero) or could not cleave past the two-cell stage at the highest end of the temperature range, leading to a very steep decline in developmental rate with increasing temperature. For 10/17 sets of embryos, in contrast, thermal sensitivity was considerably higher at the lower end of the temperature range, with  $Q_{10}$ s ranging from 128.4 to 6.2 below the breakpoint. Above the breakpoint,  $Q_{10}$ s were much lower and sometimes converged on the range of 2-4. Arrhenius breakpoints driven by high thermal sensitivities at both the upper and lower ends of a temperature range generally are interpreted as tipping points where membrane fluidity, protein synthesis, enzyme performance, and oxygen delivery systems begin to falter, and they are indicators of the end of the organisms' functional range (Pörtner et al. 2007, Peck 2016, Peck et al. 2024). Interestingly, in our data the low-end breakpoints, when present, occurred well within the normal temperature range that organisms experience in high Antarctic marine environments (Hunt et al. 2003, Cheng and Detrich 2007). Similar patterns have been found by Hoegh-Guldberg and Pearse (1995) for two Antarctic asteroids (*Odonstaster validus* and *O. meridionalis*), which had Arrhenius breakpoints at 0 °C with high  $Q_{10}$  values below the breakpoint ( $Q_{10}$ s 9.5 and 12.5 respectively) and low values above the breakpoint ( $Q_{10}$ s of 2.2 and 1.8 respectively). The same pattern was found in development of the echinoid *Sterechinus meumayeri* which had a breakpoint at +0.2 °C with  $Q_{10}$  values before the breakpoint of 13.6 and 1.1 after (Stanwell-Smith and Peck 1996). These data together suggest that despite millions of years of evolution in the cold (Peck 2018, Todgham and Mandic 2020), the cleavage phase of a broad taxonomic range of Antarctic marine organisms, including members of two protostome phyla (Arthropoda and Mollusca) and one deuterostome (Echinodermata), has not fully adapted to the extreme cold of high Antarctic waters. This may represent a lower limit to the ability of metazoan life to fully adapt to the lowest ocean temperatures (Pearse et al. 1991, Peck 2016). Such a limit is likely driven by fundamental constraints on production of proteins at near-freezing temperatures (Peck 2016, Peck et al. 2024)

## **Conclusion**

Development of Antarctic ectotherms is extremely slow, much slower than what is expected based off how temperature normally regulates development. Our data indicate that overall, the early developmental rate of Antarctic embryos is very thermally sensitive within the normal temperature range of the modern-day high Antarctic. This sensitivity suggests a fundamental cold limitation to biological adaptation of the processes of early cleavage that requires further study on a mechanistic level. Ecologically, this high sensitivity suggests that as temperatures and seasonality increase with climate change, the overall length of embryonic and larval periods may be greatly shortened with even small amounts of warming. If so, then this may lead to a mismatch between reproductive seasonality and environmental conditions for many Antarctic organisms, with larvae or juveniles entering their environment at an unfavorable time. We also found that even comparatively small amounts of warming may lead to developmental failure in some species, potentially creating a hard limit to the amount of ocean warming that these genotypes and species can tolerate.

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## Tables and Figures:

Temperature (°C) of each column												
Thermal Block	Column 1	Column 2	Column 3	Column 4	Column 5	Column 6	Column 7	Column 8	Column 9	Column 10	Column 11	Column 12
First Setup 2019-2020 10x3	-1.8	-1.4	-0.9	-0.3	0.3	1.0	1.7	2.0	2.6	3.3	N/A	N/A
Second Setup 2021-2022 12x9	-2.24	-1.77	-1.27	-0.85	-0.37	+0.07	+0.47	+0.89	+1.27	+1.65	+1.94	+2.26
Transformed temperature (K) (1/Tx10 <sup>-3</sup> ) of each column												
Thermal Block	Column 1	Column 2	Column 3	Column 4	Column 5	Column 6	Column 7	Column 8	Column 9	Column 10	Column 11	Column 12
First Setup 2019-2020 10x3	3.685	3.679	3.673	3.665	3.657	3.648	3.638	3.634	3.626	3.617	N/A	N/A
Second Setup 2021-2022 12x9	3.691	3.685	3.678	3.672	3.666	3.660	3.655	3.649	3.644	3.639	3.635	3.631

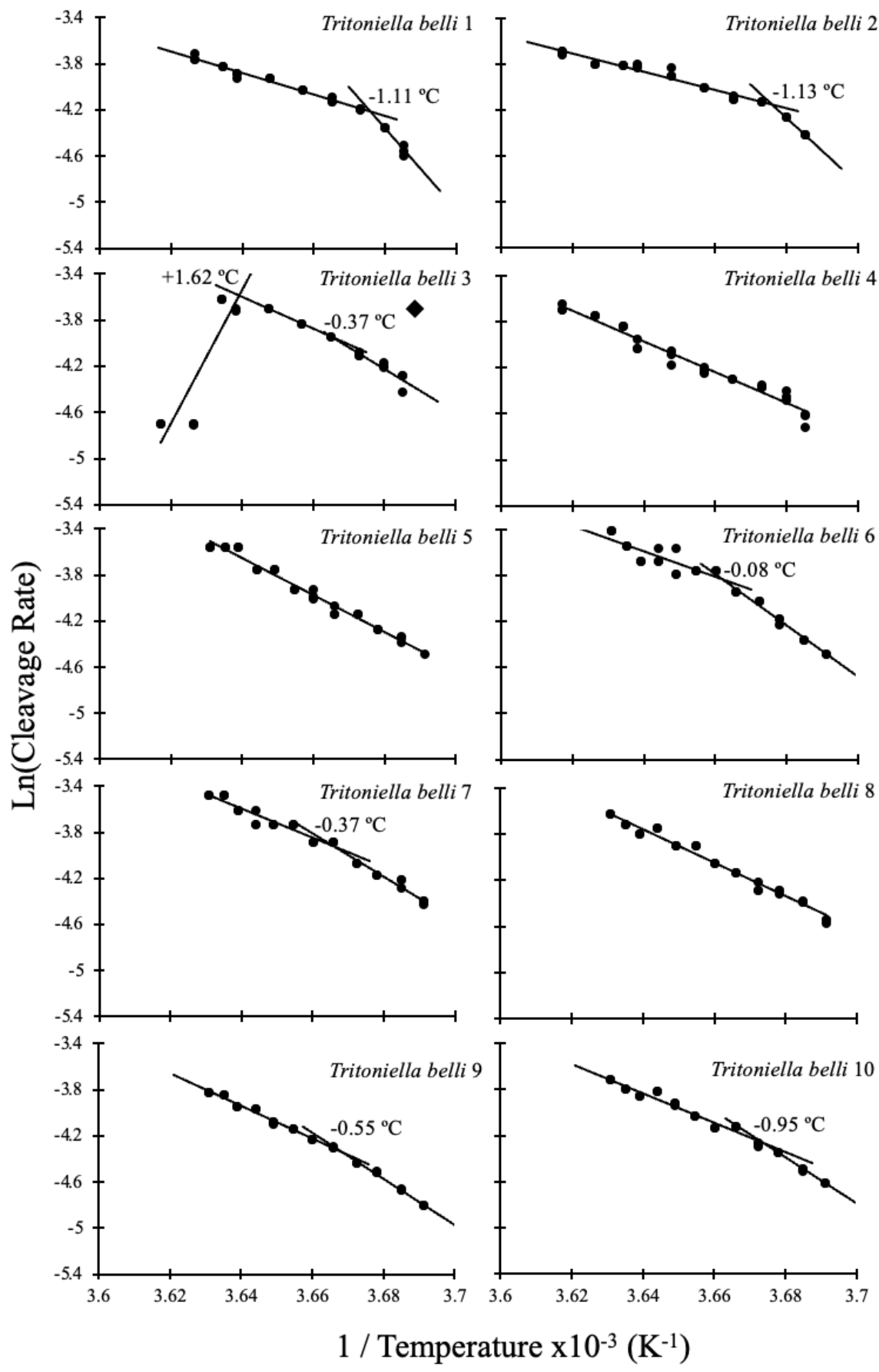
**Table 1. Thermal Block Temperatures.** Temperature of each column within the thermal gradient in both Celsius and transformed (1/Tx10<sup>-3</sup>) in Kelvin of both thermal blocks from both seasons. The first setup housed 10 columns of vials with three replicates at each temperature. The second setup housed 12 columns of vials with nine replicates at each temperature.

Time to 32-Cell (Hours)															
Species	ID	Date Collected	Starting Stage	-1.8 °C	-1.4 °C	-0.9 °C	-0.3 °C	0.3 °C	1.0 °C	1.7 °C	2.0 °C	2.6 °C	3.3 °C		
				<i>Tritoniella belli</i>	Tb 1	Tb 10/11/2019	1-Cell	>361	>361	327	304	280	267	243	243
<i>Tritoniella belli</i>	Tb 2	Tb 10/22/2019	1-Cell	>339	>339	339	315	289	265	241	241	241	217		
<i>Tritoniella belli</i>	Tb 3 *	Tb 12/6/2019	1-Cell	361	337	311	265	238	218	218	187	N/A	N/A		
<i>Tritoniella belli</i>	Tb 4	Tb 12/27/2019	1-Cell	528	462	413	389	347	293	293	245	224	204		
<i>Ammothea glacialis</i>	Ag 1	Ag 11/20/2019	1-Cell	821 (16 cells)	796	657	565	537	537	462	462	393	393		
Species	ID	Date Collected	Starting Stage	-2.24 °C	-1.77 °C	-1.27 °C	-0.85 °C	-0.37 °C	0.07 °C	0.47 °C	0.89 °C	1.27 °C	1.65 °C	1.94 °C	2.26 °C
<i>Tritoniella belli</i>	Tb 5	Tb 10/20/2021	2-Cell	335	289	289	236	236	203	185	167	167	143	143	143
<i>Tritoniella belli</i>	Tb 6	Tb 10/21/2021	2-Cell	290	264	236	204	190	169	169	144	144	144	114	114
<i>Tritoniella belli</i>	Tb 7	Tb 11/5/2021	2-Cell	262	217	217	185	167	167	142	142	142	115	115	115
<i>Tritoniella belli</i>	Tb 8	Tb 12/2/21a	1-Cell	415	370	337	312	271	250	223	223	198	198	174	174
<i>Tritoniella belli</i>	Tb 9	Tb 12/2/21b	1-Cell	602	550	463	414	394	369	336	312	271	271	248	248
<i>Tritoniella belli</i>	Tb 10	Tb 12/2/21c	1-Cell	515	448	400	351	327	298	282	259	236	236	215	215
<i>Tritonia challengeriana</i>	Tc 1 *	Tc 10/21/21	1-Cell	644	604	557	527	278	252	252	223	202	N/A	N/A	N/A
<i>Tritonia challengeriana</i>	Te 2 *	Tc 01/11/22	1-Cell	457	385	335	309	273	244	244	222	196	N/A	N/A	N/A
<i>Nymphon australe</i>	Na 1	Na 11/30/21a	1-Cell	758	665	613	543	501	453	432	408	375	350	327	327
<i>Nymphon australe</i>	Na 2	Na 11/30/21b	1-Cell	782	680	613	569	518	477	453	408	408	375	350	350
<i>Nymphon australe</i>	Na 3	Na 12/01/21	1-Cell	756	663	611	541	500	452	431	407	374	350	326	326
<i>Nymphon australe</i>	Na 4	Na 01/19/22	1-Cell	N/A	600	530	480	407	358	358	336	314	289	266	N/A

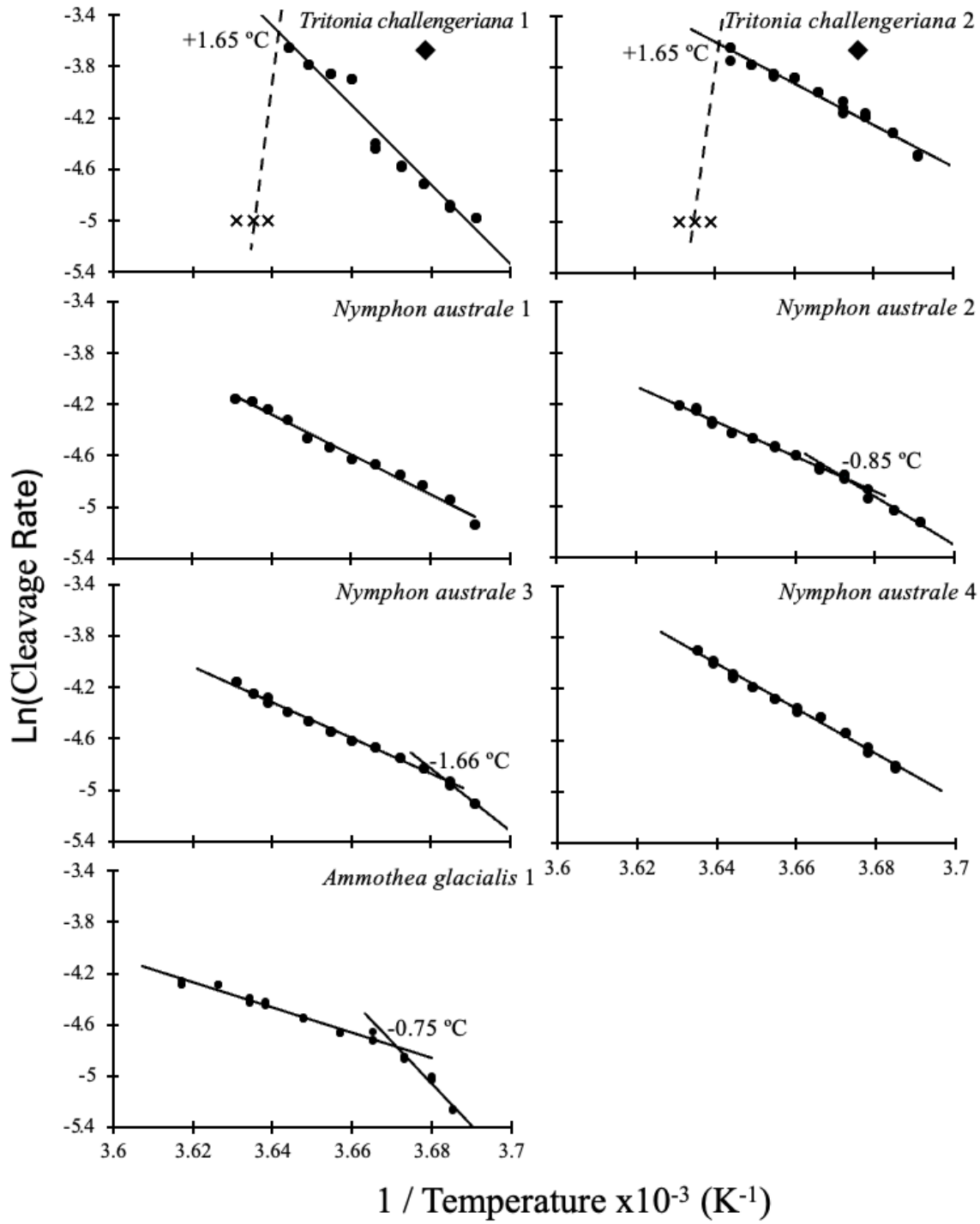
**Table 2. Time to 32-cell.** Dates (month/day/year), starting stages, experimental temperatures, and time to 32-cells stage (hours) for each set of embryos of four Antarctic species at a given temperature. An entry of N/A means that embryos did not reach that stage at the given temperature during the timeframe of the experiment. (\*) indicates treatments in which development slowed or failed at the higher end of the temperature treatment

Species	ID	Arrhenius Breakpoint Temperature (°C)	Q <sub>10</sub> Before Breakpoint	Q <sub>10</sub> After Breakpoint	E <sub>a</sub> Before Breakpoint (kJ/mol)	E <sub>a</sub> After Breakpoint (kJ/mol)	Slope Before Breakpoint	Slope After Breakpoint	Intercept Before Breakpoint	Intercept After Breakpoint
<i>Tritoniella belli</i>	Tb 1	-1.11	128.4	3.6	297.7	77.8	-35.8	-9.4	127.4	30.2
<i>Tritoniella belli</i>	Tb 2	-1.13	51.1	2.7	241.2	66.5	-29.0	-8	102.5	25.3
<i>Tritoniella belli</i>	Tb 3 *	-0.37, 1.62	12.5	6.5 , Dead	154.6	115.1, Dead	-18.6	-13.8, 57.5	64.2	46.8, -212.8
<i>Tritoniella belli</i>	Tb 4	N/A	6.2	N/A	111.5	N/A	-13.4	N/A	44.9	N/A
<i>Tritoniella belli</i>	Tb 5	N/A	8.9	N/A	134.1	N/A	-16.1	N/A	55.0	N/A
<i>Tritoniella belli</i>	Tb 6	-0.08	21.4	4.4	187.8	90.3	-22.6	-10.9	78.9	35.9
<i>Tritoniella belli</i>	Tb 7	-0.37	12.9	5.6	156.7	105.5	-18.9	-12.7	65.2	42.6
<i>Tritoniella belli</i>	Tb 8	N/A	7.3	N/A	121.6	N/A	-14.6	N/A	49.5	N/A
<i>Tritoniella belli</i>	Tb 9	-0.55	14.7	6.9	164.6	118.0	-19.8	-14.2	68.3	47.7
<i>Tritoniella belli</i>	Tb 10	-0.95	15.1	5.7	166.3	106.9	-20.0	-12.9	69.2	42.9
<i>Tritonia challengeriana</i>	Tc 1 *	1.65	66.4	Dead	257.3	Dead	-30.9	Dead	109.1	Dead
<i>Tritonia challengeriana</i>	Tc 2 *	1.65	9.0	Dead	134.8	Dead	-16.2	Dead	55.4	Dead
<i>Nymphon australe</i>	Na 1	N/A	8.3	N/A	129.7	N/A	-15.6	N/A	52.5	N/A
<i>Nymphon australe</i>	Na 2	-0.85	13.4	6.3	159.0	112.5	-19.1	-13.5	65.5	44.9
<i>Nymphon australe</i>	Na 3	-1.66	26.3	6.5	200.4	114.9	-24.1	-13.8	83.9	46.0
<i>Nymphon australe</i>	Na 4	N/A	10.6	N/A	144.8	N/A	-17.4	N/A	59.4	N/A
<i>Ammothea glacialis</i>	Ag 1	-0.75	83.0	3.8	270.9	82.1	-32.9	-9.9	114.8	31.5

**Table 3. Arrhenius Breakpoint and Q<sub>10</sub> values.** Arrhenius breakpoint temperatures and Q<sub>10</sub> values before and after breakpoint showing change in thermal sensitivity. Activation energy (E<sub>a</sub>) and change in slope and intercept used to calculate Q<sub>10</sub> values also reported. (\*) indicates treatments in which development slowed or failed at the higher end of the temperature treatment.



