BODY SIZE IN ANTARCTIC AND TEMPERATE SEA SPIDERS: THE ROLE OF TEMPERATURE AND OXYGEN

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ABSTRACT

Body size is an important part of an organism's physiology and is perhaps one of the most important traits determining an individual's performance and life history. In Antarctica, a number of marine species reach unusually large body sizes compared to relatives in temperate or tropical regions, a phenomenon known as polar gigantism. While there are many potential explanations for why these animals reach such large sizes, the hypothesis that has received the most attention is the oxygen-temperature hypothesis (OTH). This hypothesis posits that polar gigantism arose from a combination of cold-driven low metabolic rates and high oxygen availability in the polar oceans. If the oxygen-temperature hypothesis indeed underlies polar gigantism, then polar giants may be particularly susceptible to warming temperatures.

In this dissertation, I explore the effects of temperature on the physiology of Antarctic and temperate pycnogonids (sea spiders) as a way of testing an underlying principle of the OTH that large bodied animals face stricter limits to aerobic performance as temperatures warm. First, I tested the effects of temperature on performance using two genera of giant Antarctic sea spiders (Pycnogonida), *Colossendeis* and *Ammothea*, across a range of body sizes. I found no support for the oxygen-temperature hypothesis but discovered differences in thermal responses between species. I found that the porosity of the animals' cuticle increased with body size, which may allow these animals to compensate for the increasing metabolic demand from elevated temperatures and longer diffusion distances of larger animals by facilitating diffusive oxygen supply. I also tested whether temperature induced oxygen limitation in two species of temperate sea spiders by measuring oxygen consumption at a range of temperatures. Here, I found that the aerobic metabolism of temperate pycnogonids does not appear to be oxygen limited at elevated temperatures, suggesting that the generally small size of sea spiders does not reflect constraints

on oxygen supply to larger bodies in warmer environments. Finally, I measured the thermal sensitivity of the Antarctic pycnogonid, *Ammothea glacialis*, over ontogeny. Antarctic organisms are thought to be highly stenothermal meaning they can only function within a narrow temperature range, but little work has tested if this is true over ontogeny. I tested this idea by measuring the oxygen consumption of larvae, juveniles, and adults of *A. glacialis* at a range of temperatures. I found temperature sensitivity at all stages but particularly in adults. Together, this work shows that pycnogonids, both temperate and Antarctic are affected by elevated temperatures, but these effects are stronger in some taxa than others. While elevated temperature from ocean warming will undoubtedly have profound effects on the physiology of pycnogonids, giant pycnogonids appear to have found a way around these oxygen-temperature related constraints by increasing cuticle porosity. However, this work is just a piece of the larger puzzle on how climate change will affect the Antarctic benthos and emphasizes our need for a better understanding about the physiology of Antarctic ectotherms.

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CHAPTER 1: BACKGROUND

"Body size and temperature dominate the biological universe."

(Kingsolver and Huey 2008)

Introduction

Body size is perhaps one of the most important traits determining an individual's physiology, performance, and life history (Kingsolver and Huey, 2008; Ohlberger, 2013). As such an important component of an organism's physiology and ecology, organism size has long been recognized as a major field of study. In the 1980s several researchers (i.e. Peters, 1983; Calder, 1984; Schmidt-Nielsen and Knut, 1984) formalized a theoretical framework for understanding how body size influences form and function (Clarke and Fraser, 2004); this framework was called scaling. In this chapter, I will review concepts of biological scaling, how body size and temperature interact to influence an organism's physiology and life history, and finally how body size and temperature relate to the physiology of Antarctic organisms.

Body Size and Scaling

Most of the enormous literature on biological scaling assumes a power function ($Y=aM^b$) where Y is a biological process such as respiration rate, *a* is the scaling coefficient, M is body size or mass, and *b* is the scaling exponent (Huxley, 1932). When shape remains constant with growth, the constancy in shape with change in size is termed isometric growth or isometry (usually a null expectation in morphological scaling studies) and departure from isometry is termed allometry (LaBarbera, 1989). The most studied physiological variable in relation to scaling is metabolic rate. Hemmingsen (1960) identified a metabolic scaling relationship for mammals and birds which was later generalized to ectotherms (organisms whose body temperatures are determined by their surroundings), unicellular organisms, and even plants (West and Brown, 2005). In "metabolic scaling", the scaling exponent (b) has widely been accepted as ¾ (Brody and Lardy, 1946; Hemmingsen, 1960; Kleiber, 1961; McMahon *et al.*, 1983; Peters, 1983; Schmidt-Nielsen and Knut, 1984; Blaxter, 1989; Savage *et al.*, 2004; West and Brown, 2004). This ¾ relationship was even proclaimed a universal scaling law known as 'Kleiber's law' or the '3/4 power law' (Brody and Lardy, 1946; Hemmingsen, 1960; Kleiber, 1961). One important aspect of the ¾ power relationship is that for organisms which follow this rule, as log body mass (M) increases fourfold, log metabolic rate (Y) will increase threefold; thus, smaller animals have a higher

mass-specific metabolic rates than large animals (Glazier, 2006). However, the universality of the ¾ power law has repeatedly been questioned. Several researchers (i.e. Glazier, 2005, 2014; White and Kearney, 2014) have pointed out that in comparative datasets, scaling of metabolic rate can vary from 0 to 1 but most often from 2/3 to 1 under different conditions. Several studies have shown that many types of biotic (e.g. lifestyle, developmental stage, physiological status) and abiotic environmental factors (e.g. as oxygen availability, pH, and temperature) can affect the scaling of metabolic rate (reviewed in Glazier, 2018). This is important because body size scaling and temperature can actually act a fundamental constraint on characteristics of organisms (Gillooly *et al.*, 2001).

Temperature

Abiotic variables such as temperature affects nearly all aspects of an organism including behavior, physiology, and fitness (Angilletta and Dunham, 2003; Kingsolver and Huey, 2008). In large part, the ubiquitous importance of temperature stems from its direct effects on biochemical and biological processes such as oxygen consumption (Hochachka and Somero, 2002; Peck, 2016). This is especially true of ectotherms, organisms whose body temperatures closely track that of their immediate surroundings. Ectotherms are generally adapted to their characteristic thermal environments (Angilletta *et al.*, 2009; Somero, 2012) and outside of those 'normal' ranges, performance fails quickly. Ectotherms typically operate within a thermal performance curve where performance increases as temperature increases until it reaches a maximum at an optimal temperature region, then as temperatures continue to increase, performance falls rapidly until death (Huey and Stevenson, 1979; Gilchrist, 1995; Martin and Huey, 2008). Therefore, it is clear that both body size and temperature separately have profound effects on an organism's physiology and ecology, but how do these two variables interact? As a way of summarizing this complexity, Kingsolver and Huey (2008) coined three rules about body size and temperature: 1. Bigger is better, 2. Hotter makes you smaller, and 3. Hotter is better.

Body Size and Temperature

"Bigger is better" proposes that larger individuals will outperform smaller individuals within a population. Large body size has long been known to enhance many aspects of organismal performance and dominance (Peters, 1983; Litvak and Leggett, 1992; Congdon *et al.*, 1999; Kingsolver *et al.*, 2001; Kingsolver and Pfennig, 2004; Darling and Côté, 2008; Bonner, 2011). For example, in a meta-analysis Kingsolver and Huey (2008) found that larger individuals tended

to survive better, mate more frequently with a greater variety of partners, and produce more offspring. Additionally, size often plays a role in competition and predator-prey dynamics when prey can escape predation by reaching a large size (Persson *et al.*, 1996; Lundvall *et al.*, 1999) or an organism may grow large enough to prevent being overgrown by another otherwise superior spatial competitor (McFadden, 1986). While there are certainly benefits to large size, it also comes with certain costs including delayed maturation and increased energy and resource demands (Zamudio *et al.*, 1995; Gaillard *et al.*, 2000). Of course there are exceptions where bigger is not necessarily better, but these are rare; for example, alternative mating strategies (e.g. 'sneaker males') may enable smaller males to achieve greater mating success than larger males (Zamudio *et al.*, 1995; Moya-Laraño *et al.*, 2007) and poor or variable environmental conditions may prevent selection for larger body sizes in embryos through high and random mortality in the intertidal (Moran and Emlet, 2009).

The second "rule" of Kingsolver and Huey (2008), "hotter makes you smaller", is related to the temperature-size rule (TSR) which proposes that ectotherms that develop at higher temperatures will be relatively small as adults (Atkinson, 1994; Angilletta and Dunham, 2003). The TSR represents a form of phenotypic plasticity during ontogeny, in which higher temperatures increase growth rate and developmental rate but decrease the overall adult body size (Atkinson, 1994; Ohlberger, 2013). This idea can also be thought of as a "shift in size-at-age" rather than body size (Berrigan and Charnov, 1994); therefore, if the thermal sensitivity of development rate is greater than that of growth rate, then the increased temperature can lead to a reduction in body size, hence the idea that "hotter is smaller". Mechanisms underlying the TSR are not well understood but small body size at warmer temperature is thought to be caused by a decrease in cell size, a decrease in cell number, or both (Partridge *et al.*, 1994; James *et al.*, 1995; Zuo *et al.*, 2011) and differences in sensitivity for cell growth and division (Van der Have and De Jong, 1996). Additionally, in the nematode, *C. elegans*, a single nucleotide substitution can eliminate the temperature-size rule, suggesting that a simple genetic basis for the rule is possible (Kammenga *et al.*, 2007).

The temperature-size rule is often associated with Bergmann's rule, which postulates that a population or species in cooler climates tend to have larger adult body sizes (Kingsolver and Huey, 2008). Bergmann (1847) originally described a positive relationship between body size

and latitude in endotherms, but some authors have extended it to ectotherms through the temperature-size rule (Atkinson, 1994). The original Bergmann's rule described how smaller bodies have a greater capacity for heat dissipation; however, this does not apply to ectotherms as ectotherms do not have the same capacity for heat dissipation. Thus, the temperature-size rule was proposed to better explain size patters specifically in (Kingsolver and Huey, 2008; Gardner *et al.*, 2011). This however is not a universal response; as with all biological "rules" there are exceptions. For example, the opposite trend in which body size increases with warming has been reported for some marine fishes (Thresher *et al.*, 2007) and lizards (Chamaille-Jammes *et al.*, 2006). The trend of larger body size with increased temperature has also been frequently documented in species with seasonal environments that experience strong constraints on their life cycles (e.g. food scarcity) (Chown and Klok, 2003).

The TSR is also important within the context of climate change, especially climate warming; in the past few decades, warming-induced declines in mean body size have been reported in a number of organisms from both aquatic and terrestrial environments (Gardner *et al.*, 2011; Sheridan and Bickford, 2011) including crustaceans (Moore and Folt, 1993), fishes (Todd *et al.*, 2008; Genner *et al.*, 2010; Cheung *et al.*, 2013), amphibians (Reading, 2007), and birds and mammals (Yom-Tov and Geffen, 2011). This overall trend is supported by paleontological records from invertebrates which show smaller body sizes during periods of warming (Hunt and Roy, 2006) and larger body sizes during periods of cooling (Smith *et al.*, 2009). While this may not be true for all taxa (e.g. Chattopadhyay and Chattopadhyay, 2018), declines in body size have been suggested as a third "universal" response to global warming along with phenology and distributions of species (Visser and Both, 2005; Durant *et al.*, 2007; Gardner *et al.*, 2011).

The final idea of the relationship between body size and temperature explored by Kingsolver and Huey (2008) was that "hotter is better" (also known as the thermodynamic constraint hypothesis). We know that there is a strong positive relationship between optimal temperature and maximal performance or fitness (Huey and Stevenson, 1979; Gilchrist, 1995; Martin and Huey, 2008). All else being equal, higher temperatures should yield faster biochemical reaction which in turn should improve performance at the organismal level (Angilletta *et al.*, 2009). Additionally, higher temperatures also decrease both development and generation time (Gillooly *et al.*, 2002; Charnov and Gillooly, 2003) thereby increasing population growth and fitness

(Kingsolver and Huey, 2008). However, the idea that "hotter is better" is more complex than just direct effects of temperature on body size, because it describes an evolutionary pattern in which genotypes or species with relatively high optimal temperatures also have relatively high maximal performance or fitness (Hamilton, 1973; Bennett, 1987; Savage *et al.*, 2004; Frazier *et al.*, 2006). In essence, it is based on the thermodynamic argument (Savage *et al.*, 2004) that reaction rates inevitably increase with absolute temperature (because the kinetic energy of a system increases with absolute temperature); consequently maximum biochemical reaction rates of species adapted to warm temperature will be higher than those of species adapted to cold temperatures, when each is measured at its optimal temperature. There has been strong support for this idea across a diverse range of taxa including marine algae (Eppley, 1972), lizards (Garland Jr, 1994), insects (Savage *et al.*, 2004), and bacteriophages (Knies *et al.*, 2009). "Hotter is better" also says that cold-adapted species will not be able to keep up with warm-adapted species even if both were tested at their respective optimal temperature (Kingsolver and Huey, 2008) and that warm adapted species will outperform cold-adapted genotypes because high temperature inevitably accelerate chemical reactions. However, it is possible that changes in molecular and cellular structures will be able to compensate for any thermodynamic advantages of high temperature and thus, cold adapted species will still perform at the same level as warm adapted species (Angilletta *et al.*, 2009).