**Figure 1. Arrhenius Plots of *Tritoniella belli*.** Arrhenius plots linearizing the effect of temperature on cleavage rate (cleavages h<sup>-1</sup>) of ten egg masses of *Tritoniella belli*. Significant breakpoints were detected in Tb 1, Tb 2, Tb 3, Tb 6, Tb 7, Tb 9, and Tb 10 whereas Tb 4, Tb 5, and Tb 8 exhibit no breakpoint. Temperature at which breakpoint occurred is in degrees Celsius. Axes are transformed to standard metrics of 1/Tx10<sup>-3</sup> in order to calculate Q<sub>10</sub> and Activation Energy E<sub>a</sub>. (♦) indicates embryos for which development slowed or failed at the higher end of the temperature treatment.



**Figure 2. Arrhenius Plots of *Tritonia challengeriana*, *Nymphon australe*, and *Ammothea glacialis*.** Arrhenius plots linearizing the effect of temperature on cleavage rate (cleavages h<sup>-1</sup>) for two egg masses of *Tritonia challengeriana*, four egg masses of *N. australe* and one egg mass of *A. glacialis*. Breakpoints present in Tc 1, Tc 2, Na 2, Na 3, Ag 1 whereas Na 1, and Na 4 exhibit no breakpoint. Temperature at which breakpoint occurred is in degrees Celsius. Axes are transformed to standard metrics of 1/Tx10<sup>-3</sup> in order to calculate Q<sub>10</sub> and Activation Energy E<sub>a</sub>. (◆) indicates treatments in which development slowed or failed at the higher end of the temperature treatment. To visually show the breakpoints (dashed line) as no development was observed (so therefore no data), artificial data points (X's) were added to both Tc 1 and Tc 2.

## CHAPTER 3: EFFECTS OF TEMPERATURE ON THE METABOLISM OF EARLY LIFE HISTORY STAGES OF ANTARCTIC INVERTEBRATES

### Abstract

Since the formation of the Southern Ocean roughly thirty million years ago, the waters surrounding Antarctica have maintained cold and stable temperatures. Temperatures in McMurdo Sound are stable for most of the year at  $-1.8\text{ }^{\circ}\text{C}$ , with temporary summer increases to  $\sim+1.0\text{ }^{\circ}\text{C}$ . Though millions of years of evolution, many ectotherms have adapted to live within this cold and narrow temperature range. Much concern has been raised around the sensitivity of Antarctic invertebrates and their larvae to warming oceans. We investigated the thermal sensitivity of metabolism of embryos and larvae of four species of Antarctic marine invertebrates (two pycnogonids and two nudibranchs) across three developmental stages. Animals were collected by SCUBA divers from the Ross Sea surrounding McMurdo Station, Antarctica. Oxygen consumption was measured at four temperatures ( $-1.8, -0.4, +1.0, +4.0\text{ }^{\circ}\text{C}$ ), which encompass seasonal changes and predicted warming patterns. Overall, thermal sensitivities were high compared to temperate taxa, but embryos and larvae from all four species were able to sustain metabolism at the highest temperature ( $+4.0\text{ }^{\circ}\text{C}$ ) for many hours. *Tritonia challengeriana* showed the highest thermal sensitivity of all four species, with  $Q_{10}$  values for all stages exceeding the expected range of 2-4 exhibited by most biological systems. Overall, no one developmental stage appeared more sensitive to temperature than the others for any of the species.

## Introduction

As environmental temperature changes, the body temperature of ectothermic organisms follows; thus, "...changing temperature literally changes the pace of life" (Zuo et al. 2012). These changes come about because at the organismal level, temperature impacts many physiological processes (Hochachka and Somero 2002, Fields and Houseman 2004, Schram et al. 2014, Schulte 2015). At the population level, through its effects on organismal performance, temperature is one of the main factors influencing the distribution and abundance of organisms (Thorson 1950, Hofmann and Todgham 2010, Pecorino et al. 2013). Organisms live within a range of temperatures called a thermal window, defined as the range between the maximum and minimum temperatures an organism can tolerate (Sinclair et al. 2016, Flynn and Todgham 2018). Above its thermal limits, an organism's performance begins to falter as proteins begin to denature and oxygen delivery systems fail (Pörtner and Farrell 2008, Miller and Stillman 2012, Ángeles-González et al. 2020). Below thermal limits, cold temperatures slow biological processes, as increases in cellular viscosity increase the difficulty of synthesizing and folding of proteins (Tsai et al. 2002, Peck 2016, Peck 2018). Thermal windows have evolved to be as narrow as possible to minimize maintenance costs (Stillman 2006, Pörtner and Farrell 2008) and, therefore, differ between organisms living in differing regions or environments (Pörtner et al. 2006, Pörtner and Farrell 2008, Tattersall et al. 2012). Thermal windows are generally widest in temperate areas (where temperature is more variable) and narrower in the tropics and polar regions, which have less variable temperatures (Deutsch et al. 2008, Pörtner and Farrell 2008, Tattersall et al. 2012, Rezende and Bozinovic 2019).

One commonly studied metric for assessing the physiological impact of temperature on organisms is metabolism (Peck 1989, Marsh and Manahan 1999, Woods and Moran 2008, Zuo et al. 2012, Deutsch et al. 2015, Schulte 2015). Metabolism is defined as the sum of the biochemical processes within each cell that provide the organism with energy for basic functions (Clarke and Fraser 2004, Sánchez López de Nava and Raja 2022). When temperature increases, the rate at which the biochemical processes that underlie an organism's metabolism proceed also increases, raising energy demand (Hochachka and Somero 2002, Clarke and Fraser 2004, Schulte 2015). When temperatures exceed the functional range, energy must be allocated to cellular stress response mechanisms and thus be diverted from other critical processes such as growth

and reproduction (Schneider 2004, Sokolova et al. 2012, Todgham and Mandic 2020). Organisms compensate for these changes in energy demand through acclimation (occurs in the lab) or acclimatization (occurs in situ) of metabolic rate (Sokolova et al. 2012, Todgham and Mandic 2020), which decreases the energetic demand of metabolism and allows for energy demand to be met by energy supply at the higher temperature (Staples and Buck 2009, Sokolova et al. 2012, Deutsch et al. 2015, Todgham and Mandic 2020). If acclimation is not an option and the required demand for energy outpaces the energy supply, key cellular processes such as protein synthesis and ion homeostasis will fail, resulting in the death of the organism (Staples and Buck 2009, Sokolova et al. 2012). Maintaining energy homeostasis where energy supply meets energy demand is crucial for organisms to elicit a stress response while still maintaining other crucial biological functions (Sokolova et al. 2012, Todgham and Mandic 2020).

Within an organism's thermal window, and in the absence of acclimation, the changes in the rate of processes such as metabolism generally follow the Boltzmann-Arrhenius equation (Arrhenius 1915, Hoegh-Guldberg and Pearse 1995, Schute 2015), which posits that the underlying kinematics behind all biological processes are impacted by temperature in a similar way. However, it is not fully understood if these relationships are always linear or differ between processes at different levels of organization (Schute 2015). One metric of thermal sensitivity is the unitless metric  $Q_{10}$  (Hoegh-Guldberg and Pearse 1995, Crapse et al. 2021), which can be calculated using the Boltzmann-Arrhenius equation and is equal to the degree of change in any rate process standardized to a 10 °C change in temperature (Hirche 1984, Hochachka 1991, Manogaran et al. 2019). The range of  $Q_{10}$  values exhibited by most biological processes is 2-4, meaning for every 10 °C increase in temperature, rate processes are 2-4 times higher (Hochachka & Somero 2002, DeLong et al. 2016, Flynn and Todgham 2017).  $Q_{10}$ s can be much higher than the 2-4 range when temperatures are above or below an organism's functional range (Molnár et al. 2017, Crapse et al. 2021), however we lack a full mechanistic understanding of what process is causing high sensitivity as  $Q_{10}$  represent the effects of temperature on a complex suite of processes (Somero 2012, Schute 2015). In some systems  $Q_{10}$  values range from 1-2 with values exceeding 2 indicating high thermal sensitivity (Johansen et al. 2024). These high  $Q_{10}$  values at increasing temperature are often attributed to increased vulnerability because at higher

temperatures increases in oxygen demand cannot always be met by increases in oxygen supply (Schute 2015, Leiva et al. 2018), processes such as proton leaks lead to inefficient energy production (Iftikar et al. 2014, Schute 2015), proteins denature (Peck 2018), and energy may be allocated away from vital processes towards stress responses (Schneider 2004, Sokolova et al. 2012, Todgham and Mandic 2020).

Organisms with narrow thermal tolerances are thought to be among the most vulnerable to climate change (Buckley and Huey 2016, Huey et al. 2018), and some of the narrowest thermal tolerances have been measured in marine invertebrates from cold polar regions (Pörtner 2001, Pörtner 2002, Flynn and Todgham 2018). Since the formation of the Antarctic circumpolar current and the first ice sheets in the Southern Ocean ~30-34 MYA, the waters around Antarctica have been one of the coldest and most thermally stable regions on Earth (Zachos et al. 1992, Lear et al. 2000, Livermore et al. 2005, Tripathi et al. 2005). Acting as a climate buffer, the Southern Ocean stores a disproportionate amount of heat from anthropogenic warming (Antarctic Climate Change and the Environment Decadal Synopsis (ACCE)), making this region important in climate change scenarios (Peck et al. 2018, Tonelli et al. 2021). Most Antarctic marine ectotherms have evolved in near-freezing and stable temperatures for the past 4-5 million years (Pörtner et al. 2004, Peck et al. 2018); many are highly stenothermal and are therefore thought to have a limited capacity to deal with warming sea temperatures, making them potentially among the most vulnerable fauna on earth to ocean warming (Peck et al. 2014, Peck 2018, Hofmann and Todgham 2010, Bylenga et al. 2015). Sea surface temperatures in the Southern Ocean are predicted to rise between 0.5 °C and 1.75 °C by 2100 (ACCE), and this rise may profoundly impact the diverse and highly endemic fauna of the Southern Ocean.

Stenothermality has been demonstrated in a number of Antarctic invertebrate taxa, and in several ways. As some examples: (1) adults of many invertebrate species in the Antarctic are unable to survive even for short periods at elevated temperatures (Peck 1989, Pörtner et al. 1999, Peck et al. 2010, Peck 2018); (2) growth rates of the scallop *Adamussium colbecki* increased with a  $Q_{10}$  of 71 over only a +3.0 °C increase in temperature; (3) protein synthesis rates of adults of the limpet *Nacella concinna* were negatively impacted by temperatures above +1.0 °C; and (4) righting performance had a two-fold drop off for adult sea spiders (*Ammonothea glacialis*) between

-1.8 °C and +4.0 °C (Shishido et al. 2019). These studies focused on adults, but most metazoan organisms in the Antarctic and elsewhere have complex life cycles, and understanding the effects of stressors on early life stages is crucial for determining a species' resilience in the face of a changing environment (Kingsolver et al. 2011, Llopiz et al. 2014, Flynn and Todgham 2018, Todgham and Mandic 2020, Earhart et al. 2022). Early stages of development are more vulnerable to temperature stress than adults in a wide variety of invertebrates from molluscs (Brown et al. 2004, Verween et al. 2007) to echinoderms (Hoegh-Guldberg and Pearse 1995) and cnidarians (Randall and Szmant 2009), likely because these stages generally lack complex adult mechanisms for coping with thermal stress (Rombough 1997, Brown et al. 2004, Pörtner and Farrell 2008; Flynn and Todgham 2017).

Only a handful of studies have examined the thermal stress of embryonic or larval Antarctic invertebrates. Early life history stages are of particular interest in the Antarctic not only because of the high overall vulnerability of the marine ectothermic fauna to rising temperatures (Gutt et al. 2021), but also because a high proportion of invertebrates have lecithotrophic development (Peck 2018), meaning development is fueled by maternal provisioning of nutrients (Stitzel and Seydoux 2007, Liu et al. 2014). For lecithotrophic taxa, which cannot offset disruptions to energy homeostasis by feeding, if temperature-driven increases in embryonic and larval metabolism lead to an energy deficit then energy must be allocated away from key cellular processes and may lead to potential developmental failures if energetic equilibrium is not established (Hoegh-Guldberg and Emlet 1997, Staples and Buck 2009, Sokolova et al. 2012). Of the studies that have examined thermal sensitivity in early stages of Antarctic invertebrates, most have focused on a small number of experimentally tractable, planktotrophic echinoderm species. For example, Hoegh-Guldberg and Pearse (1995) showed that developmental rates of larvae of the sea stars *Odontaster validus* and *O. meridionalis* were very thermally sensitive below 0 °C but not above, and Stanwell-Smith and Peck (1998) showed that the developmental rate of larvae of the urchin *Sterechinus neumayeri* was very thermally sensitive below 0 °C but became less thermally sensitive above 0 °C. In a rare non-echinoderm example, Woods and Moran (2008) found a large effect of temperature on the metabolism of embryos of the nudibranch mollusc *Tritonia challengeriana*, with  $Q_{10}$ s ranging from 9.6 to 30 depending on the stage, and Lobert et

al. (chapter 1, MS in prep) showed that early cleavage of the same species failed above +1.65 °C. Embryos of the congener *Tritonia diomedea* from Puget Sound, Washington, showed much less thermal sensitivity with  $Q_{10}$  of oxygen consumption ranging from 2.1 to 2.6 depending on stage (Moran and Woods 2007), highlighting the high thermal sensitivity of Antarctic species.

Here, we examined the impacts of temperature on the oxygen consumption rate (as a proxy for metabolism) of embryos and larvae of four lecithotrophic Antarctic invertebrate species from two different phyla. For each species, we measured oxygen consumption at three different developmental stages and four different temperatures ranging from ambient (-1.8) to +4.0 °C, encompassing predictions for 2100 (Tonelli et al. 2021). Our goals were to (1) assess whether embryonic and larval metabolism displayed high thermal sensitivity of metabolism, which could potentially lead to energy imbalances and potential developmental failure (Hoegh-Guldberg and Emlet 1997, Sokolova et al. 2012); and to (2) determine whether thermal sensitivity changed as embryos and larvae became older and more complex. Understanding the effects of warming temperatures across the entire life cycle will be key to identifying ‘bottleneck’ stages in the life cycle how Antarctic marine ectotherms may respond to changing ocean climates (Poloczanska et al. 2016, Peck 2018, Morley et al. 2019).

## Methods

### Study Organisms

Four species of Antarctic benthic invertebrates were used in this study: two sea spiders (phylum Arthropoda, class Pycnogonida, order Pantopoda), *Nymphon australe* Hodgson 1902 and *Ammothea glacialis* (Hodgson 1907), and two nudibranchs, *Tritoniella belli* Eliot 1907 and *Tritonia challengeriana* Bergh 1884 (phylum Mollusca, class Gastropoda, order Nudibranchia). Like many other sea spiders, males of *N. australe* and *A. glacialis* carry their young on the ovigerous appendages (Mahon et al. 2008, Cano and López-González 2009, Fornshell 2019), where fertilized eggs develop through hatching and several larval stages. Both species of sea slug, *Tritoniella belli* and *Tritonia challengeriana*, produce benthic, gelatinous egg masses that contain numerous embryos (>1000) (McClintock and Baker 1997, Bryan et al. 1998, Moran and

Woods 2007) that hatch from the egg mass as swimming-crawling larvae close to metamorphosis (Woods and Moran 2008). All four species are widely distributed throughout the Antarctic (*N. australe*: Mahon et al. 2008, Zehnpfennig et al. 2024; *A. glacialis*: Cano and López González 2009; *Tritonia challengeriana*: Wägele 1995, Rossi et al. 2019; *Tritoniella belli*: Barnes & Bullough, 1996, McClintock and Baker 1997, Bryan et al. 1998) and are commonly found around our study area, McMurdo Sound. Evidence suggests that populations of *N. australe*, *Tritoniella belli*, and *Tritonia challengeriana* around the Antarctic may be cryptic species complexes (*N. australe*: Arango et al. 2011, Brenneis et al. 2017, Collins et al. 2018, *Tritonia challengeriana* and *Tritoniella belli*: Moles et al. 2021), however only one lineage was found in McMurdo Sound suggesting that the individuals within our study are most likely monospecific (as shown in Collins et al. 2018 and Moles et al. 2021)

Three discrete larval stages were used for the two pycnogonid species. Both species brood offspring on their ovigerous legs through an embryonic stage enclosed in an envelope and three subsequent hatched instar stages (*N. australe*, Brenneis et al. 2017, Cano-Sánchez et al. 2020; *A. glacialis*, Cano-Sánchez and López-González 2009, Brenneis et al. 2017). For both species, we measured the oxygen consumption of the three brooded post-hatching stages. Although it differs somewhat from the naming in previous literature, for ease of understanding in this paper, we call them N-I, N-II, and N-III, and A-I, A-II, and A-III.

The two nudibranchs progress through typical gastropod developmental stages in the egg mass, including the formation of a ciliated, shelled veliger. Development is gradual and not punctuated by molts, so *Tritoniella belli* and *Tritonia challengeriana* embryos were visually placed into one of three stages using the criteria of Woods and Moran (2008) and Moran and Woods (2010): early veliger (EV), no shell, poorly defined velar lobes and visceral mass no clear delineation of the foot; mid veliger (MV), shell apparent, with distinct and ciliated velar lobes and foot, but no eyespots or operculum; and late veliger (LV), clear, coiling shell with visible eyespots and foot that could withdraw into its shell, and an operculum.

### **Collection and Handling**

Adult sea spiders of both species bearing larval clutches and egg masses of both nudibranchs were collected by divers on SCUBA from McMurdo Sound during the 2019-2020 and 2021-

2022 field seasons. Clutches of *N. australe* and *A. glacialis* were removed from the ovigers of adults using fine forceps, and larvae were gently separated from the mass. Embryos of *Tritoniella belli* and *Tritonia challengeriana* were removed from the egg mass tubes by gently tearing the gel sleeve surrounding the embryos with fine forceps until the embryos fell freely out of the egg mass (see Moran and Woods 2007). We then rinsed larvae and embryos with 0.2- $\mu\text{m}$  freshly filtered seawater to remove particulates. Larvae and embryos were kept chilled at  $-1.8\text{ }^{\circ}\text{C}$  during handling by using saltwater ice baths and, during microscopic examination, a digital chilling plate (IC20, Torrey Pines, Carlsbad, US).

Single egg masses of *N. australe* and *A. glacialis* did not contain enough larvae for these experiments since multiple larvae were needed for measurements of oxygen consumption. Therefore, to incorporate some degree of biological replication into our measurements of metabolism, we assembled separate experimental cohorts of larvae from different sets of parents (2-13 cohorts, depending on stage and species). Each cohort consisted of  $\sim 90$  larvae from brood masses of three to four fathers; no paternal brood was used in more than one cohort. Single egg masses of *Tritoniella belli* and *Tritonia challengeriana* contained thousands of embryos, so each experimental cohort ( $\sim 1100$  larvae in each of 1-4 cohorts, depending on stage and species) consisted of embryos from one egg mass. The total number of cohorts for each stage and species is shown in Table 1. Prior to experiments, larvae and embryos were kept in the laboratory for no longer than five days (in order to reach required number of larvae/embryos for the experiment) and were stored in flow-through sea tables with temperatures between  $-1.57\text{ }^{\circ}\text{C}$  and  $+0.56\text{ }^{\circ}\text{C}$  (average:  $-1.07\text{ }^{\circ}\text{C} \pm 0.36$  (SE)  $^{\circ}\text{C}$ ). Larvae were not fed as they rely on maternally supplied yolk reserves to fuel development. Stage N-III larvae of *N. australe* were comparatively rare, and we were unable to collect enough individuals during the 2021-2022 field season to measure the oxygen consumption at  $-0.4\text{ }^{\circ}\text{C}$ . Therefore, oxygen consumption data for Stage N-III larvae of *N. australe* are only from the 2019-2020 field season.

### **Oxygen consumption**

For all species, cohorts, and stages, oxygen consumption (as a proxy for metabolic rate) was measured using the end-point determination  $\mu\text{BOD}$  method of Marsh and Manahan (1999) as

modified by Moran and Woods (2007), Woods and Moran (2008), and Whitehill and Moran (2012). In short, larvae or embryos were placed into small (514 to 783  $\mu\text{L}$ ) glass respiration vials of known volume containing 0.2- $\mu\text{m}$  freshly-filtered seawater that was air-saturated with oxygen at  $-1.8\text{ }^{\circ}\text{C}$ . A range of either 2-24 (*N. australe* and *A. glacialis*) or 50-300 (*Tritoniella belli* and *Tritonia challengeriana*) larvae/embryos were placed into each of six respiration vials. Vials were capped and incubated horizontally at each of the four temperature treatments (starting with  $-1.8$ , followed by  $-0.4$ ,  $+1.0$ , and  $+4.0\text{ }^{\circ}\text{C}$  with a 24 h rest period at  $-1.8\text{ }^{\circ}\text{C}$  between each temperature treatment) for four to ten h depending on temperature, species, and stage, timed so that oxygen levels in the vials never dropped below 80% of air saturation. During incubation, vials were inverted every two h to minimize any oxygen concentration gradients.

At the end of incubations, vials were cooled to  $-1.8\text{ }^{\circ}\text{C}$  for 30 seconds (as  $-1.8\text{ }^{\circ}\text{C}$  was the calibrated temperature for the electrode), and  $\sim 400\text{ }\mu\text{L}$  of water from each vial was removed using a temperature-equilibrated gas-tight syringe and injected into a temperature-equilibrated water-jacketed chamber (MC-100 Strathkelvin, Glasgow, UK) and read using a Clark-style oxygen electrode (Strathkelvin Model 1302) and oxygen meter (Strathkelvin Model 782). We calculated the per-embryo or per-larval oxygen consumption rate of each vial as follows. First, for each cohort at each temperature, we calculated the slope of the regression of total oxygen consumption of each vial plotted against the number of embryos in the same vial (Marsh and Manahan 1999, Moran and Woods 2007, Woods and Moran 2008). This method is robust to the common issue of non-zero y-intercepts (Marsh and Manahan 1999) but limits the power available for making comparisons between temperatures, stages, and treatments by removing information from individual vials. Therefore, following the methods of Walther et al. (2013) and Crickenberger et al. (2015), we analyzed differences between species and treatments using the per-individual oxygen consumption rates for each vial, after correcting the oxygen consumption rates for individual vials from each run by the Y-intercept for that regression.

To assess the sensitivity of oxygen consumption in response to increases in temperature, we calculated the  $Q_{10}$  across the whole temperature range (between  $-1.8$  and  $+4.0\text{ }^{\circ}\text{C}$ ) and between temperature treatments (i.e.,  $-1.8$  to  $-0.4\text{ }^{\circ}\text{C}$ , and  $-0.4$  to  $+1.0\text{ }^{\circ}\text{C}$ ).  $Q_{10}$  was calculated using the

equation  $Q_{10} = \frac{R_2^{10/(T_2-T_1)}}{R_1}$  where R is the oxygen consumption rate at given temperature T at -1.8 °C ( $R_1$  and  $T_1$ ) and +4.0 °C ( $R_2$  and  $T_2$ ). Given that the model estimated means of oxygen consumption are used to calculate  $Q_{10}$ , and contain error (and therefore error around  $Q_{10}$ ), for the purpose of this manuscript, we are reporting high thermal sensitivity as any  $Q_{10}$  higher than 4 (Peck 2018).

## Statistical Analysis

All statistical analyses were performed in the R Studio statistical package (Version 2024.04.1+748, Posit Software, PBC). Changes in oxygen consumption between temperature treatments and between stages were analyzed using one-way ANOVAs for each species separately. We assessed the effect of temperature and stage on oxygen consumption using a linear mixed effects model with the per-individual oxygen consumption rate of each vial as the dependent variable, temperature (-1.8, -0.4, +1.0, +4.0 °C), and stage (EV, MV, LV or Instar stage N-I/A-I, N-II/A-II, and N-III/A-III) as independent fixed factors, and cohort as a random factor. Models included an interaction term for temperature x stage. Prior to analysis, data were log-transformed to fit the assumption of normality. Pairwise comparisons between stages of a single species were conducted using Tukey's post hoc tests.

## Results

### *Nymphon australe*

In the overall model, temperature had a significant effect on oxygen consumption ( $F_{3, 391.34} = 141.6$ ,  $p < 0.0001$ ) (Table 2), driven by a positive relationship between the two (Fig. 1). Larval stage also had a significant overall effect on oxygen consumption rate ( $F_{2, 20.72} = 52.4$ ,  $p < 0.0001$ ), with N-III larvae having the highest oxygen consumption and N-I larvae having the lowest. The interaction between temperature and stage was also significant ( $F_{5, 394.31} = 6.9$ ,  $p < 0.0001$ ).

When all pairwise comparisons were performed, there were significant differences in oxygen consumption rates between all larval stages at each temperature except between N-I and N-II at -1.8 °C (Table 3). Post hoc tests within stage showed that for N-I larvae, oxygen consumption rose significantly between +1.0 and +4.0 °C ( $p < 0.0001$ ) but not between -1.8 and -0.4 °C ( $p = 0.9994$ ) or -0.4 and +1.0 °C ( $p = 0.3081$ ). Across the whole temperature range (-1.8 to +4.0 °C), oxygen consumption rose significantly for N-I larvae ( $p < 0.0001$ ).  $Q_{10}$  of the average oxygen consumption rate for N-I larvae across the whole temperature range was 4.6 (Table 4). For N-II larvae, oxygen consumption rose significantly between -1.8 and -0.4 °C ( $p < 0.0001$ ), -0.4 and +1.0 °C ( $p < 0.0001$ ), +1.0 and +4.0 °C ( $p = 0.0006$ ) as well as across the whole temperature range ( $p < 0.0001$ ).  $Q_{10}$  for N-II larvae across the whole temperature range was 6.2. Lastly, for N-III larvae, oxygen consumption did not significantly differ from -1.8 to +1.0 °C ( $p = 0.7954$ ), +1.0 to +4.0 °C ( $p = 1.0$ ), or -1.8 to +4.0 °C ( $p = 0.8977$ ).

### *Ammothea glacialis*

Temperature had a significant effect on the overall oxygen consumption of *Ammothea glacialis* larvae ( $F_{3, 205.505} = 82.6$ ,  $p < 0.0001$ ) (Table 2), with oxygen consumption rising as temperature increased (Figure 2). The earliest-stage larvae (A-I) had the lowest metabolic rate overall, but stage did not have a significant effect on the overall oxygen consumption rate ( $F_{2, 6.985} = 1.7$ ,  $p = 0.25$ ). Lastly, there was a significant interaction between temperature and stage ( $F_{6, 205.519} = 2.5$ ,  $p = 0.02$ ).

When all pairwise comparisons were performed, there were no significant differences in oxygen consumption rates between larval stages (Table 5). Comparing temperatures within stage, for A-I, oxygen consumption rose significantly between +1.0 and +4.0 °C ( $p = 0.0049$ ) but not between -1.8 and -0.4 °C ( $p = 0.9998$ ) nor -0.4 and +1.0 °C ( $p = 0.9994$ ). Across the whole temperature range (-1.8 to +4.0 °C), oxygen consumption rose significantly for A-I larvae ( $p = 0.0030$ ).  $Q_{10}$  of the average oxygen consumption rate for A-I larvae across the whole temperature range was 2.1 (Table 4). For A-II larvae, oxygen consumption rose significantly between +1.0 and +4.0 °C ( $p < 0.0001$ ) and across the whole temperature range ( $p < 0.0001$ ), but not between -1.8 and -0.4 °C

( $p=0.2256$ ) nor  $-0.4$  and  $+1.0$  °C ( $p=1.0000$ ).  $Q_{10}$  for A-II larvae across the whole temperature range was 3.2. Lastly, for A-III larvae, oxygen consumption rose significantly between  $+1.0$  and  $+4.0$  °C ( $p<0.0001$ ) and across the whole temperature range ( $p<0.0001$ ), but not between  $-1.8$  and  $-0.4$  °C ( $p=0.9103$ ), nor between  $-0.4$  and  $+1.0$  °C ( $p=0.1913$ ).  $Q_{10}$  for A-III larvae across the whole temperature range was 4.1.

### ***Tritoniella belli***

Overall, for all three stages of embryos of *Tritoniella belli*, temperature had a significant effect on the oxygen consumption of embryos ( $F_{3, 136.858} = 113.4$ ,  $p<0.0001$ ) (Table 2), with oxygen consumption rising as temperature increased (Figure 3). Stage also had a significant effect on the overall oxygen consumption rate ( $F_{2, 3,967} = 139.6$ ,  $p=0.0002$ ), with LV embryos having the highest oxygen consumption and EV embryos having the lowest. Lastly, there was a significant interaction between temperature and stage ( $F_{6, 136.680} = 6.1$ ,  $p<0.0001$ ).

When all pairwise comparisons were performed, there were significant differences (Table 6) in oxygen consumption rates between all stages of embryos with the exception of between MV and LV at  $-0.4$  °C ( $p=0.7832$ ). For the EV stage, oxygen consumption rose significantly between  $-1.8$  and  $-0.4$  °C ( $p=0.0046$ ),  $-0.4$  and  $+1.0$  °C ( $p=0.0058$ ),  $+1.0$  and  $+4.0$  °C ( $p<0.0001$ ) as well as across the whole temperature range ( $p<0.0001$ ).  $Q_{10}$  of the average oxygen consumption rate for the whole temperature range was 6.5 for the EV stage (Table 4). For the MV stage, oxygen consumption rose significantly between  $-0.4$  and  $+1.0$  °C ( $p=0.0065$ ), but not between  $-1.8$  and  $-0.4$  °C ( $p=0.0591$ ) nor  $+1.0$  and  $+4.0$  °C ( $p=1.0000$ ). Across the whole temperature range ( $-1.8$  to  $+4.0$  °C), oxygen consumption rose significantly for MV embryos ( $p<0.0001$ ).  $Q_{10}$  for the whole temperature range for the MV stage was 3.4. Lastly, for the LV stage, oxygen consumption rose significantly from  $-0.4$  and  $+1.0$  °C ( $p<0.0001$ ) but not between  $-1.8$  and  $-0.4$  °C ( $p=0.9878$ ) nor  $+1.0$  and  $+4.0$  °C ( $p=0.4154$ ). Across the whole temperature range ( $-1.8$  to  $+4.0$  °C), oxygen consumption rose significantly for the LV stage ( $p<0.0001$ ).  $Q_{10}$  for the whole temperature range for the LV stage was 6.7.

### ***Tritonia challengeriana***

Temperature had a significant effect on the overall oxygen consumption of embryos of *Tritonia challengeriana* ( $F_{3, 205.497} = 267.8$ ,  $p < 0.0001$ ) (Table 2), with oxygen consumption rising as the temperature increased (Figure 4). Stage also had a significant effect on the overall oxygen consumption rate ( $F_{2, 7.873} = 58.1$ ,  $p < 0.0001$ ), with the LV stage having the highest oxygen consumption and the EV stage having the lowest. Lastly, there was a significant interaction between temperature and stage ( $F_{6, 205.5} = 5.2$ ,  $p < 0.0001$ ).

When all pairwise comparisons were performed, there were significant differences in oxygen consumption rates between all larval stages except for between MV and LV at  $-1.8$  °C ( $p = 0.1078$ ),  $-0.4$  °C ( $p = 0.7832$ ), and  $+4.0$  °C ( $p = 0.4837$ ) (Table 7). For the EV stage, oxygen consumption rose significantly between  $-1.8$  and  $-0.4$  °C ( $p = 0.0002$ ), and  $+1.0$  and  $+4.0$  °C ( $p < 0.0001$ ) as well as across the whole temperature range ( $p < 0.0001$ ) but not between  $-0.4$  and  $+1.0$  °C ( $p = 0.9999$ ).  $Q_{10}$  of the average oxygen consumption rate for the whole temperature range was 7.6 for the EV stage (Table 4). For the MV stage embryos, oxygen consumption rose significantly between  $-1.8$  and  $-0.4$  °C ( $p = 0.0084$ ),  $-0.4$  and  $+1.0$  °C ( $p = 0.0002$ ), and  $+1.0$  and  $+4.0$  °C ( $p < 0.0001$ ). Across the whole temperature range ( $-1.8$  to  $+4.0$  °C), oxygen consumption rose significantly for the MV stage ( $p < 0.0001$ ).  $Q_{10}$  for the whole temperature range for the MV stage was 5.6. Lastly, for LV stage embryos, oxygen consumption rose significantly from  $+1.0$  and  $+4.0$  °C ( $p < 0.0001$ ) but not between  $-1.8$  and  $-0.4$  °C ( $p = 0.9965$ ) nor  $-0.4$  and  $+1.0$  °C ( $p = 0.8418$ ). Across the whole temperature range ( $-1.8$  to  $+4.0$  °C), oxygen consumption rose significantly for the LV stage ( $p < 0.0001$ ).  $Q_{10}$  for the whole temperature range for the LV stage was 5.1.

### **Discussion**

Much concern has been raised about how the fauna of the Southern Ocean will respond to climate change; however, very little is known about how their early life history stages, which are among some of the most vulnerable points in an organism's life cycle, will be impacted by increased temperatures (Cowart et al. 2009, Peck 2018, Todgham and Mandic 2020). Current

projections suggest climate change-driven ocean warming will increase ocean temperatures between +1.4 °C and +4.4 °C (Low vs High Emissions) by 2100 (2023 IPCC report), with the Southern Ocean warming between +0.5 °C and +1.75 °C over that time (Tonelli et al. 2021). Here, we show the effect of future relevant temperature increases on the oxygen consumption rates of four Antarctic species from two different phyla. Overall, the oxygen consumption of all larval stages of all species except for N-III increased significantly between -1.8 °C and +4.0 °C. Temperature had a large impact (as assessed from  $Q_{10}$  values) on some but not all developmental stages. Early, middle, and late stages (Stage I/EV, Stage II/MV, Stage III/LV) had similar thermal sensitivities, but later developmental stages had significantly higher oxygen consumption overall for three of the four species (*N. australe*, *Tritoniella belli*, and *Tritonia challengeriana*). When thermal sensitivities are high, indicated here by high  $Q_{10}$  values, small increases in temperature push organisms outside of their functional range, decreasing energetic efficiency (Iftikar et al. 2014, Schute 2015, Flynn and Todgham 2018) and forcing energy to be allocated away from critical processes, towards cellular stress responses (Sokolova et al. 2012, Todgham and Mandic 2020). If cellular stress responses cannot keep oxygen consumption from continually rising as shown here, climate driven temperature increases may result in energy demand outpacing energy supply, resulting in the failure of cellular processes and the eventual death of the organism (Staples and Buck 2009, Sokolova et al. 2012).

### **Oxygen consumption increases with temperature**

Temperature is known to impact a variety of processes, from oxygen consumption (Newell 1969, Pörtner et al. 1999, Moran and Woods 2007) to developmental rate (Pearse 1969, Peck 2016, Lobert et al. (chapter 1, MS in prep)), Hoegh-Guldberg and Pearse 1995, Hochachka and Somero 2002). Within an organism's thermal window, oxygen consumption and most other biological processes generally increase 2-4-fold when standardized over +10 °C temperature changes ( $Q_{10}$ ) (Hochachka & Somero 2002, Delong et al. 2016, Flynn and Todgham 2017). In our study, when we measured metabolism between -1.8 °C to +4.0 °C, we found that for eight out of 12 stages from all species combined,  $Q_{10}$ s were higher than 2-4 (range 4.1 to 7.6), suggesting that metabolism was comparatively sensitive to temperature. Few other studies have examined the thermal sensitivity of metabolism in embryos or larvae of any Antarctic invertebrates; however,

our data are consistent with similar findings by Woods and Moran (2008) for *Tritonia challengeriana*, in which the metabolism of embryos of *Tritonia challengeriana* was high and was more sensitive to temperature than the metabolism of a temperate congener, *Tritonia diomedea* (Moran and Woods 2007, Woods and Moran 2008).