Together these three ideas that 1. Bigger is better, 2. Hotter makes you smaller, and 3. Hotter is better sum up much of the research and some of the biggest ideas surrounding the interaction of temperature and body size. These ideas have major implications for predicting 'winners' or 'losers' in climate change, but these effects are not likely to be straightforward. The strength of these predictions will depend of how well we understand the mechanisms behind these effects and the ubiquity of these affects among individuals, species, and taxa. In the long term, while body size responses to climate change will be strongly dependent on species-specific thermal requirements (Portner, 2002). Kingsolver and Huey (2008) attempted to put these ideas together and explained that as the climate warms, body size will most likely be reduced as a result of warm developmental temperatures, thus yielding smaller sized adults with reduced fitness. Over time, sustained evolution may favor a higher optimal temperature thereby increasing fitness and overcoming the reduced fitness from a small body size.

Antarctica and the evolution of polar gigantism

Based on temperature effects alone, cold-adapted polar stenotherms are thought to be some of the most vulnerable organisms in the face of climate warming (Somero, 2010). Many Antarctic marine ectotherms are highly stenothermal and can only survive and function within a narrow temperature range (Somero and DeVries, 1967; Clarke and Johnston, 1996; Peck, 2002; Seebacher *et al.*, 2005; Peck *et al.*, 2007; Pörtner *et al.*, 2007). Loss of function often occurs at temperatures only 1-2°C higher than ambient (Somero and DeVries, 1967; Peck and Conway, 2000; Pörtner *et al.*, 2007) and brief excursions to 5-10°C can be fatal (Pörtner *et al.*, 2007). Similarly, (Peck *et al.*, 2004) found that at between temperatures of 2°C and 5°C, the swimming ability of the scallop *Adamussium colbecki* and the righting ability of the limpet, *Nacella concinna* significantly declined at temperature increased. This loss of performance and failure of survival at temperatures only a few degrees above ambient has also been documented in the brachiopod *Liothyrell uva* (Peck, 1989), the bivalve *Limopsis marionensis* (Pörtner *et al.*, 1999), the clam *Laternula elliptica* (Peck *et al.*, 2004), the brittlestar *Ophionotus victoriae* (Peck *et al.*, 2009), and the isopod *Paraserolis polita* (Janecki *et al.*, 2010).

Many Antarctic animals are also thought to have 'finely tuned' metabolic rates that allow them to survive in near freezing sea water temperatures. Aguera Garcia *et al.*, (2017) created a dynamic energy budget for the Antarctic bivalve, *Laternula elliptica*, in which they described *L. elliptica* as having a "metabolism specifically adapted to low temperatures, with a low maintenance cost and a high capacity to uptake and mobilize energy, providing this organism with a level of energetic performance matching that of related species from temperate regions." As early as 1916, Krogh recognized that polar ectotherms were active at cold temperatures but temperate ectotherms were not when cooled to the same temperatures. Thus, Krogh (1916) hypothesized that polar species must have elevated metabolic rates in order to overcome the effects of low temperature. This idea led to concept of 'metabolic cold adaptation or metabolic cold compensation' (MCA) (Scholander *et al.*, 1953; Wohlschlag, 1964). Numerous studies (e.g. Crockett and Sidell, 1990; Johnston *et al.*, 1998; Detrich *et al.*, 2000; Guderley, 2004; Sommer and Pörtner, 2004) support MCA and show that populations living in cooler temperatures have higher metabolic rates and usually higher cellular process rates such as enzyme and mitochondrial activity than congeners living at warmer temperatures when both are measured at the same temperature. However, when measured within their 'normal' range of temperatures,

polar organisms tend to have much lower metabolic rates than their warmer counterparts (Clarke and Johnston, 1999; Peck and Conway, 2000; Peck, 2016). White *et al.*, (2011) argued that while some compensation in cellular processes and in whole-animal metabolism is evident in Antarctic marine species, it is insufficient to move large-scale comparisons of oxygen consumption with temperate and tropical species outside Arrhenius expectations (Peck, 2018).

In addition to stenothermality and 'fine-tuned' metabolism, another unusual feature found in Antarctica is polar gigantism. Polar gigantism refer to species that reach unusually large body sizes compared to relatives in temperate or tropical regions (Wolff, 1956a; b; Arnaud, 1974; De Broyer, 1977). While there are a few other definitions for gigantism (Arnaud, 1974; De Broyer, 1977), Peck and Chapelle (1999) considered an organism a 'giant' if its body length was in the top 5% for its taxon within a particular habitat. Polar gigantism (reviewed in Moran and Woods, 2012) has been documented in numerous taxa of marine organisms including copepods (Hop *et al.*, 2006), pteropod molluscs (Weslawski *et al.*, 2008), cephalopod molluscs (Rosa and Seibel, 2010), chaetognaths (McLaren, 1966), foraminiferans (Mikhalevich, 2009), amphipod crustaceans (De Broyer, 1977), isopod crustaceans (Luxmoore, 1982), sponges (Dayton and Robilliard, 1971), polychaete annelids (Hartman, 1964), echinoderms (Dahm, 1996) and pycnogonids (Child, 1995).

Polar giants evolved in a unique ecosystem that is thought to have given rise to such large body sizes. Around 34 mya the Antarctic continent separated from South America when the Drake Passage opened which initiated a deep-water flow in the Antarctic circumpolar current (Maldonado *et al.*, 2003, 2014; Livermore *et al.*, 2004). Prior to the closing of the Drake Passage, sea temperatures around Antarctic used to be about 10-12°C warmer than at present (Zachos *et al.*, 2008), over the last 15 million years, temperatures in the Southern Ocean cooled and this region became the coldest and most temperature-stable marine environment on Earth (Barker and Thomas, 2006; Peck *et al.*, 2014). Today in the shallow near shore waters of the Ross Sea, which is a deep bay of the Southern Ocean about 200 miles from the South Pole, water temperatures vary by less than 1°C above the freezing point of water (-1.9°C) all year round (Hunt *et al.*, 2003; Orsi and Wiederwohl, 2009). Other, more northerly areas around Antarctica are somewhat warmer and more variable, especially along the Antarctic peninsula and South Shetland and South Orkney Islands; there, near-shore water temperatures each temperatures up

to 2°C (Clarke and Leakey, 1996; Aronson *et al.*, 2007; Clarke *et al.*, 2008; Martinson *et al.*, 2008; Schram *et al.*, 2015). The cooling of the Southern Ocean not only brought about physical changes to the sea and land but also profound changes to the biodiversity (Peck, 2018). Isolation, extremely cold temperatures, and repeated glaciation produced a unique marine fauna and facilitated the evolution of unusual polar adaptations such as the formation of glycoprotein antifreeze in ice fish (Devries and Lin, 1977), absence of red blood cells in channichthyid fish (Hemmingsen and Douglas, 1977), the absence of standard heat shock response in several species (Peck, 2018), and unusually large body sizes (polar gigantism, Wolff, 1956a; b; Arnaud, 1974; De Broyer, 1977) .

There are a number of hypotheses about the ecological and evolutionary factors that underlie polar gigantism (reviewed in Moran and Woods, 2012; Verberk and Atkinson, 2013). Many taxa from polar oceans share a common evolutionary history with lineages found in the deep sea where large body sizes are also common (Zinsmeister and Feldmann, 1984; Clarke, 2003). Other explanations are ecological, e.g. that habitats where food is constantly or seasonally scarce drove evolution of larger body sizes, because larger bodies are more resistant to starvation (Cushman *et al.*, 1993; Arnett and Gotelli, 2003), or a release from interspecific competition (Geist, 1987; Zeveloff and Boyce, 1988; Blackburn *et al.*, 1999). Another hypothesis focuses on developmental mechanisms related to the temperature-size rule in which organisms that are reared in cooler temperatures grow to a larger size. However, the magnitude of difference in size between polar giants and closely related nonpolar species is too great to be fully explained by the temperature-size rule (Moran and Woods, 2012). The hypothesis that received the most attention (McClain and Rex, 2001; Chapelle and Peck, 2004; Woods *et al.*, 2009; Verberk *et al.*, 2011) is the oxygen-temperature hypothesis which postulates that polar gigantism stems from a combination of cold-driven low metabolic oxygen demand and high oxygen availability (Peck and Chapelle, 1999; Pörtner, 2002) due to the upwelling of deep waters around Antarctica and low biological demand (Keeling *et al.*, 2010).

In comparison with terrestrial environments, animals in aquatic environments experience lower levels of oxygen, slower oxygen diffusion, and higher energetic costs of ventilation and circulation (Ohlberger, 2013). In addition, for ectotherms, as temperatures increase, metabolic oxygen demand increases even more steeply than oxygen supply (von Bertalanffy, 1960).

Because larger animals already have a more difficult time getting oxygen into their internal tissues (increased metabolic demand and longer diffusion distances), this mismatch of oxygen supply to demand creates a size threshold in which larger animals face greater challenges to matching oxygen uptake with whole-body oxygen requirements (von Bertalanffy, 1960; Atkinson and Sibly, 1997; Woods, 1999; Verberk and Atkinson, 2013). In aquatic environments, this challenge is further escalated because the oxygen content of water is lower than air and water is much denser and more viscous, thus raising the costs of gas exchange (Perrin, 1995; Atkinson *et al.*, 2006; Makarieva *et al.*, 2008; Verberk and Atkinson, 2013). In the Southern Ocean, the overall oxygen saturation is high compared to temperate and tropical regions because of the Southern Ocean's upwelling and low biological demand (Keeling *et al.*, 2010). However, the realized bioavailability of oxygen is lower in cold water than expected because the diffusivity of oxygen decreases with decreasing temperature due in part to effects of viscosity, outweighing the increase in solubility (Dejours, 1975; Verberk *et al.*, 2011; Verberk and Atkinson, 2013). Therefore, it is not only the high solubility and availability of dissolved oxygen in polar waters that allow for large body sizes, but also the low metabolic oxygen demand (from slowed chemical reactions involved in metabolism) resulting in a high ratio of oxygen supply to demand (Peck and Chapelle, 1999; Chapelle and Peck, 2004; Verberk and Bilton, 2011; Moran and Woods, 2012). Therefore, the mechanisms through which temperature will affect body size is also determined by oxygen and oxygen limitation is thought to be a primary determinant in setting an organism's thermal tolerance (Pörtner, 2002). If the high ratio of oxygen supply to demand in the Southern Ocean allows ectotherms to reach larger body sizes without experiencing increased oxygen deprivation (Peck and Chapelle, 1999; Chapelle and Peck, 2004; Verberk *et al.*, 2011; Moran and Woods, 2012), as temperatures continue to increase due to ocean warming then polar giants may be among some of the most vulnerable taxa to climate change (Peck and Chapelle, 1999).

Pycnogonids

Some of the best known examples of polar gigantism are found in the Class Pycnogonida (sea spiders) (Fry and Hedgpeth, 1969; Dell, 1972; Arnaud, 1974; Clarke and Johnston, 2003). There are approximately 1400 known species of sea spiders in 80 genera (Arnaud and Bamber, 1987; Gusso and Gravina, 2001; Arango and Wheeler, 2007; Peck, 2018) and they are distributed throughout the world's oceans (Dunlop and Arango, 2005), with over 20% of all species

occurring in the Southern Ocean. Sea spiders are typically small (only a few centimeters in leg span) and cryptic, hidden amongst algae, corals, sponges, and hydroids (Arnaud and Bamber, 1987; Veena *et al.*, 2010). However, in the deep sea and polar oceans, pycnogonids are quite conspicuous and can reach leg spans of over 70 cm (Fry and Hedgpeth, 1969; Arnaud and Bamber, 1987). While little is known about their behavior and ecology, pycnogonids appear to be abundant and ecologically important as predators and scavengers, especially on the Antarctic benthos (Slattery and McClintock, 1995; Clarke and Johnston, 2003; Moran *et al.*, 2018). Larvae of some pycnogonid species are internal parasites in cnidarians, while others are free-living but stay with their father until they become juveniles (Cano and López-González, 2009). Adult pycnogonids are solitary and free living, often living in close association with food hosts such as algae (Bamber and Davis 1982), anthozoans (Bamber, 1985; Arango, 2001), bryozoans (Fry, 1965), hydroids (Fry, 1965; Bain, 1991), or sedentary polychaetes (Arnaud and Bamber, 1987; Soler-Membrives *et al.*, 2011). In a recent study by Moran *et al.* (2018), Antarctic pycnogonid *Colossendeis megalonyx* were found not only to consume benthic cnidarians (Slattery and McClintock, 1995) and benthic polychaetes (Stout and Shabica, 1970), but also captured and fed on a variety of pelagic invertebrates.

Pycnogonids are group that presents a unique opportunity to study body size evolution in the context of temperature because they have a worldwide distribution genera (Arnaud and Bamber, 1987; Child, 1998; Arango and Wheeler, 2007), show size variation over several orders of magnitude (Fry and Hedgpeth, 1969; Dell, 1972; Arnaud, 1974; Clarke and Johnston, 2003), and have relatively simple gas exchange systems. Pycnogonids also lack specialized respiratory organs or pigments (Redmond and Swanson, 1968; Markl, 1986) and instead obtain oxygen and release CO_2 through diffusion either through pores in the cuticle or directly across thin parts of the cuticle (Davenport *et al.*, 1987; Woods *et al.*, 2017; Lane *et al.*, 2018). Oxygen is transported throughout the body both by a dorsal heart located in the trunk and by peristaltic contractions of the gut (Woods *et al.*, 2017), which extends into the legs and the chelifores. In addition, Woods *et al.*, (2017) demonstrated that the rate of peristalsis increased as oxygen levels were reduced, supporting the role of peristalsis in convective gas exchange.

Conclusions

Investigating the factors that underlie the evolution of polar gigantism is a potentially powerful tool for understanding the physical, ecological, and evolutionary principles that govern the

evolution of body size (Moran and Woods, 2012). This could also lead to a better understanding of the mechanistic links between body size and temperature and the consequences of this links for all levels of biological organization from species to ecosystems. Climate warming will indirectly affect an organism's body size though its effects on physiology and ecology, both over an individual's lifetime, as in the TSR, and over evolutionary time (Ohlberger, 2013). Thus, understanding the dynamics of body size with temperature (e.g. how the body size of an organism determines its response to temperature, the physiological underpinnings of these responses, and the mechanism that organisms use to overcome them) can help to identify key traits that shape the potential of different taxa to respond to climate change and provide insights into thermal tolerances, information that is currently lacking for many Antarctic species (Gardner *et al.*, 2011; Peck, 2018). Some of the fastest rates of regional warming on Earth have already been reported in the polar regions (Peck, 2018). In Antarctica, many regions have not seen significant environmental changes for thousands of years, so even small changes could have important consequences for entire ecosystems.

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CHAPTER 2: POLAR GIGANTISM AND THE OXYGEN-TEMPERATURE HYPOTHESIS: A TEST OF UPPER THERMAL LIMITS TO BODY SIZE IN ANTARCTIC PYCNOGONIDS

Abstract

The extreme and constant cold of the Southern Ocean has led to many unusual features of the Antarctic fauna. One of these, polar gigantism, is thought to have arisen from a combination of cold-driven low metabolic rates and high oxygen availability in the polar oceans (the "oxygen-temperature hypothesis"). If the oxygen-temperature hypothesis indeed underlies polar gigantism, then polar giants may be particularly susceptible to warming temperatures. We tested the effects of temperature on performance using two genera of giant Antarctic sea spiders (Pycnogonida), *Colossendeis* and *Ammothea*, across a range of body sizes. We tested performance at four temperatures spanning ambient (-1.8°C) to 9°C. Individuals from both genera were highly sensitive to elevated temperature, but we found no evidence that large bodied pycnogonids were more affected by elevated temperatures than small individuals; thus, these results do not support the predictions of the oxygen-temperature hypothesis. When we compared two species, *C. megalonyx* and *A. glacialis*, *C. megalonyx* maintained performance at considerably higher temperatures. Analysis of the cuticle showed that as body size increases, porosity increases as well, especially in *C. megalonyx*, which may compensate for the increasing metabolic demand and longer diffusion distances of larger animals by facilitating diffusive oxygen supply.

Introduction

Since the formation of the Antarctic Circumpolar Current during the Cenozoic Era, Antarctic marine species have evolved in one of the coldest and most temperature-stable marine environments on earth (Barker and Thomas, 2006; Peck *et al.*, 2014). Temperatures in the high Antarctic vary by less than 1.5°C above the freezing point of water (−1.9°C) all year round (Hunt *et al.*, 2003). This extremely cold, stable environment is thought to have driven the evolution of unusual polar adaptations such as the formation of glycoprotein antifreeze in ice fish (Devries and Lin, 1977). Many Antarctic marine organisms are also highly stenothermal and can only survive and function within a narrow temperature range (Somero and DeVries, 1967; Clarke and Johnston, 1996; Peck, 2002; Seebacher *et al.*, 2005; Peck *et al.*, 2007; Pörtner *et al.*, 2007); loss of function occurs at temperatures only 1-2°C higher than ambient (Somero and DeVries, 1967; Peck and Conway, 2000; Pörtner *et al.*, 2007), and even brief excursions to 5-10°C can be fatal (Pörtner *et al.*, 2007).

Another striking feature of the Antarctic fauna is the evolution of unusually large body sizes, a phenomenon known as polar gigantism (Wolff, 1956a; b; Arnaud, 1974; De Broyer, 1977). There are many hypotheses about the ecological and evolutionary factors that underlie polar gigantism (Moran and Woods, 2012; Verberk and Atkinson, 2013); one that has received considerable attention is the oxygen-temperature hypothesis which postulates that polar gigantism arose from a combination of cold-driven low metabolic oxygen demand and high oxygen availability (Peck and Chapelle, 1999; Pörtner, 2002) due to upwelling of deep waters around Antarctica and low biological demand (Keeling *et al.*, 2010). That body size is limited by temperature-oxygen interactions is an old idea. In 1960, von Bertalanffy (von Bertalanffy, 1960) first pointed out that for ectotherms, as temperatures increase, metabolic oxygen demand increases more steeply than oxygen supply. This creates a threshold body size above which diffusive supply cannot meet demand, meaning that at higher temperatures large animals face greater challenges to matching oxygen uptake with whole-body oxygen requirements (von Bertalanffy, 1960; Atkinson and Sibly, 1997; Woods, 1999; Verberk and Atkinson, 2013). The high ratio of oxygen supply to demand in the Southern Ocean is thought to allow ectotherms to reach larger body sizes without experiencing increased oxygen deprivation (Peck and Chapelle, 1999; Chapelle and Peck, 2004; Verberk and Bilton, 2011; Moran and Woods, 2012). However,

if the large body sizes of polar giants evolved in response to cold temperatures, then polar giants may be among the most vulnerable taxa in the face of ocean warming (Peck and Chapelle, 1999).

The pycnogonids, or sea spiders (Chelicerata, Pycnogonida), contain some of the most striking examples of polar gigantism (Fry and Hedgpeth, 1969; Dell, 1972; Arnaud, 1974; Clarke and Johnston, 2003). Pycnogonids inhabit most marine environments (Arnaud and Bamber, 1987; Dunlop and Arango, 2005) and there are approximately 1400 known species in 80 genera (Arnaud and Bamber, 1987; Child, 1998; Arango and Wheeler, 2007), with about 190 species occurring in Antarctic waters (Gusso and Gravina, 2001; Clarke and Johnston, 2003). Worldwide, most species are small as adults, with maximum leg spans of a few centimeters; Antarctic and abyssal species, however, can have leg spans of over 70cm (Arnaud and Bamber, 1987). On the Antarctic benthos, pycnogonids are abundant, diverse and ecologically important as predators and scavengers (Slattery and McClintock, 1995; Clarke and Johnston, 2003; Moran *et al.*, 2018). Pycnogonids do not have specialized respiratory organs or pigments (Redmond and Swanson, 1968; Markl, 1986), and obtain oxygen through diffusion across the cuticle (Douglas *et al.*, 1969; Davenport *et al.*, 1987; Lane *et al.*, 2018). Oxygen is transported throughout the body both by a dorsal heart located in the trunk and by peristaltic contractions of the gut (Woods *et al.*, 2017), which extends into the legs.

Woods et al. (2009) explored interactions between body size and oxygen availability in Antarctic sea spiders by measuring the righting ability of animals - a metric of physiological performance, for individuals across 12 species in five families under a range of oxygen saturations. While hypoxia strongly reduced performance, large-bodied animals were not more susceptible to oxygen limitation than small ones. Woods et al. (2009) listed three possible explanations for this surprising result. First, righting experiments were performed at near-ambient temperatures (-1.13 ± 0.02°C), and cold-induced depression of metabolic rate may have ameliorated the potential detrimental effects of large body size on oxygen supply. This interpretation is supported by results from Peck et al. (Peck *et al.*, 2007), who found interactive effects of oxygen and body size in the Antarctic clam, *Laternula elliptica*, but only at temperatures greater than 0°C. Second, large-bodied animals may have undergone evolutionary increases to their capacity for oxygen uptake and delivery to maintain performance as body size increased (i.e. symmorphosis; Weibel et al. (1991) and Woods *et al.*, (2009). Third, some species were much more sensitive to changes

in dissolved oxygen concentration than others; phylogenetic effects could have potentially masked interactions between body size, performance, and oxygen availability as their dataset combined multiple species from several different families of pycnogonids.

We directly tested the interactions between body size, temperature, and performance of Antarctic pycnogonids using animals of a range of sizes within congeneric and conspecific groups. We hypothesized that larger bodied-pycnogonids would show disproportionately poor performance compared to small bodied pycnogonids at elevated temperatures, consistent with the predictions of the oxygen-temperature hypothesis. We also predicted that like most other Antarctic ectotherms (Peck, 2002; Seebacher *et al.*, 2005; Pörtner *et al.*, 2007), Antarctic pycnogonids would be highly stenothermal and that small amounts of warming would have strong effects on performance.

Second, we explored the relationship between animal size and cuticular structure in the same taxa. Across different species and families, pycnogonids compensate for the greater diffusion distances and higher metabolic demand of larger bodies by increasing oxygen flux across the cuticle (Woods *et al.*, 2009), at least in part by increasing the porousness of the cuticle (Lane *et al.*, 2018). We examined the relationship between body size and cuticle structure (thickness and porosity) within two different species of giant Antarctic pycnogonids to test if cuticle morphology also changes to accommodate larger body size as animals grow.

Methods

Collection and Maintenance

Pycnogonids were collected on SCUBA near McMurdo Station, Antarctica (77° 51´ S, 166° 40´ E) between 10 and 40 m depth, in October and November 2015 and 2016. Pycnogonids were transported to McMurdo Station in coolers filled with chilled seawater (-1.8°C) and were kept in flow-through seawater tables maintained at 1-2°C above ambient seawater temperatures (-1.8°C) until used for experiments.

We collected animals from two genera, *Ammothea* (Ammotheidae) and *Colossendeis* (Colossendeidae). In McMurdo Sound, these are the two genera of sea spiders that attain very large body sizes as adults (leg spans > 10 cm) (Figure 1) and can be readily found in abundance and across a broad range of body sizes. Individuals were identified to species using the keys of Child (1995). When animals could not be identified to species unambiguously, we used genetic

barcoding (COI) to identify them (methods in Lane et al. 2017). In total, our dataset contained six species of *Colossendeis* (*C. australis*, n= 2, *C. hoeki*, n= 13, *C. megalonyx*, n= 20, *C. robusta*, n= 2, *C. scotti*, n= 2, and *C.* sp., n=3) and one species of *Ammothea* (*A. glacialis*, n=26).

Temperature-body size experiments

To determine if pycnogonid performance was affected by body size, temperature, and their interaction, we measured righting ability over a range of body sizes and temperatures. In 2015, we tested 30 individuals of *Colossendeis* at three different temperatures: -1.8, 4, and 9°C. Because the animals showed a sharp decline in performance between 4 and 9°C, in 2016 we added an intermediate temperature and tested an additional 12 individuals of *Colossendeis* at -1.8, 4, 7, and 9°C. Also, in 2016, we performed these tests on 26 individuals of *Ammothea glacialis* at all four temperatures. Righting experiments were carried out in a 500-L aquarium submerged in a 1000-L tank with temperature regulation provided by a submersible 'cold finger'/bath cooler (model PBC-2II, Neslab) and a heating element (immersion circulator, Fisher Scientific). This experiment was an acute temperature challenge rather than an emulation of ecologically relevant warming, so animals were moved directly from sea tables into water at the experimental temperature. The temperature of the water in the aquarium was always within 0.3°C of the target temperature. Air was constantly and gently bubbled into the aquaria to maintain 100% air saturation.

Each trial was performed with a single pycnogonid in the aquarium. Prior to each trial, each animal was allowed to adjust to its surroundings for 20 min (a length of time we had previously determined was sufficient for pycnogonids to return to their normal orientation and stance after being moved into reduced oxygen (Woods *et al.*, 2009) or a new experimental temperature (this study). Then, the animal was gently flipped upside-down and allowed to right itself; this process was repeated until we had counted the number of times the animal righted itself in a one-hour period. Each animal was tested at all three (n = 30) or four (n = 38) experimental temperatures. The order of temperature exposure was determined randomly for each individual and no animal was tested more than once per day. Each animal was assessed daily for normal appearance, movement, and behavior over the course of the experiment.

Cuticle morphometrics

After the performance of an animal had been assessed at all temperatures, the animal was blotted dry and weighed on an electronic balance (Model PE1600, Mettler Toledo) to obtain wet mass,

then photographed for morphological measurements. A subset of individuals was preserved in 100% EtOH for later genetic and cuticular analysis (preliminary tests established that EtOH preservation did not alter cuticular thickness or morphology). Cuticular analysis was performed on a combination of animals from the righting experiments (n = 10 *Colossendeis megalonyx* and n = 19 *Ammothea glacialis*) and 14 additional *C. megalonyx* and 8 *A. glacialis* collected during 2015 and 2016 at the same sites (weighed, photographed, and preserved as above). Measurements of cuticle morphometrics were not performed on other species of *Colossendeis* because of the comparatively small sample sizes. In total, we measured 24 *C. megalonyx* and 27 *A. glacialis* for four cuticular parameters: areal porosity (AP, %), cuticle thickness (CT, cm), surface area (SA, cm^2), and pore volume (PV, cm^3). All parameters were measured from digital images using Image J software (v.1.51j8, NIH) (Schneider *et al.*, 2012) (detailed methods in electronic supplementary material, S1).