### **Effects of developmental stage on oxygen consumption**

As organisms progress through ontogeny, they develop more complex physiological processes (Brown et al. 2004, Pörtner and Farrell 2008) and higher cell numbers. With these increases in complexity, generally, come increases in metabolism (Marsh et al. 1999, Koch and Britton 2008). We found this pattern in three out of our four species: *N. australe* and *Tritoniella belli* and *Tritonia challengeriana*, for whom oxygen consumption was consistently and significantly lowest at the earliest stages (N-I and EV) and highest at the latest stages (N-III and LV). Oxygen consumption rates doubled to tripled between the first two stages (N-I to N-II, EV to MV), while the metabolic rates of the oldest developmental stages (N-III and LV) were four to seven times greater than the earliest stages (N-I and EV). Larvae of *N. australe* and embryos of *Tritonia challengeriana* showed the largest jump in oxygen consumption between the stages N-II to N-III and EV to LV, whereas the largest jump for *Tritoniella belli* occurred between the EV and MV stages. These increases in oxygen consumption were not driven by an increase in mass, because embryos and larvae of all the stages studied were nonfeeding (with the possible exception of N-III, see Toh et al. in prep.) and mass decreased by roughly 29-35% between N-I/A-I and N-III/A-III (Toh et al. in prep.). Instead, these increases were mostly likely driven by increases in cell and mitochondrial number, activity, and overall physiological complexity.

In contrast to the other three species, for *A. glacialis*, the earliest stages (A-I) had the lowest rates overall, the highest rates were seen in A-II, not A-III, and oxygen consumption did not differ significantly between any of the stages at any temperature. At all three stages, larvae of *A. glacialis* were less developed than *N. australe* (See Cano-Sánchez and López-González 2009, Brenneis et al. 2017, Cano-Sánchez et al. 2020 for development descriptions) and were noticeably less active than any of the other species, including the nudibranchs. The most active

stages of *N. australe* (N-III) had oxygen consumption rates 3-4 times higher than *A. glacialis* (A-III) at any given temperature. Later-stage larvae of *N. australe* possess structures indicative of feeding, such as the presence of a mouth and anus (Brenneis et al. 2017, Cano-Sánchez et al. 2020). Complex structures such as an eye tubercle and a complete through gut are lacking in *A. glacialis* until the latest larval stage (A-III), and even then, these features are not fully developed (Cano-Sánchez and López-González 2009). The overall lower metabolic rate of *A. glacialis* as compared to *N. australe* is most likely attributed to differences in the complexity of processes (as described by Brown et al. 2004, Pörtner and Farrell 2008). A mechanistic explanation behind why A-II larvae had the highest rates and not the more complex A-III is unknown, but higher variance of oxygen consumption and limited replication lowered our power to investigate the effects of temperature on this species.

### **Thermal sensitivity of larval metabolism**

As our oceans continue to warm, understanding the effect of temperature on essential biological processes such as metabolism will allow us to understand how populations of Antarctic ectotherms and their early life history stages will be impacted by climate change. Our data showed higher than typical  $Q_{10}$  values ( $Q_{10} > 4$ ) for most but not all developmental stages between the four species. Embryos of *Tritonia challengeriana* showed the highest thermal sensitivities of the species presented here, with the EV, MV, and LV stages having  $Q_{10}$ s of 7.6, 5.6, and 5.1, respectively, across the whole temperature range. These results are consistent with the high thermal sensitivity of embryos of *Tritonia challengeriana* found by Woods and Moran (2008), who found  $Q_{10}$  for the differences in metabolic rate between  $-1.5$  and  $+1.5$  °C of 18.8 (gastrula), 30 (MV), and 9.6 (LV). The early cleavage stages of *Tritonia challengeriana* have also been shown to be very thermally sensitive, failing to develop at temperatures of  $+1.65$  °C and above (Lobert et al. (chapter 1, MS in prep)). The other nudibranch, *Tritoniella belli*, showed similar high thermal sensitivities at two of the three developmental stages, with the EV and LV stages having a  $Q_{10}$  of 6.5 and 6.7, respectively. The MV stage of *Tritoniella belli*, however, had a  $Q_{10}$  of 3.4, which is within but at the upper end of the typical 2-4 of most biological processes.

Oxygen consumption of larvae of *A. glacialis* had the lowest thermal sensitivities of all four species.  $Q_{10}$  values across the thermal regime for A-I and A-II larvae were 2.1 and 3.2, respectively, only surpassing four in A-III larvae ( $Q_{10}$  of 4.1). The only other published study of sea spider metabolism comes from the temperate species *Achelia chelata* and *Achelia gracilipes* (family Ammotheidae) (Shishido et al. 2020). Adults of *Achelia chelata* showed low thermal sensitivity of oxygen consumption with  $Q_{10}$  values ranging from 2.39 to 3.95 at temperatures between +12 and +28 °C. Adults of *Achelia gracilipes*, however, showed high thermal sensitivities over the same temperature range with  $Q_{10}$  values ranging from 3.09 to 19.24, with the highest  $Q_{10}$  value (19.24) coming between +20 and +24 °C (Shishido et al. 2020). The direct comparisons that can be made between these temperate adults and larvae of Antarctic species are limited, but it appears that some species of sea spiders (*N.australe* and *Achelia gracilipes*) show higher thermal sensitivities than others (*A. glacialis*, *Achelia chelata*) and therefore may be more vulnerable to warming temperatures.

Larvae of *N. australe* showed high thermal sensitivities for the first two developmental stages with  $Q_{10}$ s of 4.6 (N-I) and 6.2 (N-II). The most active developmental stage, *N. australe* N-III, had the lowest overall thermal sensitivity ( $Q_{10}$  of 1.6), but the difficulty in obtaining this larval stage limited our replication, so we had low power to investigate the effects of temperature on this stage. While all other larval stages of *N. australe* and *A. glacialis* were removed from the ovigerous appendages of their parent, stage III larvae of *N. australe* were rarely found still attached to the egg mass. This may be due to this larval stage having a fully formed mouth, anus, and eye tubercle (See Cano-Sánchez et al. 2020) and was presumed free living away from their parent (Cano-Sánchez et al. 2020).

We did not see any consistent pattern of increasing or decreasing in thermal sensitivity between temperature treatments (i.e. -1.8 to -0.4 °C, and -0.4 to +1.0°C) (Supplemental Tables S1-S4) that would indicate specific temperatures at which underlying mechanisms are impacted as seen in other processes such as developmental rate (Hoegh-Guldberg and Pearse 1995, Crapse et al. 2021, Lobert et al. (chapter 1, MS in prep)). There was a significant interaction between stage and temperature for all species but no consistent pattern is evident to suggest that one life stage

(Early: N-I, A-I, EV, Mid: N-II, A-II, MV, or Late: N-III, A-III, LV) between the four species was impacted more by temperature than others, suggesting that bottlenecks may not occur at specific stages, but that vulnerability occurs over all of early development. Overall, the thermal sensitivities of the vital process of oxygen consumption were high, with the majority of the developmental stages examined here (8 of 12 stages) exceeding the typical values of  $Q_{10}$ .

High thermal sensitivity of metabolism could increase a larvae/embryo's vulnerability to warming oceans, for several reasons. First, organisms that cannot maintain energy balance in warming temperatures cannot allocate energy to processes such as growth and reproduction (Schneider 2004, Sokolova et al. 2012, Todgham and Mandic 2020). Second, at higher temperatures, energy production is also less efficient, as increases in temperature increase proton permeability, therefore increasing proton leak, and decreasing the efficiency of energy production (Iftikar et al. 2014, Schute 2015). Third, higher  $Q_{10}$  values at increasing temperatures may indicate an upregulation in energy requiring processes (Shishido et al. 2020) and a greater increase in metabolic cost per temperature increase (Flynn and Todgham 2018). Therefore, at warming temperatures, larvae may have an increase in energy requiring processes that may not be met as energy production is less efficient, all while energy is allocated elsewhere for stress responses. Lastly, lecithotrophic taxa such as the ones presented here, may fall out of energetic equilibrium and may inefficiently use their limited energetic reserves with limited abilities to recuperate energy through feeding (Hoegh-Guldberg and Emlet 1997, Sokolova et al. 2012). Early life history stages, such as larvae/embryos, generally lack coping mechanisms for thermal stress, such as acclimation and induction of heat shock proteins (Rombough 1997, Brown et al. 2004, Pörtner and Farrell 2008, Flynn and Todgham 2017). This is of particular concern in the Antarctic, where organisms must fuel development for months (Hoegh-Guldberg and Pearse 1995, Peck 2018) to years (Moran et al. 2019) while potentially coping with the effects of increasing temperatures on metabolic rates over a longer period of time.

One mechanism that can help offset the negative impacts of temperature on metabolism and energy balance is acclimation (occurs in the lab) acclimatization (occurs in situ) (Peck et al. 2014, Clark et al. 2019). A classic indicator of an organism acclimating to increasing temperature

is the suppression of metabolic rate so that the organism can compensate for the increased energy demand of living at higher temperatures and maintain energy homeostasis (Newell 1969, Sokolova and Pörtner 2003, Todgham and Mandic 2020). In the majority of Antarctic taxa investigated, acclimation is highly limited (Peck et al. 2010) and was extremely slow, requiring several months to acclimate (Peck et al. 2009, Peck et al. 2014, Morley et al. 2019), which is 2-4 times greater than temperate relatives (reviewed in Peck et al. 2014). The organisms used in the present study had little time to acclimate, as they were taken freshly out of the water and used within 5 days of collection; no previously published study has shown acclimation rates shorter than two to nine months for any Antarctic marine invertebrate (Morley et al. 2012, Peck et al. 2014, Morley et al. 2024). However, we recently found that stage N-II of *N. australe* can reduce its metabolism after 24 days at +1.0 °C (Lobert et al. (chapter 1, MS in prep)). Larvae of *N. australe* (specifically N-II) showed high thermal sensitivity in the present study, but their ability to acclimate may allow them to partially buffer the negative effects of warming waters (Peck et al. 2014, Todgham and Mandic 2020). Unlike larvae of *N. australe*, larvae of *A. glacialis* may not need to be able to acclimate within these temperature ranges, as thermal sensitivity across all stages was low, but if able to acclimate, they may be more resilient to warming.

## Conclusion

Our data indicate that the larval metabolism of four species from two phyla showed an overall high thermal sensitivity ( $Q_{10} > 4$ ) relative to most other biological processes. Thermal sensitivity did not appear to increase or decrease with developmental stage, indicating that high thermal sensitivity may be present across all of early development and not limited to one bottleneck stage, such as the earliest stages, which have lower physiological complexity. Our highest experimental temperature of +4.0 °C was only 4-5 °C above the current summer maximum temperatures of McMurdo Sound (Hunt et al. 2003, Cheng and Detrich 2007), and only +2 °C warmer than the summer sea surface temperatures on the Antarctic peninsula (Barnes et al. 2006) where these organisms are also found (Wägele 1995, Barnes & Bullough, 1996, McClintock and Baker 1997, Bryan et al. 1998, Cano and López González 2009). Current warming predictions under a high-emissions scenario predict warming of 1.75 °C by 2100 (Tonelli et al. 2021). If increasing thermal sensitivity (higher  $Q_{10}$  values) at higher temperatures do indeed indicate an

increase in energy requiring processes (as described in Shishido et al. 2020) and decrease energy efficiency (Iftikar et al. 2014, Schute 2015, Flynn and Todgham 2018), then the organisms that cannot acclimate to offset increases in energy expenditures may be threatened by climate change as increased thermal stress pushes them away from metabolic equilibrium.

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**Tables and Figures:**

<b>Species</b>	<b>Cohorts of Stage I/EV</b>	<b>Cohorts of Stage II/MV</b>	<b>Cohorts of Stage III/LV</b>
<i>Nymphon australe</i>	8	13	2
<i>Ammothea glacialis</i>	2	4	4
<i>Tritoniella belli</i>	3	3	1
<i>Tritonia challengeriana</i>	4	4	3

**Table 1.** Cohorts of each species and each stage. Each cohort was divided into six individual groups ('vials') of either 2-24 (*N. australe* and *A. glacialis*) or 50-300 (*Tritoniella belli* and *Tritonia challengeriana*) individuals. Cohorts of *N. australe* and *A. glacialis* were assembled from different sets of parents, whereas each cohort of *Tritoniella belli* and *Tritonia challengeriana* came from a single egg mass.

<i>Nymphon australe</i>	SS	MS	Num <i>df</i>	Den <i>df</i>	F	p
Temperature	49.276	16.4253	3	391.34	141.5926	<0.001*
Stage	12.148	6.0740	2	20.72	52.3599	<0.001*
Temperature x Stage	3.998	0.7997	5	394.31	6.8933	<0.001*
<i>Ammothea glacialis</i>	SS	MS	Num <i>df</i>	Den <i>df</i>	F	p
Temperature	4.8022	4.9341	3	205.505	82.5857	<0.001*
Stage	0.2011	0.1005	2	6.985	1.6830	0.25319
Temperature x Stage	0.8979	0.1496	6	205.519	2.5047	0.02321*
<i>Tritoniella belli</i>	SS	MS	Num <i>df</i>	Den <i>df</i>	F	p
Temperature	20.5037	6.8346	3	136.858	113.3689	<0.001*
Stage	16.8357	8.4179	2	3.967	139.6317	<0.001*
Temperature x Stage	2.2116	0.3686	6	136.680	6.1141	<0.001*
<i>Tritonia challengeriana</i>	SS	MS	Num <i>df</i>	Den <i>df</i>	F	p
Temperature	34.714	11.5714	3	205.497	267.8354	<0.001*
Stage	5.017	2.5085	2	7.873	58.0617	<0.001*
Temperature x Stage	1.356	0.2261	6	205.546	5.2328	<0.001*

**Table 2.** Results of a two-way ANOVA testing for significant effects of temperature, stage, and the interaction between temperature and stage on oxygen consumption of *Nymphon australe*, *Ammonothea glacialis*, *Tritoniella belli*, and *Tritonia challengeriana*. Significant p values are in bold with \* ( $p < 0.05$ ).

Temperature	Stage I to Stage II	Stage II to Stage III	Stage I to Stage III
<b>-1.8 °C</b>	0.2619	<0.0001	<0.0001
<b>-0.4 °C</b>	0.0027	N/A	N/A
<b>+1.0 °C</b>	<0.0001	0.0002	<0.0001
<b>+4.0 °C</b>	0.0052	0.0153	<0.0001

**Table 3.** *Nymphon australe*; p values of pairwise comparisons (Tukey) between subsequent larval stages at each of the four temperatures.

Species	Q <sub>10</sub> of Stage I/EV	Q <sub>10</sub> of Stage II/MV	Q <sub>10</sub> of Stage III/LV
<i>Nymphon australe</i>	<b>4.6*</b>	<b>6.2*</b>	1.6
<i>Ammonothea glacialis</i>	<b>2.1*</b>	<b>3.2*</b>	<b>4.1*</b>
<i>Tritoniella belli</i>	<b>6.5*</b>	<b>3.4*</b>	<b>6.7*</b>
<i>Tritonia challengeriana</i>	<b>7.6*</b>	<b>5.6*</b>	<b>5.1*</b>

**Table 4.** Q<sub>10</sub> values across the whole temperature range (-1.8 to +4.0 °C) for oxygen consumption of three stages of *Nymphon australe*, *Ammonothea glacialis*, *Tritoniella belli*, and *Tritonia challengeriana*. Q<sub>10</sub> values associated with significant differences in oxygen consumption between -1.8 and +4.0 °C are in bold with \* ( $p < 0.05$ ).

Temperature	Stage I to Stage II	Stage II to Stage III	Stage I to Stage III
-1.8 °C	0.9901	0.9999	1.0000
-0.4 °C	0.6464	0.9920	0.9641
+1.0 °C	0.7111	1.0000	0.8395
+4.0 °C	0.7035	1.0000	0.6869

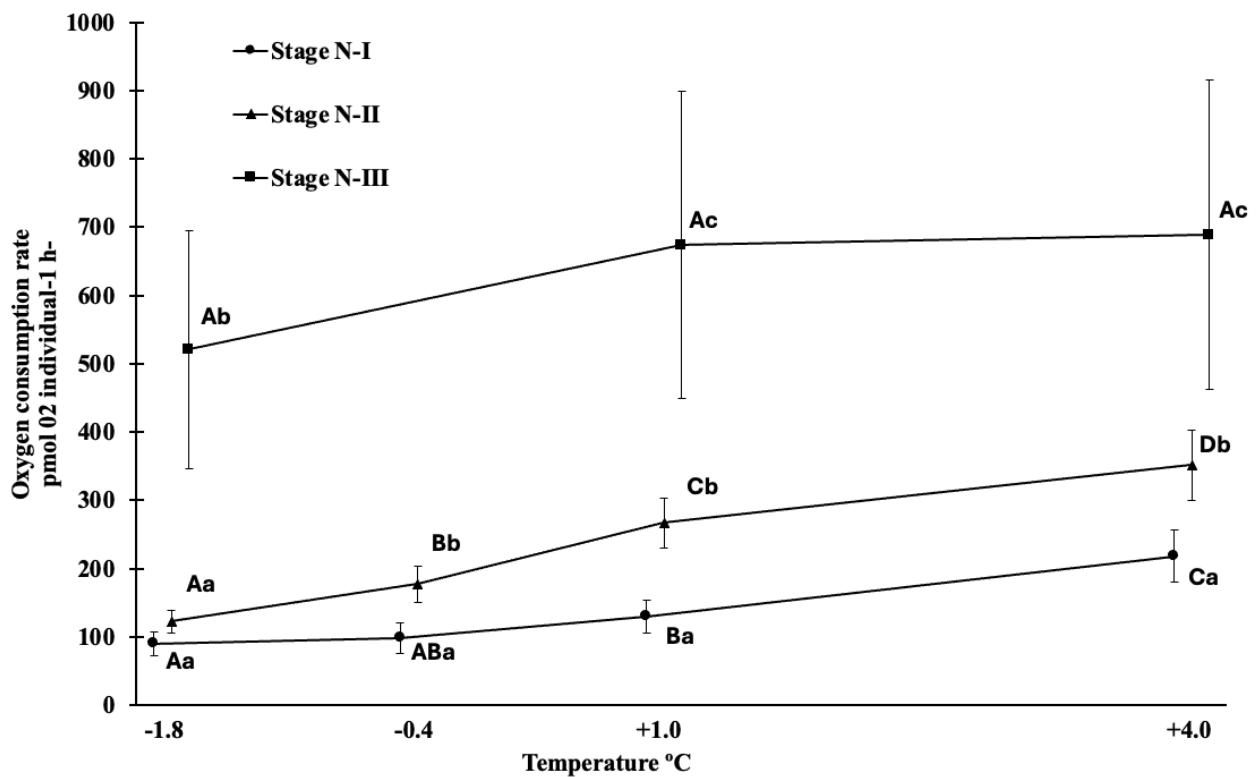
**Table 5.** *Ammothea glacialis*; p values of pairwise comparisons (Tukey) between subsequent larval stages at each of the four temperatures. No significant differences between stages were recorded at any of the temperatures.