Statistical analysis

We analyzed the performance data in three categories; all species of *Colossendeis* combined, *C. megalonyx* alone, and *A. glacialis* alone. Righting data for all groupings were strongly zero-inflated at 7 and 9 °C because some animals never righted themselves at these higher temperatures. Therefore, to determine if there was an interactive effect between mass and temperature on righting performance, we used a zero-inflated generalized linear mixed-effects model (ZIGLMM) fitted with the "glmmadmb" function in the "glmmADMB" package in R (Fournier *et al.*, 2012). The data for the masses of both *Colossendeis* and *Ammothea glacialis* were log-transformed to meet the assumptions of normality and to give reasonable distributions of the residuals. We used number of times an animal could right itself as the response variable (ZIGLMM was fitted with a Poisson distribution to account for count data), temperature treatments and size as explanatory variables, and incorporated individual pycnogonids as random effects. Pycnogonids that did not right themselves at our control temperature (-1.8°C) were excluded from the analyses (*Colossendeis hoeki* n=1, *Colossendeis megalonyx* n=4). The current *glmmADMB*package in R did not support post hoc tests; thus temperature thresholds were assessed by comparing 95% confidence intervals around mean righting rates at each temperature.

Most statistical analyses were performed in JMP (v. 13, SAS Institute Inc., Cary, NC) except the ZIGLMM which was performed in Rstudio (v.1.0.143) (RStudio Team, 2015).

To test if exposure to our highest experimental temperature (9°C) had detrimental effects on subsequent performance, we used a Student's t-test (data met all assumptions) to compare the righting performance of pycnogonids at -1.8°C between those that received -1.8°C as their first exposure (*Colossendeis* combined, n=12; *C. megalonyx*, n=5; *A. glacialis*, n=7) and those that received 9°C as their first exposure (*Colossendeis* combined, n= 11; *C. megalonyx*, n=6; *A. glacialis*, n=5). We also used an ANOVA (data met all assumptions) to determine if order of exposure and subsequently the impact of multiple days of testing, had detrimental effects on performance. We used the number of rightings as a response variable, order as an explanatory variable, and incorporated individual pycnogonids as random effects (*Colossendeis* combined, n=42; *C. megalonyx*, n=20, *A. glacialis*, n=26).

To determine how CT, PV, and SA scaled with body mass (g), we $\log_{10}$-transformed CT, PV, and SA, and fitted the data to ordinary least squares regressions. We then compared the slopes of our regressions against the scaling exponents expected from isometric geometric scaling; we considered our measured slopes to be significantly different from isometry if the 95% confidence intervals of the slope did not include the expected isometric scaling coefficient. To determine if scaling differed between species, we used ANCOVA to compare slopes between *C. megalonyx* and *A. glacialis*. We also compared the relationship between AP and body mass (g) for *C. megalonyx* and *A. glacialis* using linear regression.

Results

Temperature and righting

For all species and genera combined, temperature had a significant and negative effect on righting performance (ZIGLMM, $p<0.0001$) (Figure 2, 3; electronic supplementary material, table S2, S3). Across all individuals, righting frequency ranged from 1-75 rights per hour (RPH) at ambient temperature to 0-23 RPH at the highest temperature (electronic supplementary material, table S3). Average RPH decreased ~ten-fold for *Colossendeis megalonyx* and all *Colossendeis* combined between ambient and 9°C, and more than 20-fold for *A. glacialis*. The righting ability of individuals of *Colossendeis* combined and *C. megalonyx* was not impacted until 9°C (Figure 2; electronic supplementary material, table S4). RPH of individuals of *A. glacialis* dropped at each step increase in temperature starting at 4°C (Figure 2, electronic

supplementary material, table S4). No pycnogonids died and the behavior and activity level of animals did not visibly change over the course of the experiment. T-tests within each genus also showed no significant difference between the number of rightings at -1.8°C when we compared pycnogonids that had received their first treatment at -1.8°C to those that had received 9°C as the first exposure: *Colossendeis* (p=0.27), *Colossendeis megalonyx* (p=0.51), *Ammothea glacialis* (p=0.58). The order of exposure to experimental temperatures and impact of multiple days of testing also did not have any effect on the performance: *Colossendeis* combined (F=0.37; p=0.77), *Colossendeis megalonyx* (F=1.17; p=0.33), *Ammothea glacialis* (F=0.069; p=0.98).

Mass did not have a significant effect on RPH of *A. glacialis* (p=0.11) or on the combined *Colossendeis* individuals (p=0.44), and there was no significant interaction between temperature and body size in either group (*A. glacialis*, p=0.18; all *Colossendeis*, p=0.86) (Figure 3; electronic supplementary material, table S2). In contrast, mass did significantly affect RPH of *C. megalonyx* (p=0.002) and there was also a significant interaction between temperature and mass (p=0.005) for this group.

Cuticle morphometrics

Mass, SA, and CT all ranged over close to an order of magnitude in *C. megalonyx* and more than an order of magnitude for *A. glacialis*. No cuticle measurements were made for *Colossendeis* from other species, but mass for these ranged over two orders of magnitude from 0.21 to 21.80g.

The log-linear relationships between cuticle thickness (CT, cm) and mass (*M*, g) for the two genera were $CT_{C.megalonyx} = -2.26 \times M^{0.51}$ and $CT_{A.glacialis} = -2.21 \times M^{0.58}$. The slopes of the relationship between CT and *M* for both species were not significantly different from each other (ANCOVA, p= 0.1925). The 95% confidence intervals of the scaling exponents of the two groups (0.51 for *C. megalonyx* and 0.58 for *A. glacialis*, electronic supplementary material, table S4) overlapped substantially, but in neither case did the confidence intervals contain 1/3 (isometric scaling; electronic supplementary material, table S5). The relationship between surface area (SA, cm^2) and *M* was $SA_{C.megalonyx} = 1.45 \times M^{0.62}$ and $SA_{A.glacialis} = -1.31 \times M^{0.78}$. There was a significant difference in the slopes in the relationship of SA to *M* between *C. megalonyx* and *A. glacialis* (ANCOVA, p<0.0001*, Figure 4). The 95% confidence intervals of the scaling exponents did not include the predicted scaling exponent of 2/3 for either of the two groups (0.62 for *C. megalonyx* and 0.78 for *A. glacialis*) (electronic supplementary material, table S5). The

relationship between porosity (AP, %) and M was $AP_{C.megalonyx} = 0.18 \times M^{0.11}$ and $AP_{A.glacialis} = 0.10 \times M^{0.015}$. Because there is no expected scaling exponent for the relationship between AP and M, we could not make that comparison. However, the slope of this relationship was significantly higher for *C. megalonyx* than for *A. glacialis* (ANCOVA, p=0.0126, Figure 5). Finally, the relationship between (PV, cm^3) and M was $PV_{C.megalonyx} = -1.62 \times M^{1.50}$ and $PV_{A.glacialis} = -1.93 \times M^{1.61}$. There was no significant difference in slope of PV and mass between *C. megalonyx* and *A. glacialis* (ANCOVA, p=0.5026; Figure 4). The 95% confidence intervals of the scaling exponents for both species (1.50 for *C. megalonyx* and 1.61 for *A. glacialis*, electronic supplementary material, table S5) did not contain the expected scaling exponent of 1 (electronic supplementary material, table S5).

Discussion

Temperature strongly and negatively affected righting performance of all Antarctic pycnogonids in our study. These data are consistent with patterns seen in Antarctic bivalves (Pörtner *et al.*, 1999; Peck and Conway, 2000; Bailey *et al.*, 2005; L. S. Peck *et al.*, 2007), brachiopods (Peck, 1989), and limpets (Peck *et al.*, 2004), and with the overall paradigm of strong stenothermality of Antarctic marine ectotherms (Peck *et al.*, 2008). However, the two genera of sea spiders in our study differed in their sensitivity to temperature; for *Ammothea*, righting ability dropped at each temperature increment, and there was a two-fold drop between -1.8 and 4°C. For individuals of *C. megalonyx* alone, however, righting rates did not drop until our highest temperature treatment, 9°C. Antarctic ectotherms are typically very sensitive to small increases in temperature, so the ability of *C. megalonyx* to maintain performance up to 7°C and to survive excursions up to 9°C with no observed after effects on survival or behavior was intriguing (these temperatures are 7.5°C and 9.5°C, respectively, above the annual summer maximum of -0.5°C in McMurdo Sound (Hunt *et al.*, 2003). However, these results are consistent with Peck et al. (2009) who found that some Antarctic species such as the amphipod *Cheirimedon femoratus* survived temperatures up to 17.6°C when exposed to a rapid rate of warming (1°C day^{-1}). We used an acute thermal challenge, while previous studies (Bailey *et al.*, 2005; Peck *et al.*, 2007) that tested the effects of temperature on Antarctic invertebrates used slower ramping rates and/or longer exposure periods from 24 hrs (Peck *et al.*, 2007) to 20 days (Bailey *et al.*, 2005), both of which can increase thermal sensitivity (Peck *et al.*, 2009). It remains to be seen, therefore, whether

other types of Antarctic organisms can also survive large but short-term increases in temperature, or if the unique morphology and physiology of pycnogonids, particularly with regards to gas exchange, makes Antarctic species (particularly *Colossendeis*) unusually robust to short-term exposure to elevated temperatures.

In contrast to temperature, which had strong effects on performance, the effects of body size on performance varied. In two of three groupings (*A. glacialis* and all *Colossendeis* combined), we found no effects of body size, measured as mass, on righting performance. In *C. megalonyx*, however, righting performance increased with body size. Such an outcome could arise from several biomechanical or physiological mechanisms, e.g. a shift in the center of gravity or a hypermetric increase in muscle mass with body size. However, when data from all species of *Colossendeis* were combined, the intraspecific pattern was obscured, likely driven by the fact that the other *Colossendeis* species were much larger than *C. megalonyx* but righted themselves less frequently.

The significance of the interaction between mass and temperature also varied among groupings. Neither *A. glacialis* nor combined *Colossendeis* showed this interaction, suggesting that temperature affected righting performance similarly across all body sizes. These results are consistent with those of Woods et al. (2009) for a combined data set including 12 species from five families, in which hypoxia strongly affected performance but these effects were not size-dependent. We did, however, find an interaction between body size and temperature for *C. megalonyx*; compared with smaller animals, larger animals were more strongly affected by warm temperatures. This pattern was driven by a change in the relationship between body size and righting rates at the highest temperature, 9°C. At the three lower temperatures, larger animals righted themselves more times per hour than small animals; at 9°C, in contrast, RPH was reduced to close to 0 for all body sizes. Two interpretations of these results are that (1) larger animals were in fact more strongly affected by the highest temperature, supporting the temperature-size hypothesis, or (2) 9°C exceeded the performance threshold of most animals regardless of size. We think the latter is more likely , since more than half of the *C. megalonyx* did not right themselves at all at 9°C, and 9°C is higher than, or in some cases very close to, the thermal limits that have been observed in other Antarctic marine ectotherms (Bailey *et al.*, 2005; Peck *et al.*, 2007; Pörtner *et al.*, 2007). In general, these results suggest that temperature-size

interactions should be interpreted cautiously when measurements are made close to the physiological limits of performance.

Our experiments testing the temperature-size rule are consistent with previous work (Woods *et al.*, 2009) in showing that these organisms do not meet the predictions of the oxygen-temperature hypothesis; limitations of oxygen diffusion on performance do not increase with body size, regardless of whether oxygen is manipulated in isolation (as in Woods *et al.*, 2009)) or when metabolic oxygen demand increases with rising temperature (present study). One way to get around the constraints that limitation of oxygen diffusion sets on body size is to increase the surface-area-to-volume ratio as body size increases (Peck and Chapelle, 1999; Woods, 1999; Verberk and Atkinson, 2013). In the case of our two species, the scaling of surface area to mass was very close to geometric isometry for *C. megalonyx* (0.62) and not much greater than isometry for *A. glacialis* (0.78), similar to the interspecific relationship between SA and mass reported in Woods et al. (2009). Because pycnogonids acquire oxygen across the cuticle (Douglas *et al.*, 1969; Davenport *et al.*, 1987; Lane *et al.*, 2018), an alternative mechanism could be to increase the permeability of the cuticle to oxygen diffusion as body size increases. We found that cuticle thickness, pore volume, and areal porosity all increased with body mass for both *A. glacialis* and *C. megalonyx*. These results match an interspecific comparison (Lane *et al.*, 2018) demonstrating that larger-bodied species of sea spiders have proportionally greater pore volume than smaller species. Our results confirm that these patterns also occur across body sizes; as animals grow, the increasing metabolic oxygen demand of a larger body, and the longer diffusion distances within the body, are offset by increasing cuticular porosity. This increase in porosity likely allows pycnogonids to maintain their activity levels as they grow and may also in part explain why large-bodied pycnogonids are able to cope well with elevated temperatures (though other mechanisms may also be at play, such as ontogenetic changes to the circulatory system or a reduction in the impact of boundary layers with increased size (Statzner and Holm, 1989; Verberk *et al.*, 2011; Verberk and Atkinson, 2013). The increase in porosity with body size is also a potential example of ontogenetic symmorphosis, in that it represents a change in body structure that meets the increasing functional demands associated with larger body size.

Although both species showed an increase in porosity with body size, we also found differences in cuticle porosity between *A. glacialis* and *C. megalonyx*. Overall, the cuticle of *C. megalonyx*

was considerably more porous than *A. glacialis*, and this difference became more pronounced at larger body sizes. All else being equal, this suggests that *C. megalonyx* has a greater capacity for gas exchange, and therefore may be capable of higher levels of aerobic activity, than *A. glacialis*. Indeed, in >200 hours of casual observation in the laboratory and field, we most often saw *C. megalonyx* walking across the substrate, while other species were more stationary. Likewise, *C. megalonyx* is capable of rapid and active capture of pelagic prey (Moran *et al.*, 2018), and maximum escape speeds of *C. megalonyx* were almost twice as fast as those of *A. glacialis* (0.023 cm sec^{-1} vs 0.013 cm sec^{-1}) (Lane *et al.*, 2018). Greater cuticle porosity may be one mechanism that allows *C. megalonyx* to evolve both large size and maintain high levels of activity.

Porosity also scaled more steeply with size in *C. megalonyx* than in *A. glacialis*. This suggests that larger *C. megalonyx* may have a greater capacity for maintaining aerobic activity than small ones, which is consistent with the overall increase we found in righting performance with body size, although other factors might have affected this well. Ecologically, larger body size may confer a fitness benefit on *C. megalonyx* because larger, more mobile animals have increased probabilities of encountering and capturing food (Moran *et al.*, 2018). The disproportionate increase in porosity with size may also reflect an ontogenetic shift in ecological feeding modes from comparatively stationary, parasitic juveniles to more mobile and actively predatory adults. Mobile, active organisms are likely to have a higher aerobic scope than less active ones, which may confer greater thermal tolerance and a greater capacity to cope with warming sea temperatures (Pörtner *et al.*, 2007). Linkages between cuticle structure, functional performance, and behavior of *C. megalonyx* vs. *A. glacialis* strongly suggest that the two species inhabit different niches in the Antarctic benthos and may have different vulnerabilities to warming oceans.

Like other Antarctic ectotherms, pycnogonids are sensitive to elevated temperatures; but we did not find evidence that larger-bodied pycnogonids were more strongly affected than small ones. We propose that Antarctic pycnogonids can attain giant sizes not only because their metabolic rates are limited by cold temperatures, but also because the porosity of the cuticle increases as they get larger. Future ocean warming will undoubtedly have profound effects on Antarctic marine organisms and ecosystems. However, even among these polar giants, whose large body

size is thought to confer a particular vulnerability to climate change (Peck and Chapelle, 1999; Woods *et al.*, 2009), predicting ‘winners’ or ‘losers’ requires a more nuanced, whole-organism approach that integrates across many levels of a species’ ecology, life history, and physiology.

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Figures

Figure 1. Top Left: *Ammothea glacialis* Photo: Timothy R. Dwyer, PolarTREC 2016/courtesy of ARCUS, Bottom Left: cuticle cross-section of *A. glacialis*, Top Right: *Colossendeis megalonyx* Photo: Timothy R. Dwyer, PolarTREC 2016/courtesy of ARCUS, Bottom Right: cuticle cross-section of *C. megalonyx.* Arrows on bottom pictures indicate pores.

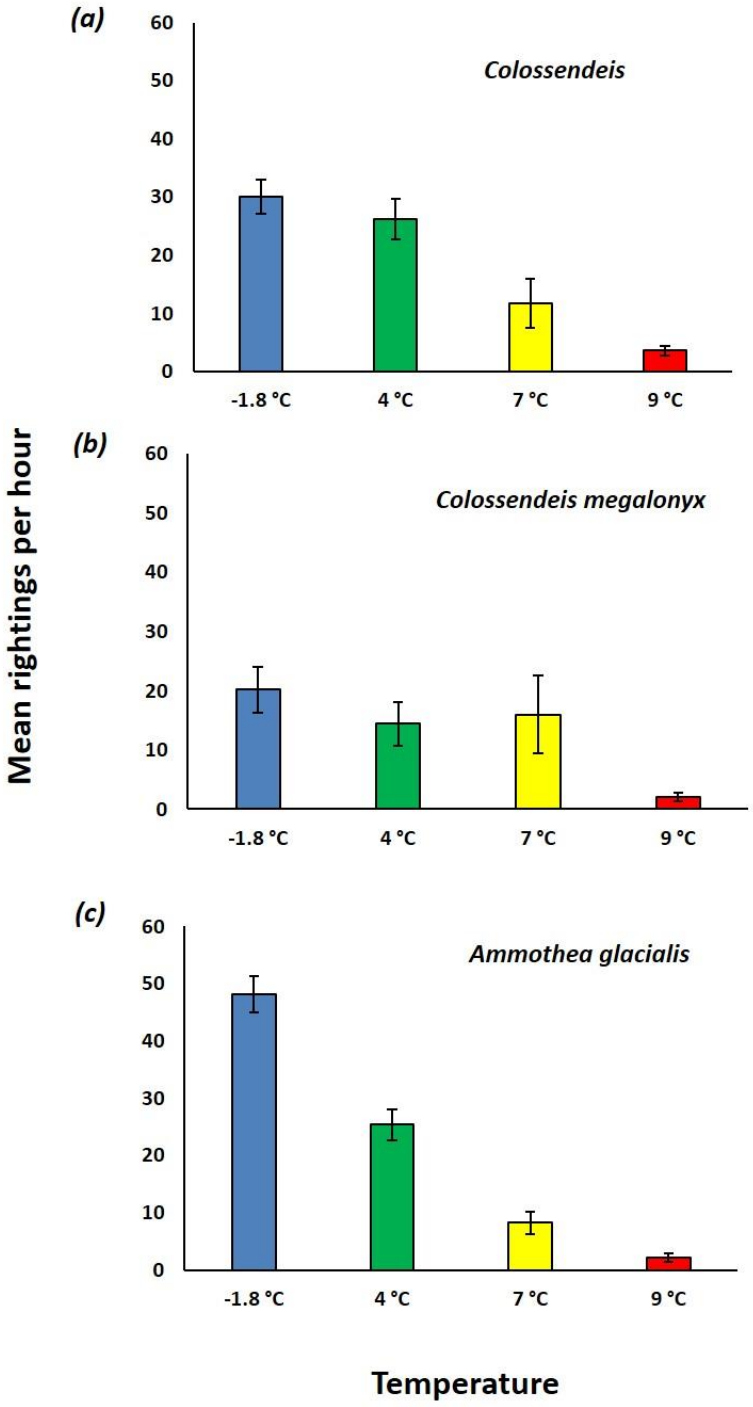

Figure 2. Rate of self-righting (mean ±SE) rightings per hour for all species of (a) *Colossendeis* $p<0.0001$, (b) *Colossendeis megalonyx* $p<0.0001$ and (c) *Ammothea glacialis* $p<0.0001$, at ambient (-1.8°C) and three elevated (4°, 7°, and 9°C) temperature treatments. See electronic supplementary material, table S4 for summary of 95% confidence intervals.

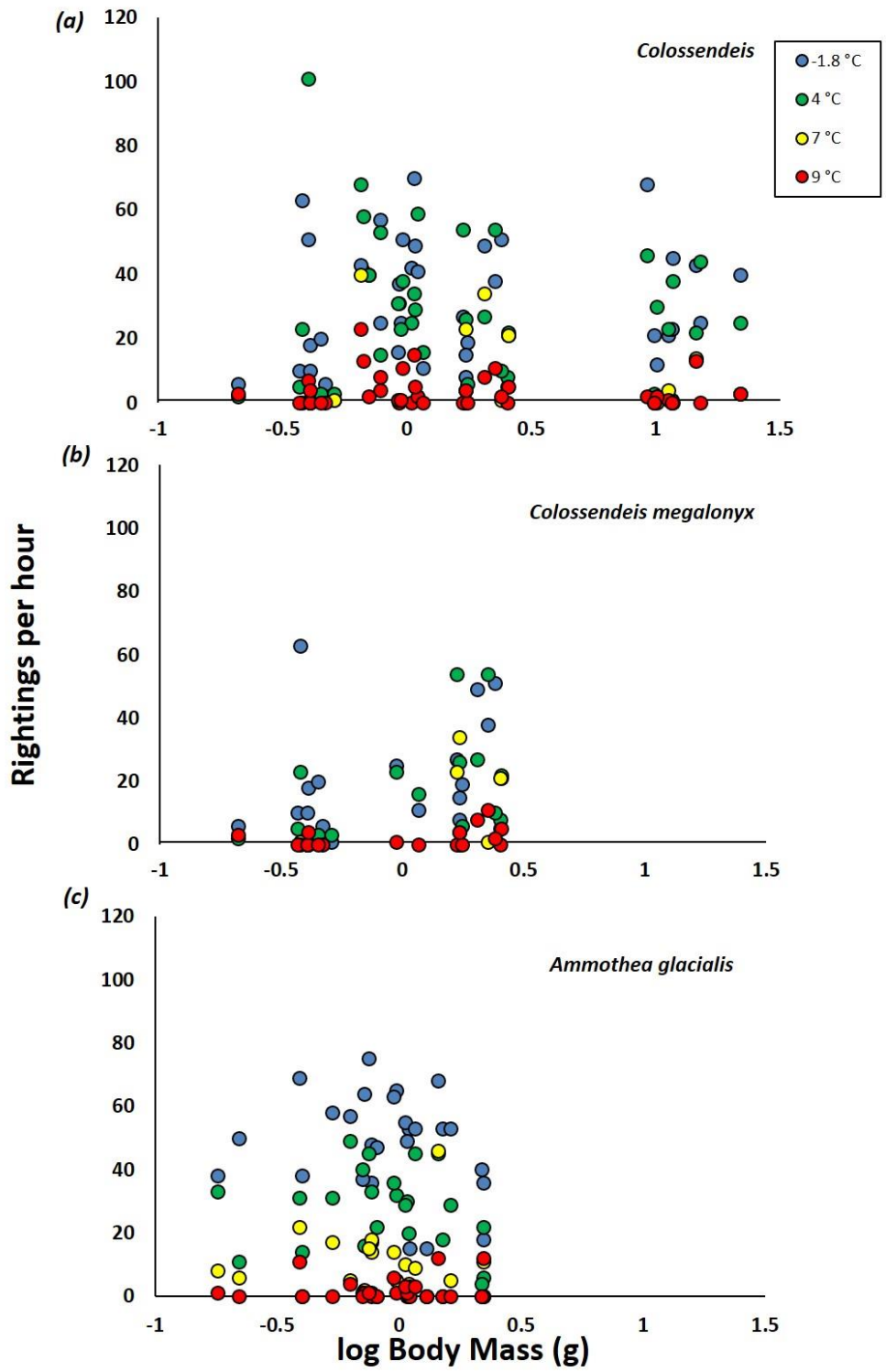

Figure 3. Righting performance (number of rightings per hour) compared to body mass (g) of (a) *Colossendeis*, (b) *Colossendeis megalonyx* and (c) *Ammothea glacialis*. See electronic supplementary material, table S2 for results of ZIGLMM.