Temperature	EV to MV	MV to LV	EV to LV
-1.8 °C	<0.0001	0.0110	<0.0001
-0.4 °C	<0.0001	0.7832	0.0001
+1.0 °C	<0.0001	0.0086	<0.0001
+4.0 °C	0.0009	0.0002	<0.0001

**Table 6.** *Tritoniella belli*; p values of pairwise comparisons (Tukey) between subsequent larval stages at each of the four temperatures. All comparisons are significant with the exception of that between MV and LV at -0.4 °C.

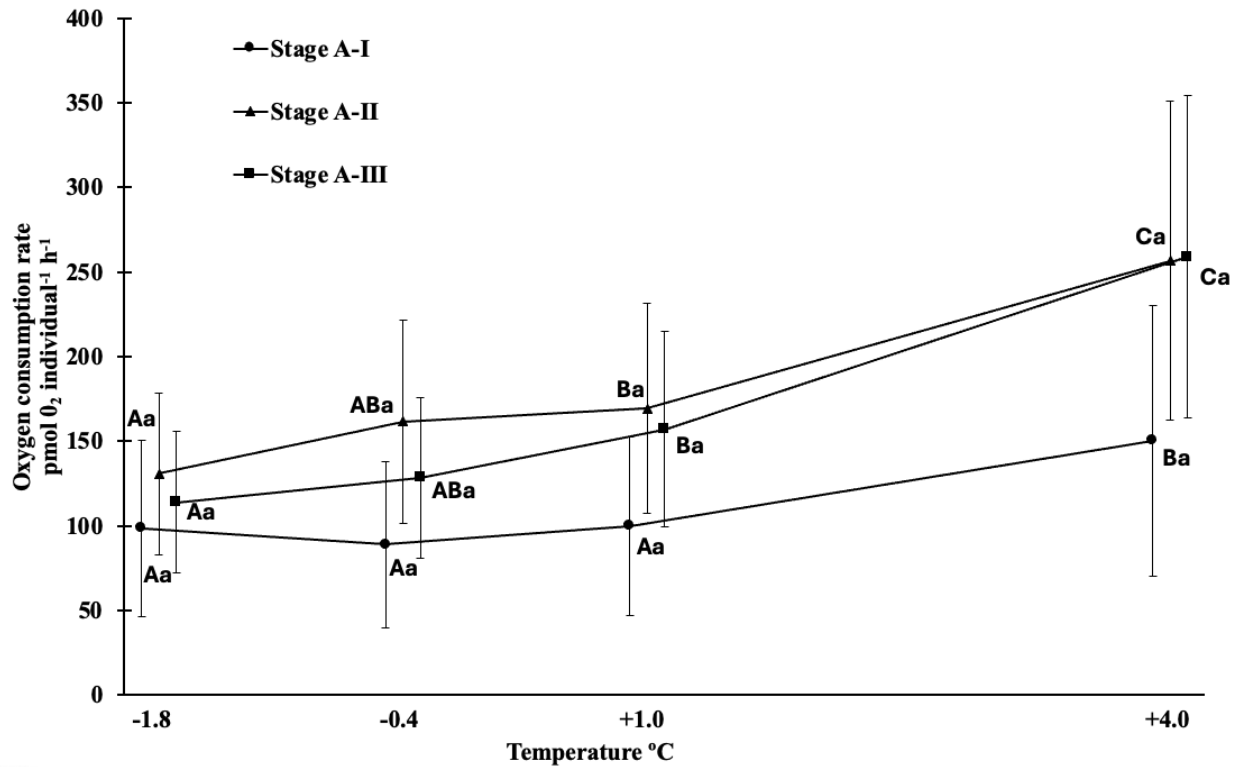
Temperature	EV to MV	MV to LV	EV to LV
-1.8 °C	0.1078	0.0005	<0.0001
-0.4 °C	0.1933	0.0014	<0.0001
+1.0 °C	0.0126	0.0078	<0.0001
+4.0 °C	0.4837	0.0007	0.0001

**Table 7.** *Tritonia challengeriana*; p values of pairwise comparisons (Tukey) between subsequent larval stages at each of the four temperatures.

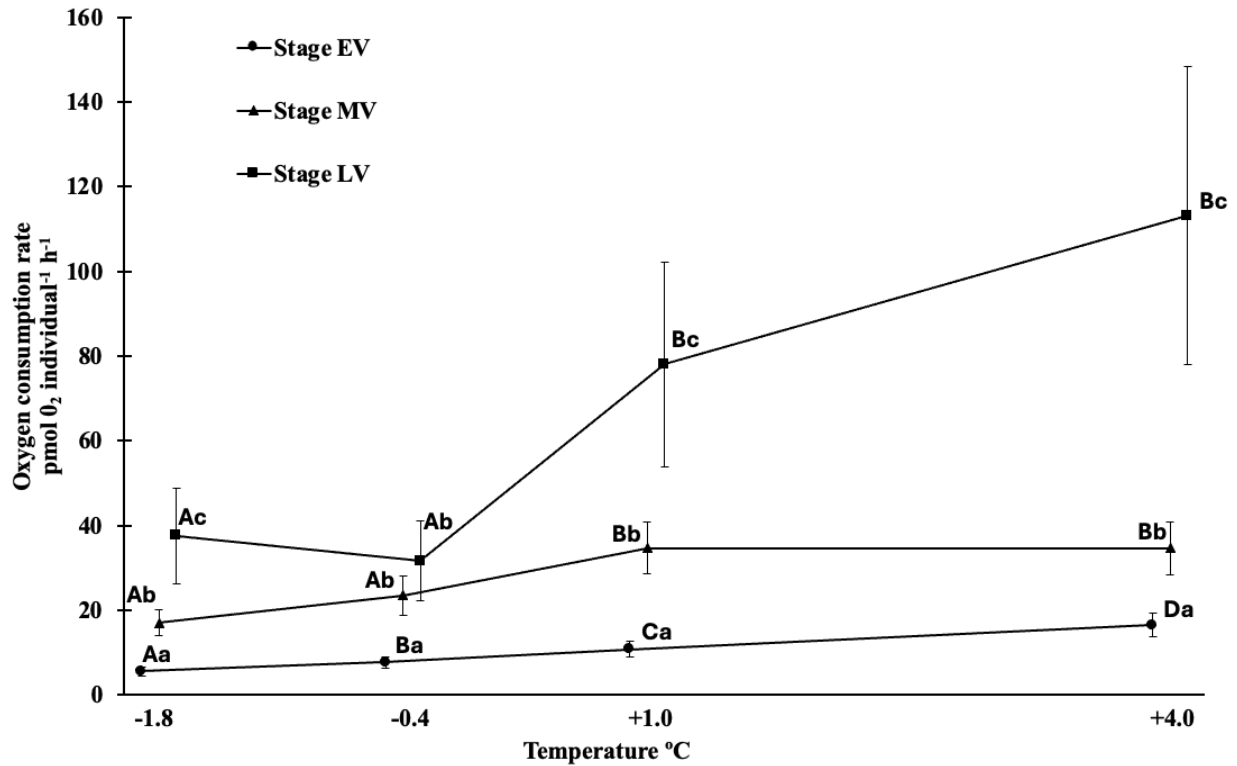


**Figure 1.** *Nymphon australe*. Oxygen consumption rates of three developmental stages (N I, II, and III) at four experimental temperatures (-1.8, -0.4, +1.0, +4.0 °C). Each point is the estimated means of the oxygen consumption rate ( $\pm 95\%$  CI) of n= 2-13 (N-I: 8, N-II: 13, N-III: 2) cohorts of larvae measured at each stage at the given temperature. Significant differences in oxygen consumption rate between temperatures within a developmental stage are indicated by capital

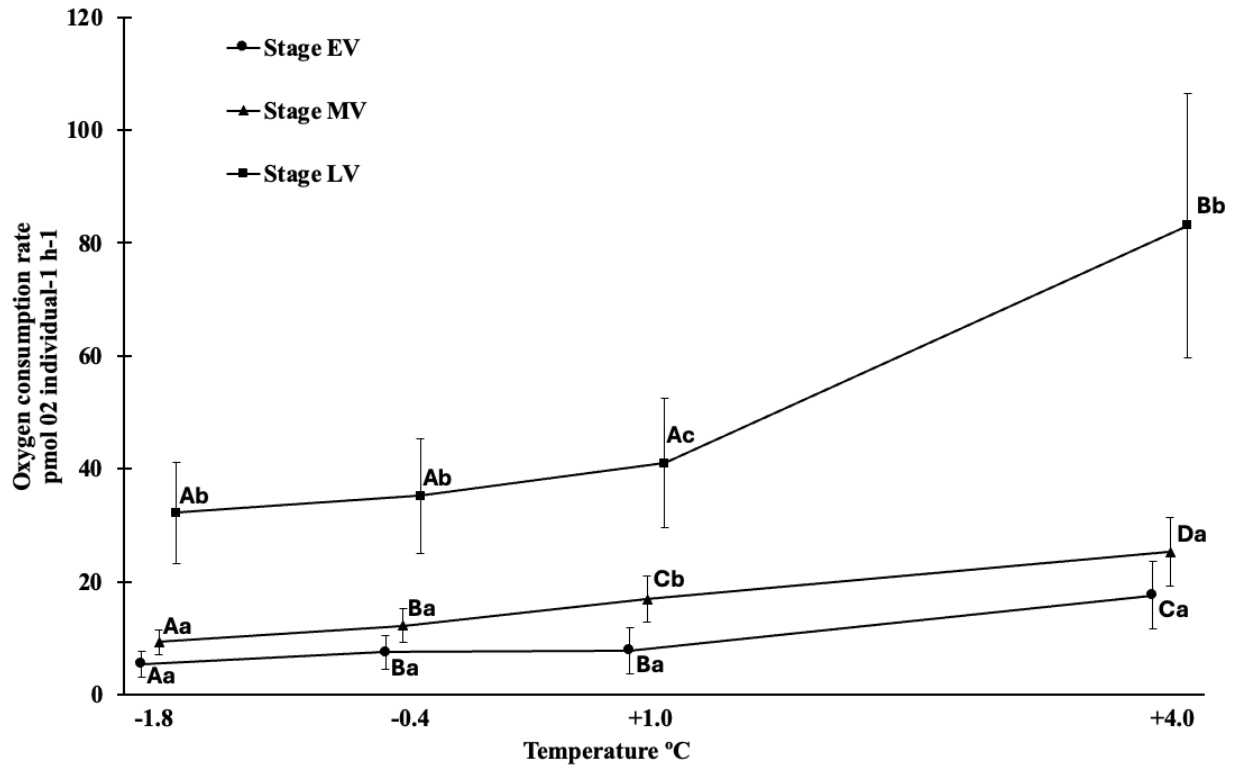
letters (A-D), while significant differences between developmental stages within a single temperature are indicated by lowercase letters (a-c). Temperature is staggered  $\pm 0.1$  °C around experimental temperature (-1.8, -0.4, +1.0, +4.0 °C) for graphical representation to avoid overlaps of error bars.



**Figure 2.** *Ammonoetea glacialis*; model output of the oxygen consumption rates of three developmental stages (Stage A-I, A-II, and A-III) at four experimental temperatures (-1.8, -0.4, +1.0, +4.0 °C). Each point is the estimated means of the oxygen consumption rate ( $\pm 95\%$  CI) of  $n = 2-4$  (Stage A-I: 2, Stage A-II: 4, Stage A-III: 4) cohorts of larvae measured at each stage at the given temperature. Significant differences in oxygen consumption rate between temperatures within a developmental stage are indicated by capital letters (A-D), while significant differences between developmental stages within a single temperature are indicated by lowercase letters (a-c). Temperature is staggered  $\pm 0.1$  °C around experimental temperature (-1.8, -0.4, +1.0, +4.0 °C) for graphical representation to avoid overlaps of error bars.



**Figure 3.** *Tritoniella belli*; model output of the oxygen consumption rates of three developmental stages (EV, MV, LV) at four experimental temperatures (-1.8, -0.4, +1.0, +4.0 °C). Each point is the estimated mean of the oxygen consumption rate ( $\pm 95\%$  CI) of  $n= 1-3$  (EV: 3, MV: 3, LV: 1) cohorts of embryos measured at each stage at the given temperature. Significant differences in oxygen consumption rate between temperatures within a developmental stage are indicated by capital letters (A-D), while significant differences between developmental stages within a single temperature are indicated by lowercase letters (a-c). Temperature is staggered  $\pm 0.1$  °C around experimental temperature (-1.8, -0.4, +1.0, +4.0 °C) for graphical representation to avoid overlaps of error bars.



**Figure 4.** *Tritonia challengeriana*; model output of the oxygen consumption rates of three developmental stages (EV, MV, LV) at four experimental temperatures (-1.8, -0.4, +1.0, +4.0 °C). Each point is the estimated means of the oxygen consumption rate ( $\pm 95\%$  CI) of n= 3-4 (EV: 4, MV: 4, LV: 3) cohorts of embryos measured at each stage at the given temperature. Significant differences in oxygen consumption rate between temperatures within a developmental stage are indicated by capital letters (A-D), while significant differences between developmental stages within a single temperature are indicated by lowercase letters (a-c). Temperature is staggered  $\pm 0.1$  °C around experimental temperature (-1.8, -0.4, +1.0, +4.0 °C) for graphical representation to avoid overlaps of error bars.

## CHAPTER 4: ACCLIMATION AND ACCLIMATIZATION OF LARVAE OF THE ANTARCTIC PYCNOGONID *Nymphon australe*

### Abstract

Global climate change is warming our oceans at unprecedented rates, with predicted warming of +1.4 °C to +4.4 °C by 2100. These increasing temperatures cause organisms to experience thermal stress, as they are subjected to temperatures outside of their thermal range. In response to stress, organisms can migrate away from the stressor, adapt to new conditions through natural selection, physiologically acclimate to the warmer waters, or die. In the ecosystems of the high Antarctic regions of the Southern Ocean, organisms are already inhabiting the coldest waters on Earth, so migration to cooler waters is unlikely as these waters warm. The long generation times and slow development that are common in Antarctic fauna make the possibility of adaptation unlikely as well, limiting the endemic and highly diverse fauna in the Southern Ocean to rely on acclimatory responses. Most fish in the Antarctic do show signs of the ability to acclimate to warmer temperatures, but take 2-4 times longer than their temperate relatives to do so. Unfortunately, the majority of studies on Antarctic invertebrates indicate that the ability to acclimate is limited, either taking several months to do so or not occurring at all. Here, I show that larvae of *Nymphon australe* are able to acclimate to temperatures of +1.0 °C after 24 days of exposure. Oxygen consumption rates of larvae acclimated to +1.0 °C had lower overall oxygen consumption rates than those of larvae incubated at -1.8 °C, a classic sign of acclimation. These data are the first that show acclimation in larvae of Antarctic invertebrates, a life history stage that was previously thought to lack stress response mechanisms such as acclimation responses. These larvae not only were able to acclimate but could do so in a biologically relevant time period, able to account for seasonal increases in temperature.

## **Introduction:**

The three main adaptive responses that organisms can deploy in response to climate change are migration, evolution, and acclimation; or more simply put, move, acclimate, adapt, or die (Peck et al. 2010, Habary et al. 2017, Bernatchez et al. 2023, Donelson et al. 2023). One major aspect of climate change is ocean warming, and the 2023 IPCC report predicts the world's oceans will warm between +1.4 °C and +4.4 °C (Low vs High Emissions) by 2100. Temperature, through its effects on the chemical reactions underlying biological functions, is a key factor impacting the physiology, distribution, and abundance of organisms (Thorson 1950, Hoegh-Guldberg and Pearse 1995, Fields and Houseman 2004, Pecorino et al. 2013, Détrée et al. 2023). One important impact of ocean warming is that it can push marine organisms away from their thermal optimum towards areas of increased stress and potentially death (Hofmann and Todgham 2010, Flynn and Todgham 2017, Peck 2018), meaning as the world's oceans continue to warm, organisms will face growing challenges (Peck et al. 2010, Sandblom et al. 2014).

For millions of years the waters of the Southern Ocean surrounding the Antarctic continent have remained at near constant, frigid temperatures (Peck et al. 2014, Todgham and Mandic 2020). The Southern Ocean is of global importance because it acts as a climate buffer, storing a disproportionate amount of heat from anthropogenic warming (ACCE Decadal Report). The Southern Ocean is also biologically important because it contains a diverse and highly endemic fauna (Reviewed in Peck 2018). A major paradigm of Antarctic biology is that most species, particularly ectotherms, are highly adapted, stenothermal cold-temperature specialists and as such will be particularly vulnerable to ocean warming (Peck 2002, Peck et al. 2010, Détrée et al. 2023). Current predictions suggest sea surface temperatures in the Southern Ocean will rise between +0.5 °C and +1.75 °C by 2100 (ACCE Decadal Report), and while this may seem like a small change for organisms in temperate regions, it may have large effects on Antarctic organisms if they are unable to mount successful responses at the organism or population levels (Peck et al. 2006, Pörtner et al. 2007, Peck 2018).