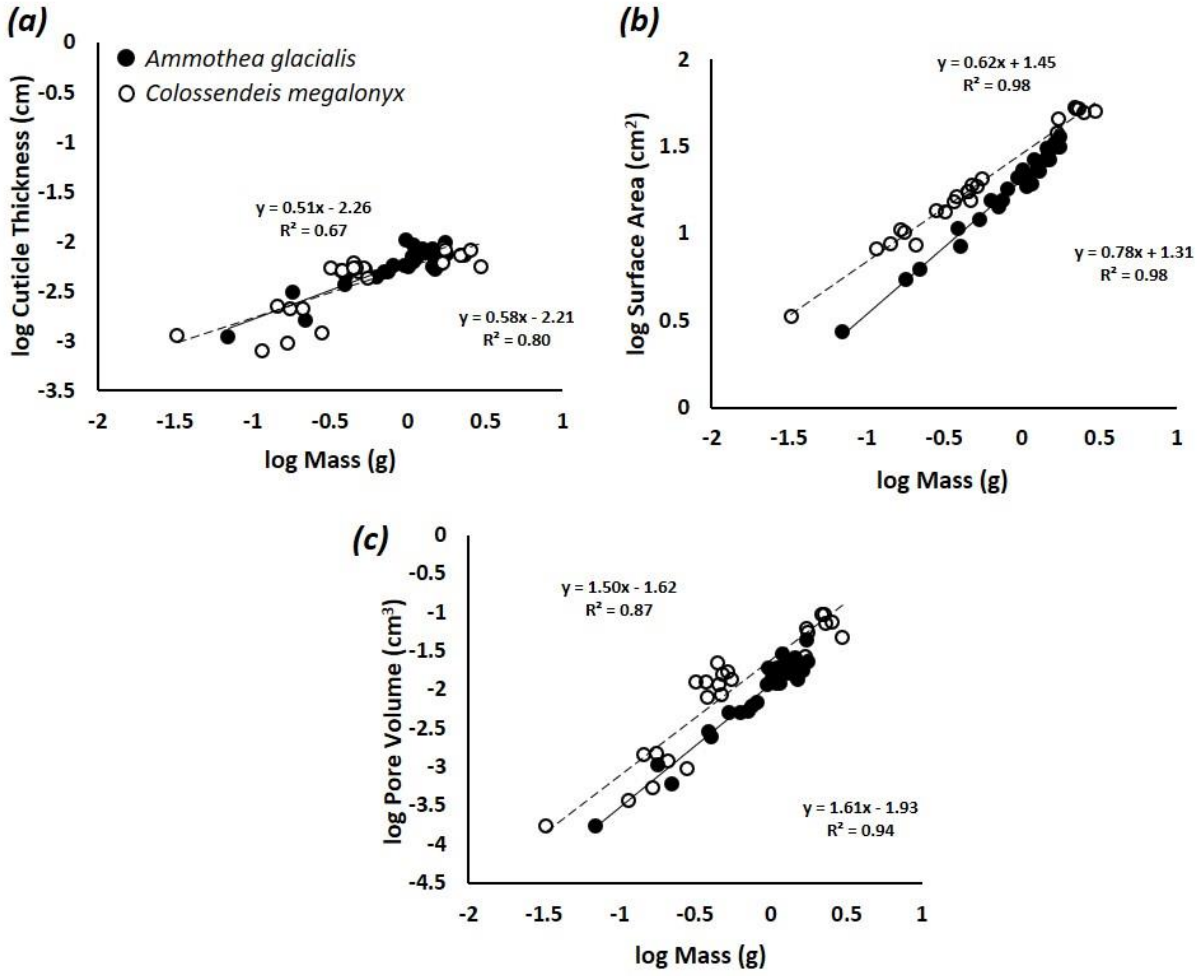

Figure 4. Regression lines for scaling relationships between (a) cuticle thickness (cm), (b) surface area (cm^2), and (c) pore volume (cm^3). Individuals of *Ammothea glacialis* are represented by closed circles and *Colossendeis megalonyx* are represented by the open circles. See electronic supplementary material, table S5 for summary of scaling coefficients.

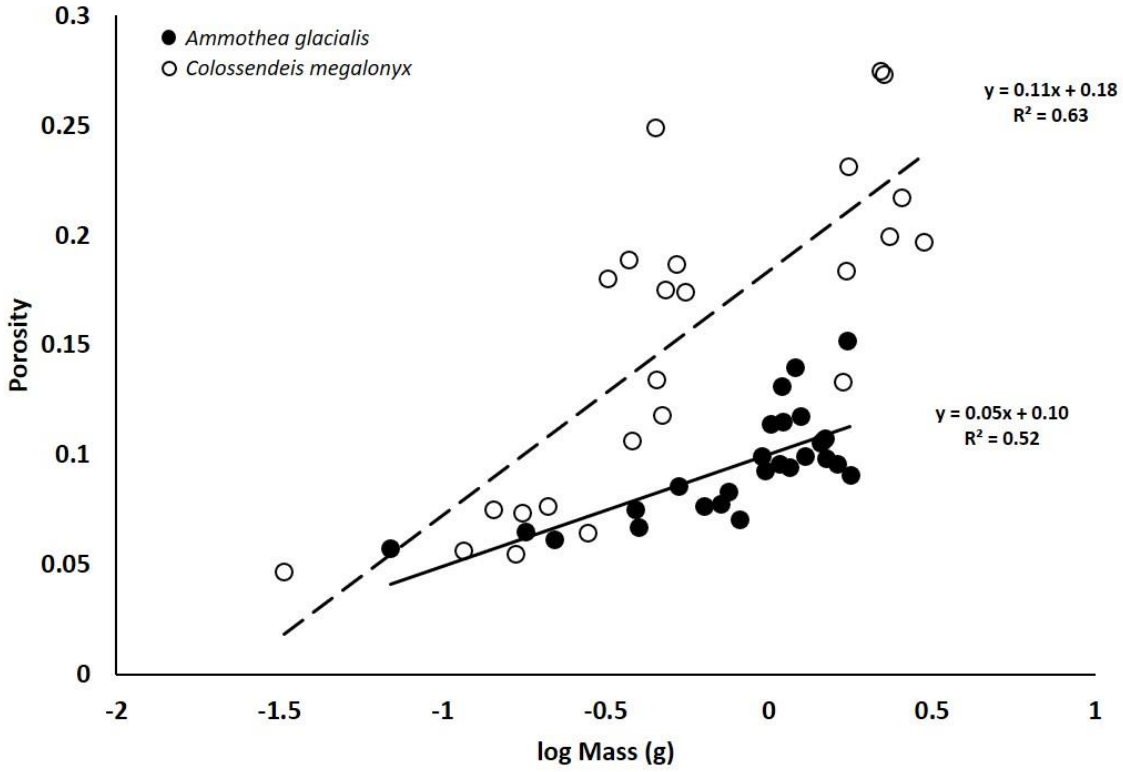

Figure 5. Regression lines for scaling relationships between areal porosity and mass (g). Individuals of *Ammothea glacialis* are represented by closed circles and *Colossendeis megalonyx* are represented by the open circles.

CHAPTER 3: BODY SIZE OF TEMPERATE PYCNOGONIDS: NO EVIDENCE OF OXYGEN-TEMPERATURE LIMITATIONS

Abstract

Oxygen limitation has been proposed as one of the key factors that limits body size at high temperatures (the 'oxygen-temperature hypothesis'). Geographic patterns in body size are thought to be driven in part by the effects of temperature on oxygen supply and demand, particularly when the increased oxygen demand of tissues at higher temperatures outpaces the ability of large organisms to supply internal tissues with oxygen. We tested the effects of temperature on the metabolic rate of two temperate sea spider (Pycnogonida) species, *Achelia chelata* and *Achelia gracilipes*, across a range of body sizes. We measured metabolic rate at five temperatures: 12, 16, 20, 24, and 28°C. The metabolic rates of both species increased significantly with temperature, but the effect was size-independent; thus, we found no evidence to support the oxygen-temperature hypothesis. While previous interspecific studies on Antarctic pycnogonids have found that larger-bodied animals have more porous cuticles, thus potentially offsetting their higher metabolic demand by increasing oxygen diffusivity, the porosity of our two temperate species did not change with body size. This suggests that the generally small size of warm-water sea spiders may arise from selective factors other than oxygen limitation; alternatively, warm-water animals may use other, unknown pathways for increasing oxygen supply.

Introduction

Almost all aspects of an organism's physiology are influenced by body size (Kingsolver and Huey, 2008). As a result, the physiological and environmental factors that underlie evolutionary or acclimatory changes to organismal mass, volume, or other metrics of size have received considerable attention (reviewed in Verberk *et al.*, 2011;Verberk and Atkinson, 2013). In both aquatic and terrestrial systems, environmental oxygen level correlates closely with body size in a wide range of taxa, including mammals (Falkowski *et al.*, 2005), reptiles (VandenBrooks, 2007), fish (Pauly and Kinne, 2010), insects (Harrison *et al.*, 2010), and many aquatic invertebrates (Peck and Chapelle, 1999; Chapelle and Peck, 2004; reviewed in Verberk *et al.*, 2011). In ectotherms, this pattern is thought to be driven in part by the effects of temperature on the dynamics of oxygen supply and demand; warmer temperatures increase the rate of diffusive supply to tissues, but organismal oxygen demand also increases as metabolic rates increase with temperature (Gillooly *et al.*, 2001; Jacobsen *et al.*, 2003; Makarieva *et al.*, 2005; Verberk *et al.*, 2011). The latter, effect generally outweighs the former (Woods, 1999; Woods *et al.*, 2009; Verberk and Atkinson, 2013); thus, oxygen limitation has been proposed as one of the central factors limiting body sizes in ectotherms at high temperatures (the 'oxygen-temperature hypothesis') (von Bertalanffy, 1960; Atkinson *et al.*, 2006; Audzijonyte *et al.*, 2019). At an even broader scale, mismatches between oxygen supply and demand have become a leading hypothesis for why body sizes of adults of many aquatic and marine environments appear to be shrinking as the environment warms (Sheridan and Bickford, 2011; Cheung *et al.*, 2012; Verberk *et al.*, 2016). However, recent studies have argued that at least for organisms with specialized gas exchange structures, such as gills, oxygen-based constraints on body size may not apply because these organisms can and do modify their oxygen uptake and distributional systems to meet increasing metabolic demand (Lefevre *et al.*, 2018; Audzijonyte *et al.*, 2019).

Pycnogonids, or sea spiders, present a unique opportunity for testing the oxygen-temperature hypothesis in marine ectotherms. First, pycnogonids are distributed throughout the world's oceans across a wide geographic range of environmental temperatures, from the tropics to the deep sea and the Southern Ocean, with approximately 1400 known species in 80 genera (Arnaud and Bamber, 1987; Child, 1998; Arango and Wheeler, 2007). Second, both within and among species, pycnogonids show size variation over several orders of magnitude. Body size broadly tracks latitude and environmental temperature; tropical and temperate pycnogonids typically get

no larger than a few centimeters, but in the Antarctic ocean, animals can reach leg spans of over 70 cm (Fry and Hedgpeth, 1969; Dell, 1972; Arnaud, 1974; Clarke and Johnston, 2003). The large size of Antarctic sea spiders exemplifies the phenomenon known as polar gigantism (Arnaud, 1974; De Broyer, 1977), which has been attributed to the high ratio of oxygen supply to demand due primarily to the very low cold-driven metabolic oxygen in polar waters (Wolff, 1956a; b; McClain and Rex, 2001; Chapelle and Peck, 2004; reviewed in Moran and Woods, 2012).

Lastly, pycnogonids have relatively simple gas exchange systems. They lack specialized respiratory structures (Redmond and Swanson, 1968; Markl, 1986; Rehm *et al.*, 2012) and instead obtain oxygen through diffusion either directly across the cuticle, or through cuticular pores (Douglas *et al.*, 1969; Davenport *et al.*, 1987; Lane *et al.*, 2018). Respiratory pigments are also lacking (Redmond and Swanson, 1968; Markl, 1986; Rehm *et al.*, 2012), and oxygen is distributed through the simple open circulatory system by contractions of a weak dorsal heart in the trunk (Tjonneland *et al.*, 1985) and peristaltic contractions of the gut (Woods *et al.*, 2017). Because pycnogonids are solely "skin breathers," unlike organisms with gills or lungs, and their body mass and surface area scale with close to geometric isometry (Woods *et al.*, 2009; Shishido *et al.*, 2019), mechanisms for increasing the capacity for oxygen supply are few compared to most metazoans that reach similar body sizes.

Shishido et al. (2019) recently tested the oxygen-temperature hypothesis in two species of giant Antarctic pycnogonids by comparing the effects of body size and temperature on performance of individuals that ranged over two orders of magnitude in size. For both species, the effects of temperature were equivalent across all body sizes, showing no support for the predictions of the oxygen-temperature hypothesis. However, like the interspecific pattern found by Lane et al. (2017) (for 12 species), Shishido et al. (2019) found that within species, the porosity of the cuticle increased as body size increased. Thus, at least for giant Antarctic taxa, pycnogonids display (both within and between species) a novel mechanism through which larger animals can increase their capacity for diffusive oxygen uptake without changing shape.

In this study, we further tested the oxygen-temperature hypothesis using pycnogonids from temperate, rather than Antarctic, waters. Under this hypothesis, constraints on body size limits are more stringent in warmer environments (Forster *et al.*, 2012; reviewed in Ohlberger, 2013);

the mismatch between tissue oxygen demand and the organisms' ability to acquire and transport oxygen, therefore, may be an important factor underlying the characteristically small body sizes of sea spiders in temperate and tropical regions. To test this idea, we measured oxygen consumption rates of two species of temperate pycnogonids across a range of body sizes and temperatures to determine whether larger-bodied individuals were more strongly affected by temperature than smaller-bodied ones. To determine whether temperate sea spiders compensate for larger body sizes or warmer temperatures by increasing the porosity of the cuticle, we also measured cuticle porosity in two temperate species to determine whether, as in Antarctic taxa, the cuticles become more porous as these temperate species grow.