While global warming threatens all life across all biomes (Bernatchez et al. 2023), organisms in the Antarctic may be particularly vulnerable due to proportionally greater limits (compared to temperate species) on their ability to migrate, adapt, or acclimate in response to warming. In other ecosystems, many studies have identified patterns of organismal retreat from warmer areas near the equator poleward towards cooler environments (Parmesan et al. 1999, Kortsch et al. 2015, Maire et al. 2019, Todgham and Mandic 2020); however, migration or movement to colder areas is not an option for organisms in the high Antarctic since there is no colder, higher latitude to move to (Todgham and Mandic 2020). Along with migration, adaptation through natural selection is also an unlikely mechanism for resilience of the Antarctic marine fauna due to the long generation time and slow development of most organisms there (Flynn and Todgham 2017, Peck 2018, Todgham and Mandic 2020).

Given the low likelihood of migration or adaptation to climate change, acclimation may be the most important process for coping with increasing temperatures (Peck 2018, Todgham and Mandic 2020). Acclimation occurs on shorter time scales than evolution or migration and occurs at the individual not population level (Leroi et al. 1994, Morley et al. 2019). Overall ability of individual organisms to adaptively change their physiology to cope with warming is thought to be highly limited in the Antarctic (Peck et al. 2010). Relative to the high diversity of Antarctic marine ectotherms, only a handful have been investigated for their ability to acclimate to temperature (Pörtner et al. 2007). Most studies of acclimation have focused on Antarctic fish. These studies indicate that, most fish in the Antarctic are able to acclimate some aspects of their physiology to elevated temperatures in the range of +1 to +4 °C (Franklin et al. 2007, Robinson and Davidson 2008, Peck et al. 2014). However, Antarctic fish take 2-4 times longer to acclimate to similar temperature changes than temperate species (reviewed in Peck et al. 2014). Marine invertebrates are highly diverse and ecologically important in the Southern Ocean (Peck et al. 2006, Griffiths 2010) and have been studied much less than fishes. Existing work suggests that species many have very little ability to acclimate to higher temperature; for those that could, acclimation was extremely slow, requiring 2-4 months of exposure (Peck et al. 2009, Peck et al. 2014, Morley et al. 2019).

Increasingly, researchers have identified a need for experiments involving extended experimental exposures of several months to capture the full acclimatory ability of Antarctic invertebrates (Peck et al. 2018, Morley et al. 2019, Morley et al. 2024). However, even if animals are able to acclimate to warmer conditions in a matter of months, there remains the question of whether such a long scale of acclimation is relevant in the current or near-future high Antarctic. McMurdo Sound, which has the southern-most seasonally ice-free water in the world, is one of the most historically and extensively studied regions of the Antarctic next only to the Antarctic Peninsula. The waters of McMurdo Sound remain at or sometimes below the freezing temperature of sea water ( $<-1.8\text{ }^{\circ}\text{C}$ ) for the majority of the year, with seasonal, irregular, non-monotonic increases of one to two degrees (total) beginning in December and ending around March (Hunt et al. 2003, Cheng and Detrich 2007). If acclimation in Antarctic invertebrates does indeed take four months (or longer), organisms in the high Antarctic may spend much of the year physiologically mismatched to the environment. When the pace of acclimation lags significantly behind the pace of environmental change, acclimatization comes with few benefits and high energetic costs (Sandblom et al. 2014). Thus, at least in the high Antarctic, slow temperature acclimation could lead to vulnerability, particularly as summer warming becomes more prominent with climate change (Peck et al. 2009).

In this study we examined the ability of larvae of the Antarctic sea spider *Nymphon australe* Hodgson 1902 (phylum Arthropoda, class Pycnogonida, order Pantopoda) to acclimate to higher temperatures over a timeframe that is ecologically relevant under modern seasonal temperature regimes. We used oxygen consumption rate (as a proxy for metabolic rate) as a metric to assess acclimation in laboratory experiments, because the suppression of metabolic rate at higher temperatures is a classic indicator of beneficial physiological adjustments that compensate for the increased energy demand of living at higher temperatures (Newell 1969, Sokolova and Pörtner 2003, Todgham and Mandic 2020). We also measured oxygen consumption rates of larvae collected directly from the field in the late austral winter and the austral summer to test for evidence of seasonal acclimatization in the field. We chose to work with the larval stages because they were experimentally tractable and because the acclimatory ability of larvae is rarely tested (Flynn and Todgham 2017), though early life history stages like embryos and larvae are often

considered to be the most physiologically vulnerable points in the life cycle (Bressan et al. 1995, Cowart et al. 2009, Walther et al. 2013). These data provide what is to our knowledge the first evidence for acclimation by larval stages of any Southern Ocean marine invertebrate.

## **Methods**

### **Organism**

*N. australe* are the most common pycnogonid in the Southern Ocean (Mahon et al. 2008, Arango et al. 2011, Soler-Membrives et al. 2017, Collins et al. 2018). While described as a singular species with circumpolar distribution, distinct populations have been documented and hypothesized to be separate species (Mahon et al. 2008, Collins et al. 2018, Zehnpfennig et al. 2024). Populations of *N. australe* have been reported outside of the Southern Ocean off the coast of Argentina and Chile, and in the Indian Ocean (Munilla and Membrives 2009). Like many other pycnogonids, male *N. australe* brood their young on specialized ovigerous legs through hatching and several larval molts (King 1973, Bain 2003, Cano and López-González 2009, Fornshell 2019). Post-embryonic development of *N. australe* is consistent with type five protonymph development, hatching from the egg membrane as an advanced larva with two pairs of walking legs (Brenneis et al. 2017, Cano-Sánchez et al. 2020).

### **Collection and Maintenance**

Adult *N. australe* bearing egg masses (Figure 1A) were collected by divers on SCUBA at a nearshore dive site off McMurdo Station, Antarctica called the Intake Jetty (77° 51.069' S, 166° 39.855' E) in two different field seasons, 2019-2020 and 2021-2022. *N. australe* are locally abundant at the Intake Jetty and were collected at depths of ~20-25 m. We used larvae at the postlarval instar two stage (Figure 1B and C) for these experiments because they were the most abundant stage in our collections.

## Laboratory Acclimation and Field Acclimatization

To assess the ability of larvae to acclimate to temperature in the laboratory, we measured the metabolic rate of larvae of *N. australe* incubated at either -1.8 °C or +1.0 °C for 24 days. Oxygen consumption of larvae from both treatments was measured across four measurement temperatures (-1.8, -0.4, +1.0, +4.0 °C). -1.8 °C was chosen as the lower experimental temperature as it is the most common temperature experienced throughout the year (Hunt et al. 2003, Cheng and Detrich 2007). +1.0 °C was chosen as the upper experimental temperature because: 1) we knew from previous experiments that stage two larvae of *N. australe* could survive for extended time periods at +1.0 °C without any visible negative impacts, 2) temperatures of 1.0 °C to 2.0 °C above the summer maximum temperature (maximum summer for McMurdo Sound -0.1 °C to -0.5 °C) have been shown to cause acclimation in other Antarctic taxa (Peck et al. 2010, Morley et al. 2019) and 3) +1.0 °C is very warm for McMurdo Sound but is within the temperature range that animals can experience, though rarely, at the Intake Jetty site (data in prep.).

Larvae for the laboratory acclimation experiments were collected November 15<sup>th</sup> through November 20<sup>th</sup> during the 2021-2022 field season. At that time, water temperatures at the collection site had been at winter temperatures close to -2.0 °C, in equilibrium with ice temperatures, since the previous April. Clutches containing stage-two larvae were removed from adult ovigers using fine forceps and larvae were gently separated. We then washed the larvae by pipetting over them with 0.2- $\mu$ m freshly filtered seawater to remove any biofouling. Larvae were kept chilled at -1.8 °C during handling. Prior to experiment, larvae were kept in the laboratory for no longer than five days (in order to reach required number of larvae for the experiment) in flow-through sea tables with temperatures between -1.5 °C and -0.5 °C, or in incubators at -1.8 C (ambient field).

Single egg masses did not contain enough Stage II larvae for these experiments, since multiple larvae were needed for measurements of oxygen consumption. Therefore, to incorporate some degree of biological replication, we assembled three separate experimental cohorts of larvae

from different sets of parents. Each cohort consisted of 230 larvae from brood masses of four to eight fathers; no paternal brood was used in more than one cohort. In total, we used 690 larvae from 19 different fathers for the three cohorts. The 230 larvae from each cohort were split evenly into two groups of 115, making sure that larvae from each father were evenly distributed in the two groups. Then, each group was randomly assigned to either  $-1.8\text{ }^{\circ}\text{C}$  or  $+1.0\text{ }^{\circ}\text{C}$  as a temperature treatment. For the thermal exposure period, larvae were distributed evenly into separate wells of six-well plates filled with 5 ml of  $0.2\text{-}\mu\text{m}$  freshly filtered seawater at temperature. Larvae were maintained at a concentration of  $\sim 4\text{ larvae ml}^{-1}$ . Trays were then placed in either a  $-1.8\text{ }^{\circ}\text{C}$  incubator (LT-36VL, Percival, Perry, US) or a  $+1.0\text{ }^{\circ}\text{C}$  incubator (Precision 818, Thermo Scientific, Waltham, US) for 24 days. Water was replaced weekly with  $0.2\text{-}\mu\text{m}$  freshly filtered seawater, cooled to treatment temperature. Larvae were not fed as they rely on maternally supplied yolk reserves to fuel early development. Throughout the 24-day incubation, a few larvae ( $<5\%$ ) molted from instar II to instar III. Any larva that molted was removed from the experiment to avoid potential effects of stage-dependent changes in metabolic rate or acclimation potential. After the 24-day incubation, oxygen consumption of larvae from both temperature groups were measured and compared at four different temperature treatments:  $-1.8$ ,  $-0.4$ ,  $+1.0$ , and  $+4.0\text{ }^{\circ}\text{C}$ . Oxygen consumption was first recorded at  $-1.8$ , followed by  $-0.4$ ,  $+1.0$ , and  $+4.0\text{ }^{\circ}\text{C}$ .

To test for evidence of seasonal acclimatization in the field, larvae of *N. australe* were collected from the field at two contrasting times, the end of austral winter (collection dates between October 28<sup>th</sup> and November 19<sup>th</sup>) and the austral summer (between January 7<sup>th</sup> and January 31<sup>st</sup>) (Figure 2). Water temperature data was obtained during both 2019-2020 and 2021-2022 field seasons from dataloggers (Hobo U26-001, Onset, Inc.) deployed close to the bottom at 30 m depth by the seawater intake pipe at McMurdo Station. Loggers recorded ambient temperature every 30 minutes. Average temperature during collection for each cohort was calculated using temperature data from the first collection date to the last for each cohort (Table 1). For temperatures 24 days prior to each cohort's collection period, the same period was used for averaging simply shifted backwards in time from the initial collection date. As for acclimation experiments, no one egg mass contained enough larvae to run the experiment, so larvae from

three to five egg masses collected during the same collection period were pooled into cohorts. Several cohorts were collected during the same collection period but no paternal brood was used in more than one cohort. To minimize the opportunity for acclimatization to lab temperatures, animals from the austral winter were stored less than 24 hours in flow through sea tables before counting and then were moved to an incubator at  $-1.8\text{ }^{\circ}\text{C}$  (ambient temperature in austral winter) until use. As animals from the austral summer were collected over a much shorter time scale, animals were stored no longer than three days (in order to reach required number of larvae for the experiment) in flow-through sea tables with temperatures between  $-1.5\text{ }^{\circ}\text{C}$  and  $-0.5\text{ }^{\circ}\text{C}$ . Oxygen consumption was measured at four temperatures ( $-1.8$ ,  $-0.4$ ,  $+1.0$ ,  $+4.0\text{ }^{\circ}\text{C}$ ) as for the laboratory acclimation experiments. In total we measured the oxygen consumption rates of six cohorts of larvae from the austral winter and seven cohorts from the austral summer.

### **Oxygen consumption**

For both the lab acclimation and field acclimatization trials, oxygen consumption (as a proxy for metabolic rate) was measured using an end-point determination  $\mu\text{BOD}$  method (Marsh and Manahan 1999, Moran and Woods 2007, Woods and Moran 2008). In brief, larvae were placed into small (514 to 783  $\mu\text{L}$ ) glass respiration vials of known volume containing  $0.2\text{-}\mu\text{m}$  freshly filtered seawater that was air-saturated with oxygen at  $-1.8\text{ }^{\circ}\text{C}$ . A range of 2-24 larvae were placed into each of six respiration vials. Vials were capped and incubated horizontally at each of the four temperature treatments (starting with  $-1.8$ , followed by  $-0.4$ ,  $+1.0$ , and  $+4.0\text{ }^{\circ}\text{C}$  with a 24 h rest period at  $-1.8\text{ }^{\circ}\text{C}$  between each temperature treatment) for four to seven h depending on temperature, with oxygen saturation never dropping below 80%. During incubation vials were inverted every two h to minimize oxygen concentration gradients. At the end of incubations, vials were cooled to  $-1.8\text{ }^{\circ}\text{C}$  for 30 seconds (as  $-1.8\text{ }^{\circ}\text{C}$  was the calibrated temperature for the electrode) and  $\sim 400\text{ }\mu\text{L}$  of water from each vial was removed using a temperature-equilibrated gas tight syringe and injected into a temperature-equilibrated water-jacketed chamber (MC-100 Strathkelvin, Glasgow, UK) and read using a Clark-style oxygen electrode (Strathkelvin Model 1302) and oxygen meter (Strathkelvin Model 782). For each cohort at each temperature, oxygen consumption per larva was calculated as the slope of the regression of total oxygen consumption of each vial plotted against the number of larvae in the same vial (Marsh and Manahan 1999,

Moran and Woods 2007, Woods and Moran 2008). This method is robust to the common issue of non-zero y intercepts (Marsh and Manahan 1999), but limits the power available for making comparisons compare between temperatures, stages and treatments by removing information from individual vials. Therefore, following the methods of Walther et al. (2013) and Crickenberger et al. (2015), we corrected the oxygen consumption rates of each individual within a vial using the intercept for of the regression line for that set of vials.

## **Statistical Analysis**

All statistical analyses were performed in the R Studio statistical package (Version 2024.04.1+748, Posit Software, PBC). Changes in oxygen consumption were analyzed using one way ANOVAs. For the lab acclimation experiment we assessed the effect of acclimation temperature using a linear model with oxygen consumption as the dependent variable with independent variables of respiration temperature, acclimation temperature, the interaction between them, with cohort as a blocking factor. For the seasonal acclimatization experiment a linear model was used with oxygen consumption as the dependent variable with independent variables of respiration temperature, season (austral winter or austral summer) and the interaction between them. Pairwise comparisons between treatments (incubation temperature or season) were conducted for both experiments using Tukey's post hoc test.

## **Results**

After 24 days at controlled experimental temperatures in the laboratory, the oxygen consumption rate of larvae kept at  $-1.8\text{ }^{\circ}\text{C}$  was  $\sim 12\%$  higher overall across the four measurement temperatures than the oxygen consumption rates of larvae kept at  $+1\text{ }^{\circ}\text{C}$  and this difference was significant ( $F_{1,3} = 12.4$ ,  $p = 0.0006$ ) (Figure 3A). Temperature also had a significant overall effect on oxygen consumption rate ( $F_{3,3} = 57.2$ ,  $p < 0.0001$ ), with overall higher consumption rates at higher temperatures. There was a significant interaction between experimental temperature treatments and respiration measurement temperature ( $F_{3,3} = 23.6$ ,  $p < 0.0001$ ). When all pairwise comparisons were performed (two treatment groups, four measurement temperatures), differences between treatment groups were significant at the two middle measurement

temperatures (-0.4 °C and +1 °C) but not at -1.8 °C or +4 °C (Table 2). For larvae maintained at -1.8 °C, oxygen consumption rose significantly between -1.8 °C and -0.4 °C ( $p < 0.0001$ ), -0.4 °C and +1 °C ( $p = 0.0081$ ), but not between +1 °C and +4 °C ( $p = 0.8447$ ). For larvae maintained at +1 °C, oxygen consumption was not significantly different between -1.8 °C and -0.4 °C ( $p = 0.9998$ ) or between -0.4 °C and +1 °C ( $p = 1.0$ ) but increased significantly between +1 and 4 ( $p < 0.0001$ ).

Larvae collected from January 7<sup>th</sup> to January 31<sup>st</sup> (austral summer) had a ~8% higher oxygen consumption rate ( $F_{1,3} = 8.2$ ,  $p < 0.0001$ ) than those collected between October 28<sup>th</sup> and November 19<sup>th</sup> (austral winter) (Figure 3B). There was a significant interaction between the season and measurement temperature ( $F_{3,3} = 14.0$ ,  $p < 0.0001$ ). When all pairwise comparisons were performed (two seasons, four measurement temperatures), differences between seasons were significant at -1.8 °C, +1.0 °C, and +4.0 °C, but not at -0.4 °C (Table 3). Unlike at the lower temperatures, at +4.0 °C oxygen consumption of larvae from the austral winter was higher. Temperature also had a significant overall effect on oxygen consumption rate ( $F_{3,3} = 190.3$ ,  $p < 0.0001$ ), with overall higher consumption rates at higher temperatures. For larvae from the austral winter, oxygen consumption rose significantly between -1.8 °C and -0.4 °C ( $p < 0.0001$ ), -0.4 °C and +1.0 °C ( $p = 0.0017$ ), and +1.0 °C and +4.0 °C ( $p < 0.0001$ ). For larvae from the austral summer, oxygen consumption was not significantly different between -1.8 °C and -0.4 °C ( $p = 0.9663$ ) but rose significantly between -0.4 °C and +1.0 °C ( $p < 0.0001$ ) and +1.0 °C and +4.0 °C ( $p = 0.005$ ).

The temperatures experienced by larvae in the field during the austral summer collection period averaged  $-1.54 \text{ °C} \pm 0.07$  (SD) across all summer cohorts; temperature during the austral winter collection period averaged  $-2.14 \text{ °C} \pm 0.006$  across all winter cohorts. 24 days prior to austral summer collection period the temperatures experienced by larvae averaged  $-1.55 \text{ °C} \pm 0.04$  across all summer cohorts; temperature 24 days prior to the austral winter collection period averaged  $-2.13 \text{ °C} \pm 0.007$  across all winter cohorts (Table 1, Figure 2).

## Discussion

As global climate change continues to disrupt ecosystems worldwide, thermally sensitive organisms and populations must move, adapt, or acclimate to persist in a warming world (Peck et al 2010, Donelson et al. 2019, Bernatchez et al. 2023). Here we found that larvae of the Antarctic pycnogonid *Nymphon australe* showed strong evidence of acclimation of metabolic rate to an increase in temperatures of 2.8 °C over ambient; larvae maintained at +1 °C for 24 d had lower metabolic rates across a range of temperatures than members of the same cohort maintained at -1.8 °C for the same period. Furthermore, this acclimation occurred over an environmentally relevant timeframe of 24 days. However, larvae collected from the field in the late austral winter and austral summer did not show evidence of acclimatization, in that larvae collected in the austral summer had higher metabolic rates overall than larvae collected at the end of the winter. Field temperatures measured in the 24 d prior to collection differed by at most 0.75 °C, suggesting that lack of acclimatization may have been due, in part, to seasonal variation in the magnitude and timing of the summer temperature peak which led to small temperature differences between our summer and winter collection times. The laboratory data suggest that larvae of *N. australe* have a greater capacity for short-term acclimation than has been previously demonstrated in most Antarctic invertebrates. If the ability to acclimate confers resilience to ocean warming (Sandblom et al. 2014, Morley et al. 2019), then early life history stages of *N. australe* may be more robust to changing temperatures than previously thought.

Maintaining metabolic equilibrium confers greater resilience in the face of stressors such as temperature increases due to climate change (Todgham and Mandic 2020), and the ability to acclimate to warmer water by suppressing oxygen consumption to pre-stress levels or lower helps organisms to remain in equilibrium where energy supply meets energetic demand (Newell 1969, Sokolova and Pörtner 2003, Flynn and Todgham 2017). Most (if not all) studies of acclimation in Antarctic invertebrates have focused on adults (Peck et al. 2010, Morley et al. 2019), and our understanding of larval acclimation in the Antarctic comes largely from studies of fish (Franklin et al. 2007, Windisch et al. 2011, Flynn and Todgham 2017). Early life history stages like embryos and larvae are generally considered to be the most vulnerable points in the life cycle (Bressan et. al 1995, Cowart et. al 2009, Walther et al. 2013) with comparatively

limited capacity to acclimate (Rombough 1997); thus, the impacts of climate warming on early life history stages may determine the overall vulnerability of a species (Del Rio et al. 2021). In lecithotrophic taxa that rely on maternally supplied yolk to fuel development, such as *N. australe*, energy maintenance is one of the major factors that determines the rate of yolk utilization and the energetic state of later stages of life (Hoegh-Guldberg and Emlet 1997). In our study, larvae acclimated to warmer temperatures by suppressing oxygen consumption below that of larvae acclimated to cooler temperatures, potentially keeping them in a metabolic equilibrium and conserving yolk energy. The proper maintenance of energy expenditure (metabolic equilibrium) is particularly important in the Antarctic where seasonal blooms of nutrient and light availability limit food resources for periods of several months (Peck et al. 2006, Peck 2018, Caputi et al. 2020). Understanding the mechanistic role of metabolism in early development in a changing climate may give insight into population level resilience. If larvae of *N. australe* show signs of thermal resilience through acclimation such as shown here, this resilience may occur in adulthood as well (Bizuayehu et al. 2015, Flynn and Todgham 2017).

Unlike laboratory acclimation, we found no evidence for field acclimatization when we compared larvae of *N. australe* that were collected in the late austral winter vs early austral summer. The oxygen consumption rate of *N. australe* during the warmer austral summer was significantly higher than the oxygen consumption rate of larvae during the colder austral winter across all experimental temperatures, the opposite of what one would expect if larvae were acclimating to warmer temperature. One explanation for the lack of evidence for acclimation may be the small amount of temperature differences that animals experienced in the field in different seasons relative to our two laboratory temperatures. Whereas larvae were removed and placed into incubators at either -1.8 °C or +1.0 °C (difference of 2.8 °C), larvae for the field acclimatization had experienced a difference in temperature of only ~0.60 °C in the 24 d prior to the collection period. This small difference in temperature may not have been enough to elicit a measurable acclimation response. Acclimatization may still occur in the field, but because the timing and duration of peak temperatures vary considerably from year to year, it may be difficult to capture given the constraints of performing these types of collections and experiments in the Southern Ocean.

Another interesting pattern in the acclimatization data was that the oxygen consumption rate of ‘summer’ larvae, which had experienced slightly higher temperatures, had higher (rather than lower) rates of oxygen consumption than larvae collected at the end of winter. This not only failed to support acclimatization, but followed the opposite pattern than expected if larvae were lowering their metabolism to maintain energy balance under warmer thermal regimes.

Reproduction is highly seasonal for many species in the Antarctic (Pearse et al. 1991, Freire et al. 2005). If that is also the case for *N. australe*, and larvae were all produced at around the same time of year, then summer larvae would have been several months older, and potentially more physiologically complex than winter larvae and with a higher oxygen demand.

We performed these experiments on animals that live in the southernmost open ocean of the high Antarctic, where temperatures rarely reach our highest experimental temperature of +1.0 °C. At this specific location, unless acclimatization occurs on an even shorter time scale than 24 d (which we did not measure) and at lower temperatures, the benefits of maintaining physiological capacity for acclimatization to +1.0 °C may be limited. However, *N. australe* is a very broadly distributed species in the Southern Ocean (Mahon et al. 2008, Arango et al. 2011, Soler-Membrives et al. 2017, Collins et al. 2018) with a range that extends into the Subantarctic (Munilla and Membrives 2008). Populations are found at lower latitudes (Mahon et al. 2008, Collins et al. 2018) where water temperatures are at or above +1.0 °C for roughly three months of the year before returning back to near freezing temperatures (Barnes et al. 2006, Cardenas et al. 2018). Thus, in parts of its range, larvae of *N. australe* may incur substantial energetic benefits from acclimatization. Likewise, it is possible that the required acclimation period is shorter than 24 d, which would increase the ability of larvae to match their metabolic rate to the variable temperature regimes in the high Antarctic summer, varying by at least a degree over a single month (Figure 2.). Future studies should test even shorter timeframes to better understand the timing of acclimatory capacity of *N. australe* and other Antarctic taxa.

Previous studies examining acclimation by Antarctic ectotherms have used metrics such as swimming rate (Wilson et al. 2002), cardiovascular output (Seebacher et al. 2005), and upper lethal temperatures (Peck et al. 2010, Carter et al. 2023), in addition to oxygen consumption (Robinson and Davison 2008, Peck et al. 2010). While the use of multiple metrics is preferred to single metrics for definitively identifying acclimation (Johansen et al. 2021), metabolic compensation is a common and well-established mechanism for acclimation to high temperature (Coggins et al. 2021) and is often used to evaluate acclimation in the context of climate change (Robinson and Davison 2008, Peck et al. 2010, Terblanche and Hoffmann 2020, Enriquez-Urzelai and Gvoždík 2024). One downside of metabolism as an indicator of acclimation is that other processes besides acclimation may change its temperature-dependence. For example, if embryos and larvae accumulated temperature-related physiological damage during the 24-d incubation at +1.0 °C, then their lower metabolism could have represented temperature-driven partial failure of cellular processes (O’Brein et al. 1991, Iftikar and Hickey 2013, Schulte 2015). At temperatures above of an organism’s thermal performance window oxygen, delivery systems begin to fail and proteins begin to denature (Pörtner and Farrell 2008, Miller and Stillman 2012, Ángeles-González et al. 2020). However, we saw no mortality in our trials (in either treatment), and in a longer-term experiment performed on larvae from the same collections, larvae of all stages (including N-II) survived for 200+ days at +1.0 °C (Toh in prep.). We also saw no discernable difference in activity between treatments in terms of overall activity (no larvae were very active). Another possibility is that larvae incubated at the colder temperature showed higher metabolic costs because they were synthesizing enzymes or proteins needed for maintaining function at temperatures close to the freezing points, such as the antifreeze proteins found in notothenoid fishes (Harding et al. 2003, Durman 2015). However, few if any Antarctic marine invertebrate taxa utilize antifreeze proteins (Durman 2015). Also, different aspects of organismal performance are likely to acclimate at different rates; in tropical fishes, standard metabolic rate acclimates more rapidly than other performance metrics like aerobic scope, maximum metabolic rate, and red blood cell production (Johansen et al. 2021). Other commonly-used metrics like righting ability (Woods et al. 2009, Shishido et al. 2019), and heart rate (Stenseng et al. 2005, Jayasundara et al. 2009), were not feasible for larvae of *N. australe*. Due to the small size and limited mobility of larvae of *N. australe* we were limited in our ability to measure acclimation using most metrics other than metabolic rate. These findings suggest that larvae *N. australe* have

lower oxygen consumption rates when incubated at higher temperatures due to acclimation responses and not due to system failure or increased protein expression. Future studies utilizing additional physiological and biochemical metrics (i.e. mitochondrial membrane potential), along with expanding out to more life stages are needed to better understand the thermal resilience of acclimation for the species.

In order for acclimation to be beneficial for a species, organisms need to be able to respond to increases in temperature in a timeframe that allows them to match the acclimated phenotype to the appropriate environmental condition (Tattersall et al. 2012, Sandblom et al. 2014). Seasonal increases in water temperature around McMurdo Sound occur over a 2–3-month time period during the austral summer, after which temperatures return to near freezing for the winter (Hunt et al. 2003, Cheng and Detrich 2007). Temperature changes are also not monotonic, with frequent peaks and valleys occurring within days of each other throughout the warm period. As one example, our data from 2020 at the Intake Jetty showed that temperature ranged from  $-1.11^{\circ}\text{C}$  to  $-0.63^{\circ}\text{C}$  over the course of one day (01/15/20) with changes as great as  $0.42^{\circ}\text{C}$  occurring in a single hour. Thus, if acclimation occurred over an extended time period ( $>2$  months) as for previous studies of Antarctic invertebrates (Peck et al. 2010, Morely et al. 2019), this would likely result in a large cumulative mismatch between acclimatization state and the thermal environment for organisms in McMurdo Sound over the course of a year. Generally speaking, slow acclimation is particularly disadvantageous in varying thermal regimes because slow acclimators will accumulate an excessive amount of energetic costs as they attempt to continually acclimate to new temperatures (Sandblom et al. 2014, Morely et al. 2019). Future winners in terms of a species resiliency to changes in temperature due to climate change may come down to not who can acclimatize, but who can do so in time (Sandblom et al. 2014).

### **Conclusion:**

As the oceans continue to warm, the ability for organisms to acclimate to changing water temperatures may be vital for the success of vulnerable populations. Given the unlikelihood that migration or evolution will occur on relevant time scales in the Antarctic, it is vital to understand

the extent to which the diverse ectothermic marine fauna of the Antarctic can acclimatize, and whether they can acclimatize rapidly enough to benefit from their capacity. Our results show that unlike most other adult Antarctic marine invertebrates studied to date, larvae of *N. australe* in the coldest parts of their range are able to adjust their metabolic rate in a potentially beneficial way in response to increases in temperatures similar to those predicted by climate forecasts from the IPCC and ACCE. Given the broad ranges of this species, the generality of the finding is uncertain. For a broader understanding of the role that early life history stages may play in the resilience or vulnerability of Antarctic taxa, future studies should test for acclimatory capacity and the pace of acclimation of embryos and larvae of a wide variety of marine ectotherms in the Southern Ocean, particularly species with more restricted thermal habitats.

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**Tables and Figures:**

Cohort(s)	Austral Season	Collection Period (M/D/Y)	Average Temperature (°C) During Collection Period	Average Temperature (°C) 24 Days Prior to Collection Period
1	Summer	01/7/20	-1.39 ± 0.049 °C	-1.90 ± 0.024 °C
2-4	Summer	01/31/20	-1.50 ± 0.027 °C	-1.39 ± 0.049 °C
5-7	Winter	10/28/21 to 11/09/21	-2.15 ± 0.006 °C	-2.12 ± 0.009 °C
8-10	Winter	11/01/21 to 11/19/21	-2.15 ± 0.006 °C	-2.14 ± 0.005 °C
11-13	Summer	01/24/22 to 01/27/22	-1.63 ± 0.113 °C	-1.59 ± 0.049 °C

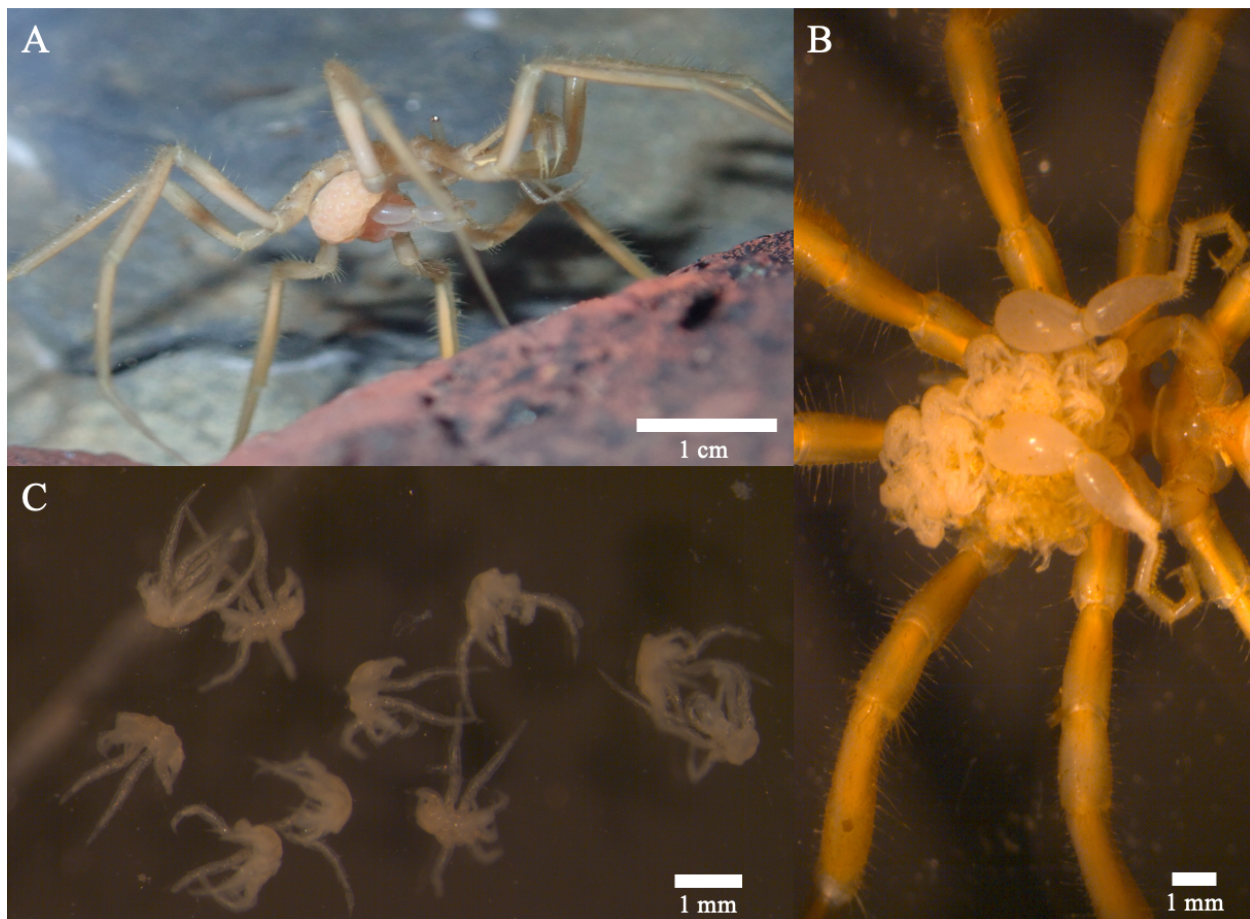
**Table 1:** Collection period of each cohort and average ( $\pm$  SD) water temperature during collection period and 24 days prior to entire collection period (24 days before first and last collection date).

		Incubated at -1.8 °C			
Incubated at +1.0 °C	Measurement Temperature	-1.8 °C	-0.4 °C	+1.0 °C	+4.0 °C
	-1.8 °C	0.3119	0.0001	<0.0001	<0.0001
	-0.4 °C	0.1336	0.0009	<0.0001	<0.0001
	+1.0 °C	0.0674	0.0037	<0.0001	<0.0001
	+4.0 °C	<0.0001	0.0004	0.9880	0.3169

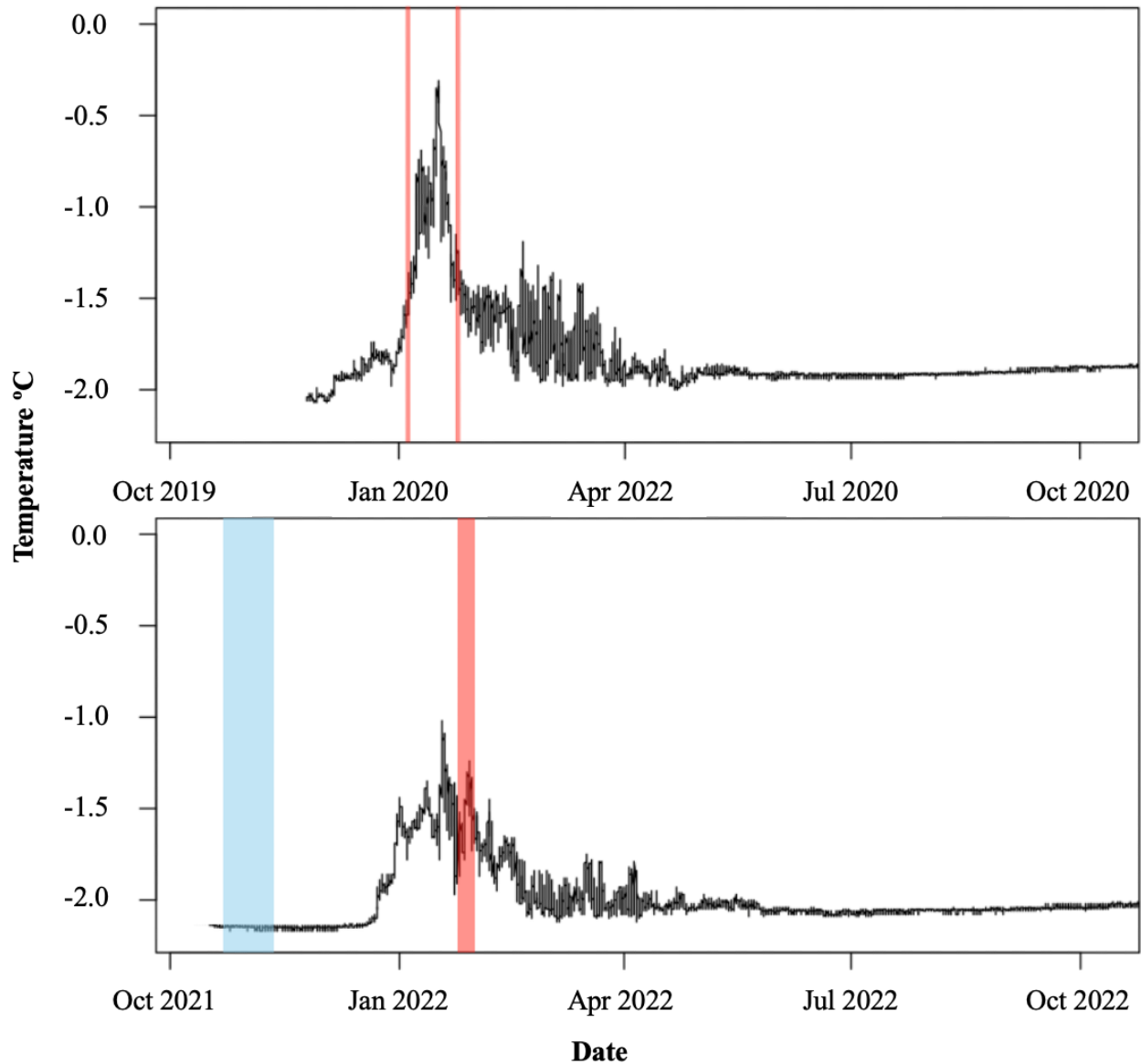
**Table 2:** p values of pairwise comparisons (Tukey) between two treatments (-1.8 °C and +1.0 °C) across four different measurement temperatures (-1.8 °C, -0.4 °C, +1.0 °C, +4.0 °C). Grey highlights indicated comparisons between the two treatment groups at the same measurement temperature.