Methods

Collection and Maintenance

Pycnogonids were collected from two different temperate localities in the northeastern Pacific, in two different years: Friday Harbor, WA, USA (48°53´N, 123°02´W), near the Friday Harbor Laboratories (FHL), in 2005; and Lighthouse Beach, Charleston, Oregon, USA (43°34´N, 124°33´W), near the Oregon Institute of Marine Biology (OIMB), in 2006. Collections at FHL were made on SCUBA in June 2015 from hydroids at depths of 3-20 m. Most experimental animals were collected on the large submersed ballast blocks suspended from the FHL docks at a depth of approximately 5 m. At OIMB, pycnogonids were collected intertidally in June 2016 from one end of a sandy, wave exposed beach, where the animals were found clinging to turf algae and seagrass on the tops of large rocks that were partially embedded in the sand. Our focal species from the two sites were *Achelia chelata* (FHL) (Hilton, 1939) and *A. gracilipes* (OIMB) (Cole, 1904). Individuals were identified to species using Kozloff and Price (1996) and Carlton (2007); identifications were confirmed by Dr. Claudia Arango (Queensland Museum). Near the time of collection, seawater temperatures at FHL averaged 11.4°C ± 0.9 (mean ± SD; daily average from 6/1-6/30/15) (NOAA National Data Buoy Center, Station FRDW1). At OIMB, seawater temperatures on the coast near Coos Bay averaged 12.6°C ± 1.0 (mean ± SD; daily average from 6/1-6/30/16) (NOAA National Data Buoy Center, Station CHAO3). After collection, animals were kept in flow-through sea water tables at FHL or OIMB until used for experiments. All experiments were performed within two weeks of collection.

Temperature-body size experiments

To determine how the metabolic rates of *Achelia chelata* and *A. gracilipes* were affected by body size, temperature, and their interaction, we measured oxygen consumption rates of individual sea spiders across a range of body sizes and temperatures using an end-point determination method. Individual pycnogonids were incubated in small (700-800 µl) gas-tight respiration vials of known volume that were filled with temperature-equilibrated, air-saturated, 0.2 µm-filtered seawater. Temperature control was achieved by immersing respiration vials in a refrigerating/heating water bath set to the experimental temperature (EcoGold, Lauda Brinkmann). Incubation times ranged from 30 minutes at 12°C to 5 minutes at 28°C; the length of incubation was determined by pilot experiments at each temperature, in which we established durations that never resulted in ending oxygen concentrations below 80% of air saturation at that temperature. At the end of the incubation, a 300 µl subsample was taken from each vial with a temperature equilibrated gas-tight syringe and oxygen tension was measured with a polarographic oxygen sensor (Model 1302, Strathkelvin Instruments, UK) to determine total oxygen consumption. Respiration rates of each individual pycnogonid were measured sequentially at 12, 16, 20, 24, and 28°C (FHL, n=32 (n=7 for 28°C)); OIMB, n=34 for all temperatures). Between measurements, pycnogonids were returned to ambient conditions in the sea table for at least 3 h before being exposed to the next higher temperature. Each animal was assessed daily for normal appearance, movement, and behavior over the course of the experiment. After the highest temperature, we also measured the oxygen consumption of each animal again at 12°C (after a recovery period, as above) to assess whether respiration at ambient was impacted by the previous high temperature exposures.

After all metabolic measurements were complete, each animal was blotted dry and weighed on an electronic balance (Cahn Model 4400, Ventron Corp and AE163, Mettler-Toledo) to obtain wet mass and then preserved in 100% EtOH for later genetic and cuticular analysis (preliminary tests established that ethanol preservation did not alter cuticular morphology).

Cuticle morphometrics

To determine whether cuticles became more porous on larger animals, we measured the porosity of the cuticle from a subset of individuals from the respiration measurements at FHL and OIMB (n=8 *A. chelata*, n=23 *A. gracilipes*). To measure porosity, we mounted the femur of the second left leg of each individual (to avoid artifacts from any differences across legs; as in Lane et al.

2017) under a compound microscope (Olympus BX41TF, Tokyo, Japan) with an attached camera (Q Color 3, Olympus America, Pennsylvania, USA) and imaged each individual using the Qcapture program (version 2.9.13, Quantitative Imaging Corporation, Surrey, B.C., Canada). Three pictures along the surface of the cuticle of the femur of each animal were taken (to capture entire length of the leg); within each image, one 100μm x 100μm virtual quadrat was placed on a part of the image that was completely in focus (i.e. in one focal plane) and the total pore surface area within the quadrat was measured (Figure 1). Average porosity was then calculated as the area of the quadrat divided by the mean of the total pore area of the three quadrats. All image analyses were performed using Image J software (v.1.51j8, NIH).

Statistical analysis

To estimate the effects of mass, temperature, and their interaction on metabolic rate, we used linear mixed effect models in JMP (SAS Institute Inc., Cary, NC, USA) for the two species separately. We used metabolic rate as the response variable, temperature treatments and mass as explanatory variables, and we incorporated individual pycnogonids as random effects. Metabolic rate and mass data were log-transformed to meet the assumption of normality and to give reasonable distributions of the residuals. Individuals that did not measurably draw down oxygen at a particular temperature were excluded from the analysis for that temperature trial (*A. chelata*, n=3; *A. gracilipes*, n=2). As another way of looking at the interactive effects of temperature and body size on metabolism, and to compare the scaling of mass and metabolism of our species to relationships in the literature, we log10-transformed mass and metabolism and fitted the data to ordinary least-squares regressions. The scaling exponent was determined from the slope of each regression line. Lastly, as an estimate of thermal sensitivity of metabolism across the temperature range, we also calculated Q_{10} values for each individual pycnogonid for both the overall temperature range (12 to 28°C) and for each temperature step (i.e. 12 to 16, 16 to 20, 20 to 24, and 24 to 28°C). We calculated Q_{10} values using the standard equation:

$$Q_{10} = \frac{R_2^{\left(\frac{10}{T_2 - T_1}\right)}}{R_1}$$

where R_1 and R_2 are oxygen consumption rates at temperature T_1 and T_2, respectively. To test if cumulative exposure to the higher experimental temperatures (which were warmer than animals generally experience at either site) had detrimental effects on metabolic rates, we used a paired t-

test to compare the metabolic rates of pycnogonids at 12°C before the high temperature trials, to the metabolic rates measured again at 12°C after all higher exposures were complete. Finally, to determine if porosity changed with body size, we performed linear regressions for *A. chelata* and *A. gracilipes* separately, with areal porosity (%) as the dependent variable and body mass as the explanatory variable (after Shishido *et al.*, 2019)).

Results

Temperature-body size experiments

Increasing, temperature had a significant and positive effect on metabolic rate for both *A. chelata* and *A. gracilipes* (LMM, $p<0.0001$, Figure 2). Q_{10} values from 12 to 28°C were 2.91 ±0.23 for *A. chelata* and 3.09 ± 0.28 (mean ± SE) for *A. gracilipes* (Table 2). When Q_{10} values for each species were calculated more finely between each temperature step, the values for *A. chelata* were consistently between 2 to 4, but values for individuals of *A. gracilipes* were considerably higher and ranged from 4.7 ± 1.6 between 16-20°C to 19.2 ± 6.3 between 20 to 24°C (mean ± SE) (Table 2).

No pycnogonids died and the behavior and activity level of animals during rest periods at ambient temperature did not visibly change over the course of the experiment. *A. chelata* did not show a significant difference in metabolic rates taken at 12°C before or after the elevated temperature exposures (0.012 ± 0.009 (mean ± SE) µmol O_2 individual^{-1} h^{-1} for both) (Paired T-test, p=0.26). In contrast, the metabolic rate of *A. gracilipes* at 12°C decreased threefold between the initial measurements and measurements taken after the high-temperature exposures (from 0.012 ± 0.004 to 0.004 ± 0.003 µmol O_2 individual^{-1} h^{-1} (mean ± SE)) and this difference was significant (Paired T-test, p=<0.0001).

Individual masses ranged over more than an order of magnitude for both *A. chelata* (0.00036 to 0.006 g) and *A. gracilipes* (0.0009 to 0.0021 g). Mass had a positive effect on metabolism in both species (Figure 3); this effect was significant for *A. chelata* (LMM, $p<0.0001$) but not *A. gracilipes* (p=0.18) (Figure 3). We found no evidence for an interaction between temperature and body size in either *A. chelata* (LMM, p=0.32) or *A. gracilipes* (p=0.68) (Figure 3, Table 1). The scaling exponents of metabolism with body size were all close to 1 for *A. chelata* at each

temperature treatment (Table 3). By contrast, the scaling exponents for *A. gracilipes* were highly variable and ranged from (-0.08 to 0.86) (Table 3).

Cuticle Porosity

The average porosity (P) of *A. chelata* was 0.12± 0.02 (mean ±SE), and of *A. gracilipes* was 0.07± 0.01 (mean ±SE). The log-linear relationships between porosity (P) and mass (M) for the two species were $P_{A.chelata}= 1.65\times M^{0.67}$ and $P_{A.glacialis}= -0.026\times M^{-0.032}$. The slope of this relationship was not significantly different from zero for either *A. chelata* ($R^2= 0.25$; p=0.18) or *A. gracilipes* ($R^2=0.01$; p=0.52), Figure 4).

Discussion

In agreement with previous studies of pycnogonids and many other taxa (Gillooly *et al.*, 2001; reviewed in Glazier, 2005, 2018; Verberk *et al.*, 2011; Lane *et al.*, 2018), respiration rate of both *Achelia chelata* and *Achelia gracilipes* increased with body size and temperature. However, counter to the predictions of the oxygen-temperature hypothesis, we did not find a significant interaction between temperature and body size in either species; temperature affected metabolic rate similarly across body sizes. This outcome is comparable to those of Shishido et al. (2019) for Antarctic pycnogonids, in which the effect of temperature on performance was equivalent across two orders of magnitude in body size. However, Shishido et al. (2019) also proposed a functional explanation for their result: cuticles of the two Antarctic species, both of which display gigantism, become more porous as they grow. Because pycnogonids acquire oxygen via diffusion across their cuticle (Douglas *et al.*, 1969; Davenport *et al.*, 1987), and pores greatly increase oxygen conductance (Lane *et al.*, 2018), the increase in porosity with size may enable polar giants to overcome the oxygen deficit that would otherwise accompany the increasing metabolic oxygen demand of a larger body and longer diffusion distances within the body.

Like the Antarctic species, the temperate species in our study had numerous pores distributed broadly across the cuticle of the legs and body. The mean cuticle porosity of the two temperate species was comparatively high, however; while variable among individuals, average cuticle porosity was 12% ± 2.3 and 7% ± 0.7 (mean ± SE), for *A. chelata* and *A. gracilipes*, respectively. For the confamilial Antarctic *Ammothea glacialis* (Shishido *et al.*, 2019, Fig. 5), similar levels of porosity were not reached until animals were ~30x as large (see Shishido *et al.*, 2019, Fig. 5).

Temperate animals had much greater mass-specific oxygen demands (*A. chelata*, 4.55 ± 0.38; *A. gracilipes*, 8.70 ± 1.16 μmol h^{-1} g^{-1} (mean ± SE) than Antarctic ones (*A. glacialis*, 0.05 ± 0.004 μmol$h^{-1}g^{-1}$ (mean ± SE) in Shishido *et al.*, 2019), so a higher cuticle porosity with respect to body size is consistent with the physiological need for greater cuticular conductance (Lane *et al.*, 2018). However, the high variance in porosity among individuals, which was not correlated with individual metabolic rates (supplementary material), supports a more diversified role than gas exchange alone. Storch and Welsch (1972) observed that some pores in pycnogonid cuticle led to putative mucus-producing cells, indicating a secretory role. King (1973) suggested that if the pores are secretory, they may aid in desiccation reduction (for intertidal species) or protection from nematocysts of hydroid hosts. Pores could also have a more generalized transport function in taking up or excreting small particles across the cuticle (Tomaschko and Brückmann, 1990; Tomaschko, 1992). While our limited range of body sizes, especially with *A. gracilipes*, may have reduced our ability to detect a significant relationship between porosity and body size, without a better understanding of the roles of pores in particular species or environments, it is difficult to interpret the high degree of variance in porosity among individuals. One possible explanation, however, lies in the molt cycle. Pycnogonids, like other arthropods, molt their exoskeleton as they grow (reviewed in King, 1973). This molting process includes molt-stage dependent structural changes which involves a cycle of thinning and thickening of the cuticle (Chung *et al.*, 2012). As cuticle thickness varies, so may porosity; thus, depending on stage in the molt cycle, similarly-sized pycnogonids may have cuticles that are very different. This would likely be more apparent in temperate and tropical environments than in the Antarctic, where the extreme cold temperatures and slow growth rates (Peck 2018) probably make molting highly infrequent.

Despite the high variability in porosity, the metabolism of *A. gracilipes* and *A. chelata* did not appear to be oxygen limited, even at the largest body sizes and highest temperatures. This raises the question of how temperate sea spiders adjust their oxygen uptake to meet increasing metabolic demands. We currently have no clear answer, but there are several possibilities. First, pores may not be an important component of cuticular conductance in small-bodied sea spiders. Across 10 species of Antarctic pycnogonids, Lane et al. (2018) showed that for smaller-bodied taxa, the cuticle was generally thin enough to allow for sufficient diffusive oxygen supply even in the absence of pores. Without knowing the thickness or conductance of the cuticle of the two

temperate species, it is difficult to determine whether, like Antarctic species, pycnogonids from warmer-water environments can supply their internal oxygen needs by diffusion directly across the cuticle, at least within the range of body sizes we tested. The temperate species in our study were three orders of magnitude smaller than the Antarctic species, and their small size may have allowed for adequate diffusive supply through the cuticle regardless of temperature, body size, or porosity. In contrast to temperature, which had strong effects on both species, the effects of body size on metabolism varied. While metabolic rate significantly increased as *A. chelata* got larger, there was no significant effect of body size on the metabolic rate of *A. gracilipes*. However, is possible that because we used a much smaller size range than *A. chelata*, we were simply not able to detect an effect of body size on metabolism in *A. gracilipes*.