		Late Austral Winter			
Austral Summer	Measurement Temperature	-1.8 °C	-0.4 °C	+1.0 °C	+4.0 °C
	-1.8 °C	0.0036	0.8359	<0.0001	<0.0001
	-0.4 °C	0.0011	1.0	0.008	<0.0001
	+1.0 °C	<0.0001	<0.0001	0.0002	<0.0001
	+4.0 °C	<0.0001	<0.0001	<0.0001	0.0067

**Table 3:** p values of pairwise comparisons (Tukey) between two seasons (austral winter and austral summer) across four different measurement temperatures ( -1.8 °C, -0.4 °C, +1.0 °C, + 4.0 °C). Grey highlights indicated comparisons between the two treatment groups at the same measurement temperature.

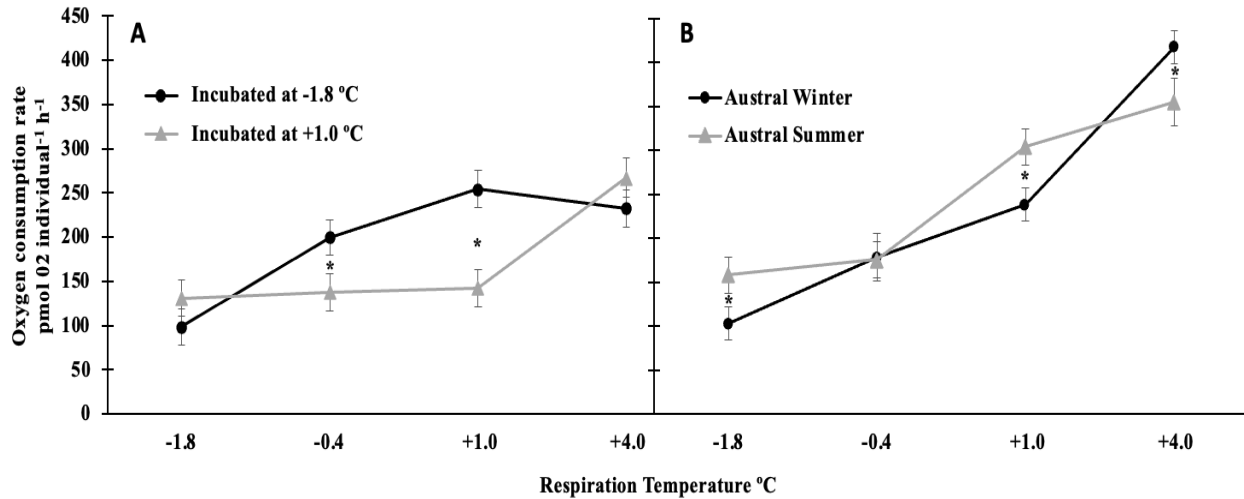


**Figure 1:** Photographs of *Nymphon australe*. A. Adult male *N. australe* carrying egg mass on ovigers. B. ventral view of adult *N. australe* carrying stage II larvae on ovigers. C. Stage II larvae of *N. australe* removed from egg mass. Scale indicated by white bar in lower left corner of photograph.



**Figure 2:** Seasonal temperature variation at the Intake Jetty at McMurdo Station in 2019-2020 (top) and 2021-2022 (bottom). Blue highlights indicate the times when animals were collected in the late austral winter (October - November) and red highlights show the collection periods in

the austral summer (January). Collection periods are indicative of temperature regimes for larvae used in seasonal acclimatization study.



**Figure 3: A)** Oxygen consumption (pmol O<sub>2</sub> individual<sup>-1</sup> h<sup>-1</sup>) of Stage II larvae of *Nymphon australe* from the intake jetty at McMurdo Sound after being maintained in the laboratory for 24 d at -1.8 °C (black circles, black line) or +1.0 °C (grey triangles, grey line), measured at four different temperatures. Asterisks (\*) indicate a significant difference ( $p < 0.001$ ) between the oxygen consumption rates of cohorts at a particular temperature. Error bars are the 95% confidence intervals around the grand mean of oxygen consumption of three cohorts at -1.8 °C and three cohorts at +1.0 °C. **B)** Oxygen consumption rate of stage II larvae of *Nymphon australe* from the intake jetty at McMurdo Sound collected during the late austral winter (October 28<sup>th</sup> to November 19<sup>th</sup>) (black circles, black lines) and the austral summer (January 7<sup>th</sup> to January 31<sup>st</sup>) (gray triangles, gray lines), measured at four different temperatures. Asterisks (\*) indicate a significant difference ( $p < 0.001$ ) between the oxygen consumption rates of seasons at a particular temperature. Error bars are the 95% confidence intervals around the grand mean of oxygen consumption of seven cohorts from the late austral winter and seven cohorts from the austral summer.

## Chapter 5: Conclusions and Future Directions

This dissertation aimed to understand the effects of increasing ocean temperatures on early life history stages of Antarctic invertebrates. Early life history stages such as embryos and larvae are often considered to be the most vulnerable points in an organism's life cycle (Bressan et al. 1995, Cowart et al. 2009, Walther et al. 2013), as they are thought to lack in physiological complexity (Brown et al. 2004, Pörtner and Farrell 2008), and are limited in their ability to respond to stressors using coping mechanisms such as acclimation (Brown et al. 2004, Pörtner and Farrell 2008, Rombough 1997, Flynn and Todgham 2018). Here, I show the effects of temperature on early embryonic cleavage and metabolism, processes that are not only vital for success but common to all metazoans. Overall, thermal sensitivity was high for both metrics as indicated by high  $Q_{10}$  values. High thermal sensitivity may indicate high vulnerability to climate change, as increasing temperatures, increase the metabolic costs of development (Flynn and Todgham 2018), decrease the efficiency of energy utilization (Iftikar et al. 2014, Schute 2015), and require energy to be allocated for stress responses (Schneider 2004, Sokolova et al. 2012, Todgham and Mandic 2020). Acclimation is one mechanism that organisms have to compensate when faced with stressors such as increasing temperatures, but this process is thought to be highly limited in Antarctic ectotherms (Pörtner et al. 2007, Peck et al. 2010, Peck et al. 2014, Peck 2018). Here, I present for the first time evidence of metabolic acclimation in larvae of Antarctic invertebrates, a process that infers greater resiliency in the face of warming oceans.

In Chapter 2, I investigated the effects of temperature on the early embryonic cleavage phase of two species of pycnogonid (*Nymphon australe* and *Ammothea glacialis*) and two species of nudibranch (*Tritoniella belli* and *Tritonia challengeriana*). Overall, temperature increased cleavage rate across the whole temperature range. Thermal sensitivity (as indicated by  $Q_{10}$ ) was high throughout the temperature range ( $Q_{10} > 4$ ), but was highest at the lowest end of the temperature range which may represent a lower limit to the ability of metazoan life to fully adapt to the lowest ocean temperatures (Pearse et al. 1991, Peck 2016). Significant Arrhenius breakpoints were seen in 10 of the 17 experiments, suggesting a transition between rate-limiting processes as temperature increases (Hoegh-Guldberg and Pearse 1995, Clarke 2017, Crapse et al. 2021). In future studies, I would like to expand the temperature range to capture not only

breakpoints at the lowest temperatures but also the upper thermal limits of early cleavage. Upper thermal limits were only seen in *Tritonia challengeriana* at temperatures above +1.62 °C, and one egg mass of *Tritoniella belli* at temperatures above +2.6 °C. These upper thermal limits indicate the failure of basic biological processes such as oxygen delivery systems, and denaturing proteins (Pörtner and Farrell 2008, Miller and Stillman 2012, Ángeles-González et al. 2020), and are important to understand how much warming early cleavage stages can withstand if they do not acclimate. The current study was limited to 12 temperature treatments due to the limitations of the thermal block. In future studies, I would like to increase the number of temperatures to more accurately capture the location of the Arrhenius breakpoint temperature. Future studies looking at the underlying mechanisms, such as gene expression, protein folding and stability, mitochondrial respiration, and increases in viscosity, may allow us to understand what processes are being impacted at certain temperatures that are dictating the changes I show here.

In Chapter 3, I investigated the effects of temperature on the oxygen consumption (as a proxy for metabolism) of early life history stages of two species of pycnogonid (*Nymphon australe* and *Ammothea glacialis*) and two species of nudibranch (*Tritoniella belli* and *Tritonia challengeriana*). Overall, increases in temperature increased oxygen consumption across all stages of development, with high thermal sensitivity in 8 of the 12 developmental stages across all species. If not met with an acclimatory response, the increased oxygen consumption rates of lecithotrophic organisms may push organisms outside of energetic equilibrium with limited abilities to recuperate energy through feeding (Hoegh-Guldberg and Emlet 1997, Sokolova et al. 2012). At these increased temperatures, it is believed that there is a greater increase in metabolic cost per temperature increase (Flynn and Todgham 2018), along with an increase in proton leaking, which decreases the efficiency of energy production (Iftikar et al. 2014, Schute 2015). The combination of these mechanisms may push thermally sensitive organisms out of energetic equilibrium and may increase their vulnerability to climate change if stress responses such as acclimation are not induced. Similar to Chapter 2, in future studies, I would like to expand the temperatures at which oxygen consumption was recorded (to more than four) to better capture not only the effects of temperature but also the temperatures at which Arrhenius breakpoints occur, indicating changes in rate-limited processes. With increased numbers of temperature

treatments, we may be able to see if oxygen consumption is affected by temperature in a similar way that early cleavage is specifically at the lowest temperatures.

In Chapter 4, I investigated the acclimatory ability of a single larval stage of *N. australe* to increasing temperatures. Acclimation is thought to be highly limited in Antarctic fauna, with the acclimatory ability of most fish in the Antarctic taking 2-4 times longer than temperate relatives (reviewed in Peck et al. 2014). Acclimation in Antarctic invertebrates is rarely studied, although the few studies that have investigated it show either extended acclimation periods of several months or no signs of acclimation (Peck et al. 2009, Peck et al. 2014, Morley et al. 2019), which increases the vulnerability of Antarctic invertebrates to a changing climate. Here, I show for the first time that acclimation is not only possible in vulnerable early life history stages but also occurs in ecologically relevant time frames. Larvae of *N. australe* were able to acclimate to temperatures of +1.0 °C after 24 days of exposure by decreasing their oxygen consumption at any given temperature below those acclimated to the ambient -1.8 °C. The present study did not see signs of seasonal acclimatization in larvae of *N. australe*, potentially due to the low temperature difference (~0.60 °C) between austral winter and austral summer not being enough to elicit a stress response. However, the ability for larvae to acclimate to increases in temperatures above what is seen in the high Antarctic suggests that vulnerable early life history stages may be able to compensate for increases in temperature during future warming scenarios and during seasonal temperature increases. In future studies, I would like to investigate other larval stages of *N. australe* to investigate if all larval stages are capable of acclimating or if certain stages, which are either more or less physiologically advanced, may be bottlenecks for populations if they cannot acclimate. I would also like to investigate early life history stages of other taxa to see if acclimation is limited in all Antarctic invertebrates as suggested by Peck (2018).

Overall, my PhD work shows high thermal sensitivity of metabolism and early embryonic cleavage in early life history stages of Antarctic invertebrates. These high thermal sensitivities are often attributed to high vulnerability to increasing temperatures as climate change warms our oceans. However, this dissertation shows hope as early life history stages, such as larvae of *N.*

*australe*, which are considered to be particularly vulnerable to stressors, show signs of being able to acclimate to warming temperatures. Although it is difficult to predict which species may be able to compensate for increasing temperatures, future winners may come down to not who can acclimatize, but who can do so in time (Sandblom et al. 2014). These studies are part of a larger picture as we try to understand which species may be threatened by increasing global temperatures.

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Appendix

Supplemental Material for Chapter 3

<i>N. australe</i>			
	Comparison	Q <sub>10</sub>	p-value
N-I	-1.8 to -0.4 °C	1.85	0.994
	-0.4 to +1.0 °C	7.28	0.3081
	+1.0 to +4.0 °C	5.59	<0.0001
	-1.8 +4.0 °C	4.56	<0.0001
N-II	-1.8 to -0.4 °C	14.19	<0.0001
	-0.4 to +1.0 °C	18.34	<0.0001
	+1.0 to +4.0 °C	2.51	0.0006
	-1.8 +4.0 °C	6.16	<0.0001
N-III	-1.8 to +1.0°C	2.71	0.7954
	+1.0 to +4.0 °C	1.07	1.0000
	-1.8 +4.0 °C	1.62	0.8977

S1: Q<sub>10</sub> values between each subsequent temperature for three stages of *N. australe* (N-I, N-II, N-III) and significance differences in oxygen consumption between temperatures (p-values).

<i>A. glacialis</i>			
<b>Comparison</b>		<b>Q<sub>10</sub></b>	<b>p-value</b>
<b>A-I</b>	<b>-1.8 to -0.4 °C</b>	0.47	0.9998
	<b>-0.4 to +1.0 °C</b>	2.31	0.9994
	<b>+1.0 to +4.0 °C</b>	3.90	0.0049
	<b>-1.8 +4.0 °C</b>	2.07	0.0030
<b>A-II</b>	<b>-1.8 to -0.4 °C</b>	4.55	0.2256
	<b>-0.4 to +1.0 °C</b>	1.41	1.0000
	<b>+1.0 to +4.0 °C</b>	3.99	<0.0001
	<b>-1.8 +4.0 °C</b>	3.20	<0.0001
<b>A-III</b>	<b>-1.8 to -0.4 °C</b>	2.35	0.9103
	<b>-0.4 to +1.0 °C</b>	4.22	0.1913
	<b>+1.0 to +4.0 °C</b>	5.29	<0.0001
	<b>-1.8 +4.0 °C</b>	4.12	<0.0001

**S2:** Q<sub>10</sub> values between each subsequent temperature for three stages of *A. glacialis* (A-I, A-II, A-III) and significance differences in oxygen consumption between temperatures (p-values).

<i>Tritoniella belli</i>			
	<b>Comparison</b>	<b>Q<sub>10</sub></b>	<b>p-value</b>
<b>EV</b>	<b>-1.8 to -0.4 °C</b>	11.12	0.0046
	<b>-0.4 to +1.0 °C</b>	10.32	0.0058
	<b>+1.0 to +4.0 °C</b>	4.14	<0.0001
	<b>-1.8 +4.0 °C</b>	6.55	<0.0001
<b>MV</b>	<b>-1.8 to -0.4 °C</b>	9.84	0.0591
	<b>-0.4 to +1.0 °C</b>	16.25	0.0065
	<b>+1.0 to +4.0 °C</b>	0.99	1.0000
	<b>-1.8 +4.0 °C</b>	3.39	<0.0001
<b>LV</b>	<b>-1.8 to -0.4 °C</b>	0.30	0.9878
	<b>-0.4 to +1.0 °C</b>	629.02	<0.0001
	<b>+1.0 to +4.0 °C</b>	3.46	0.4154
	<b>-1.8 +4.0 °C</b>	6.70	<0.0001

**S3:** Q<sub>10</sub> values between each subsequent temperature for three stages of *Tritoniella belli* (EV, MV, LV) and significance differences in oxygen consumption between temperatures (p-values).

<i>Tritonia challengeriana</i>			
	<b>Comparison</b>	<b>Q<sub>10</sub></b>	<b>p-value</b>
<b>EV</b>	<b>-1.8 to -0.4 °C</b>	9.99	0.0002
	<b>-0.4 to +1.0 °C</b>	1.40	0.9999
	<b>+1.0 to +4.0 °C</b>	14.67	<0.0001
	<b>-1.8 +4.0 °C</b>	7.58	<0.0001
<b>MV</b>	<b>-1.8 to -0.4 °C</b>	6.80	0.0084
	<b>-0.4 to +1.0 °C</b>	10.50	0.0002
	<b>+1.0 to +4.0 °C</b>	3.79	<0.0001
	<b>-1.8 +4.0 °C</b>	5.58	<0.0001
<b>LV</b>	<b>-1.8 to -0.4 °C</b>	1.89	0.9965
	<b>-0.4 to +1.0 °C</b>	2.97	0.8418
	<b>+1.0 to +4.0 °C</b>	10.49	<0.0001
	<b>-1.8 +4.0 °C</b>	5.10	<0.0001

**S4:** Q<sub>10</sub> values between each subsequent temperature for three stages of *Tritonia challengeriana* (EV, MV, LV) and significance differences in oxygen consumption between temperatures (p-values).