If the animals in our experiments never reached a size threshold at which temperature-driven oxygen limitation become apparent, this suggests the small size of temperate and tropical pycnogonids may be maintained by selective factors other than oxygen supply. The giant size of Antarctic sea spiders has been attributed to many factors in addition to oxygen, including a release from predation pressure and/or a reduction in competition in the Southern Ocean (Aronson *et al.*, 2009; Moran and Woods, 2012). In temperate regions, where predators like crabs and fish are common, small body size may be selectively favored because it reduces attractiveness and visibility (Bain, 1991; Bamber and Costa, 2009; Veena *et al.*, 2010). Another factor that might act to keep body size small is the physical flow environment. Large-bodied Antarctic pycnogonids experience generally low flow (3.6 cm s^{-1} over 10 months in McMurdo Sound; Tobalske et al., unpublished data reported in Moran et al. 2018), whereas both temperate species in this study were collected in regions of much higher free-stream current velocity. *A. chelata* was collected from the shallow subtidal in Friday Harbor, WA where currents in subtidal channels average 74.0 ± 15.2 cm s^{-1} (mean ± SD, based on 2018 daily maximum, NOAA tides and currents); *A. gracilipes* was collected intertidally from a wave-swept site on the outer coast of the Pacific Northwest, a region where intertidal organisms experience very high water velocities and accelerations (Denny *et al.*, 1985). These high water velocities are likely to select for a smaller body size (e.g. Denny *et al.*, 1985; Carsen *et al.*, 1996; Perry *et al.*, 2014) since getting larger would mean living outside the boundary layers of substrates including those provided by small hydroids, and, therefore, allocating a greater amount of energy to remaining in place as well as a higher chance of getting swept away by currents.

Finally, even if pores are important in oxygen uptake, temperate sea spiders may also deploy other strategies to supply oxygen to internal tissues in the face of higher metabolic demands (driven either by temperature or body size). Pycnogonids transport oxygen internally via peristaltic contractions of the gut which are shown to increase as temperatures increase (Woods *et al.*, 2017) and thus could potentially offset increasing metabolic demand by increasing the rate of contractions, at least in the short term. However, as a long-term response, this mechanism would likely come at a high energy cost that might not be sustainable as an adaptive strategy in warm-water environments.

This study also gave us the opportunity to examine the relationship between metabolic rate and body mass, which has been a central question in physiological ecology because it provides a powerful tool to predict biological processes at all levels of organization from individuals to ecosystems (Nisbet *et al.*, 2000; Brown *et al.*, 2004; Whitfield, 2004; Burgess *et al.*, 2017). The relationship between metabolic rate (Y) and body mass (M) can be expressed as a power function, $Y=aM^b$ (Krough, 1916; Kleiber, 1932; Schmidt-Nielsen and Knut, 1984). In many cases the scaling exponent (b) is 3/4 where log body mass increases 4-fold, and log metabolic rate increases 3-fold (Brody and Lardy, 1946; Hemmingsen, 1960; Schmidt-Nielsen and Knut, 1984; Gillooly *et al.*, 2001). This pattern, also known as the ¾ power law or Kleiber's law, is not universal; comparing both within and among a wide range of taxa, Glazier (2005) noted that scaling exponents varied from ~0 to >1 but typically fell within the range of 2/3 to 1. The scaling exponent for one species in our study, *A. chelata* did not match the ¾ power law, being above 1 at all temperatures; the confidence intervals around the scaling exponent included ¾ only at the two highest temperatures. A scaling exponent of close to one is typical of species with low overall oxygen consumption (Glazier, 2005), which appears to be true of pycnogonids in general (Scholander *et al.*, 1953; Douglas *et al.*, 1969) and with *A. chelata* in our study, whose mass-specific metabolic rates at ambient temperatures were ~2x lower than the predicted value for aquatic benthic crustaceans in general (Figs, 2 and 3, Seibel and Drazen, 2007). The scaling exponents for *A. gracilipes*, in contrast, had wide confidence intervals at all temperatures. While most pycnogonids employ low energy-use strategies (Douglas *et al.*, 1969; Clarke and Johnston, 2003), some are more active (*Anoplodactylus californicus* in Bain, 1991; *Colossendeis* spp. in Moran *et al.*, 2018) and their scaling relationships between metabolism and mass may differ.

Underlying scaling in *A. gracilipes* may also have been obscured by the smaller range in mass values and the high variation in mass-specific metabolic rate among individuals of that species.

In contrast to the scaling exponent, both *A. chelata* and *A. gracilipes* had similar metabolic responses to temperature. As has been found for most ectotherms, metabolic rate increased with temperature (Cossins and Bowler, 1987; Somero, 1997; Gillooly *et al.*, 2001; Clarke, 2004) and this was found even between the two highest temperature increments, where our highest temperature treatment (28°C) was >10°C higher than the maximum recorded water temperature at either FHL (15.1°C) or OIMB (18.2°C) (NOAA National Data Buoy Center, based on historical readings from 2005-2012). Even at the highest temperature, neither species showed signs of aerobic failure. Q_{10} values calculated across the full temperature range (12 to 28°C) fell between 2-3, which is typical of most biological processes (Clarke, 1983; Hochachka, 1991). This was also largely true between each temperature increment for *A. chelata*, indicating that for this species, the thermal sensitivity of aerobic metabolism did not change greatly with temperature. For *A. gracilipes* Q_{10} values were considerably higher at the upper temperature increments, reaching 19.24 between 20 and 24°C, possibly indicating an active upregulation of energy-requiring processes. Our specimens of *A. gracilipes* were collected intertidally, in a region where they experience brief excursions to air temperatures up to 31°C (NOAA National Data Buoy Center, based on historical readings from 2005-2012, Station CHAO 3). Warmer temperatures are likely to coincide with areal exposure during low tide; because of the reduced risk of dislodgement from waves and sand scour, low tide could be an advantageous time for high-energy activities such as feeding, mating, or migration. However, aerobic metabolism is a complex process that integrates biological networks at multiple levels, which makes it challenging to interpret experimentally-determined patterns of thermal sensitivity (Schulte, 2014).

While no pycnogonids died and behavior did not visibly change over the course of the experiment, individuals of *A. gracilipes* (but not *A. chelata*) showed a significant decrease in metabolic rate when reassessed at ambient (12°C) after the elevated temperature trials. This may suggest that of the two species, *A. gracilipes* accumulated more physiological damage from the short-term exposure to elevated temperatures. However, this seems unlikely because intertidal organisms are exposed to constant fluctuations in temperature and are therefore generally more

eurythermal than subtidal congeners (Somero, 2002; Stillman, 2002). Another possibility is that the lower mass-specific metabolic rates of *A. gracilipes* (at ambient) after high-temperature exposures indicated rapid acclimation to higher temperatures (Marshall *et al.*, 2011; Pörtner, 2012). Little is known about the acclimatory capacities of pycnogonids, but several species are found intertidally (Utinomi, 1951; Child and Hedgpeth, 1971; Stock, 1974; Child, 1982) and these capacities may be important in helping these species to persist.

Overall, we found that temperature drove increases in metabolic rate of both species, but we found no evidence to support the oxygen-temperature hypothesis in either species even at elevated temperatures. This suggests that in both *A. chelata* and *A. gracilipes*, body size is not constrained by the capacity for oxygen uptake and transport to internal tissues. Unlike Antarctic pycnogonids, neither temperate species showed an increase in the porosity of their cuticle with increasing body size. It is possible that these temperate pycnogonids do not need to increase their cuticular porosity with body size because they are able to utilize other pathways for oxygen supply such as increasing gut peristalsis or diffusion across a very thin cuticle. Alternatively, these small-bodied animals may be below a size threshold for oxygen limitation, and size limits may be set by other biotic or abiotic aspects of their environment. These hypotheses could best be tested with modelling approaches, and with additional experimental studies on pycnogonid species that inhabit tropical, even warmer-water environments.

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Tables

Table 1. Summary of linear mixed-effects models of metabolic rate (μmol h^{-1}) compared to body mass (g).

Species	n	df	F Ratio	p
Achelia chelata	32			
Temperature		4	32	<0.0001*
Mass		1	160.59	<0.0001*
Temperature*Mass		4	1.19	0.32
Achelia gracilipes	34			
Temperature		4	13.75	<0.0001*
Mass		1	1.95	0.1792
Temperature*Mass		4	0.58	0.6752

Table 2. Q_{10} values calculated over entire temperature range from 12 to 28°C (bolded) and at each temperature step for individuals of *A. chelata* and *A. gracilipes*.

Species	Temperature Range °C	Mean Q_{10}	Std Error
Achelia chelata			
	12 to 28	**2.91**	**0.23**
	12 to 16	3.95	1.12
	16 to 20	3.93	1.03
	20 to 24	3.71	0.46
	24 to 28	2.39	1.14
Achelia gracilipes			
	12 to 28	**3.09**	**0.28**
	12 to 16	5.92	1.47
	16 to 20	4.70	1.56
	20 to 24	19.24	6.26
	24 to 28	9.59	3.18

Table 3. Scaling of metabolism with body size in pycnogonids. log(Metabolic rate) vs log(Mass)

Models	n	a	95 % CI	P	b	95 % CI	P	R^2
Achelia chelata								
12°C	32	0.88	(0.25,1.51)	0.0074	1.10	(0.87,1.33)	*<0.0001	0.76
16°C	29	1.03	(0.52,1.55)	0.0003	1.11	(0.91,1.31)	*<0.0001	0.83
20°C	30	1.64	(1.03,2.24)	<0.0001	1.30	(1.07,1.53)	*<0.0001	0.83
24°C	30	1.16	(0.37,1.95)	0.0057	1.05	(0.75,1.34)	*<0.0001	0.65
28°C	7	1.38	(-0.18,2.94)	0.0728	1.07	(0.52,1.63)	*<0.0041	0.83
Achelia gracilipes								
12°C	20	-0.41	(-2.10,1.28)	0.6175	0.57	(-0.02,1.16)	0.0564	0.19
16°C	19	-0.67	(-4.11,2.78)	0.6875	0.41	(-0.78,1.61)	0.4749	0.03
20°C	18	-2.04	(-5.07,0.99)	0.1726	-0.08	(-1.14,0.97)	0.8715	0.002
24°C	19	0.92	(-0.82,2.66)	0.2803	0.83	(0.23,1.44)	*0.0097	0.33
28°C	20	1.15	(-2.94,5.25)	0.5612	0.86	(-0.56,2.28)	0.2181	0.08

n =number of individuals used in each analysis. Regression coefficients: intercept ("a"), scaling exponent calculated from the slope of the regression line (“b”).

Figures

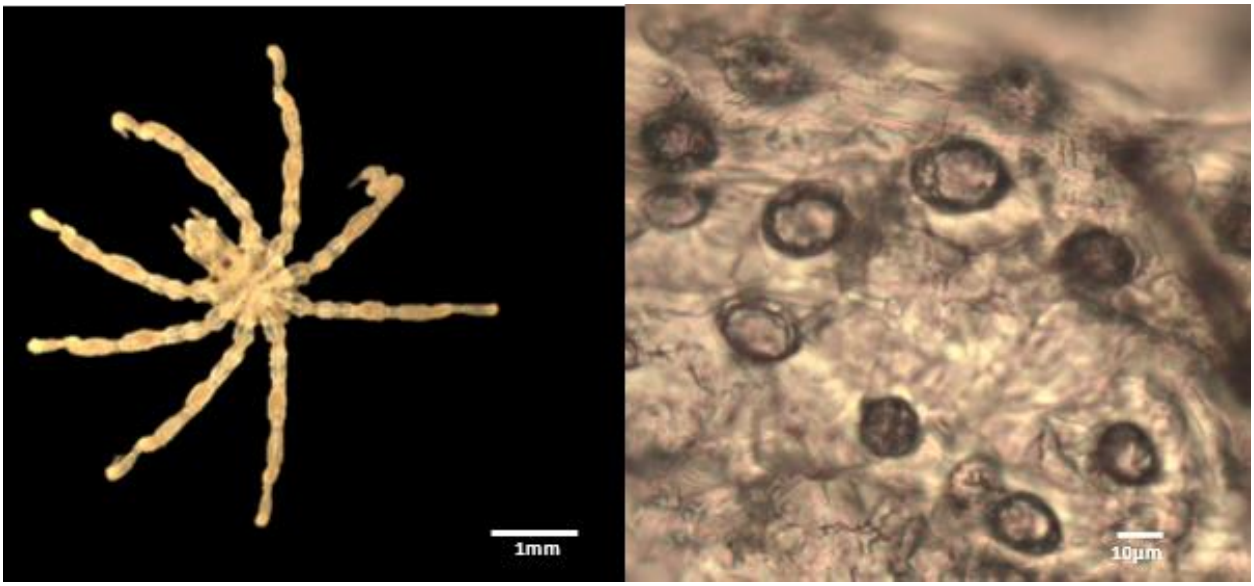

Figure 1. Adult specimen of *A. gracilipes* (left), cuticle pores of *A. gracilipes* (right).

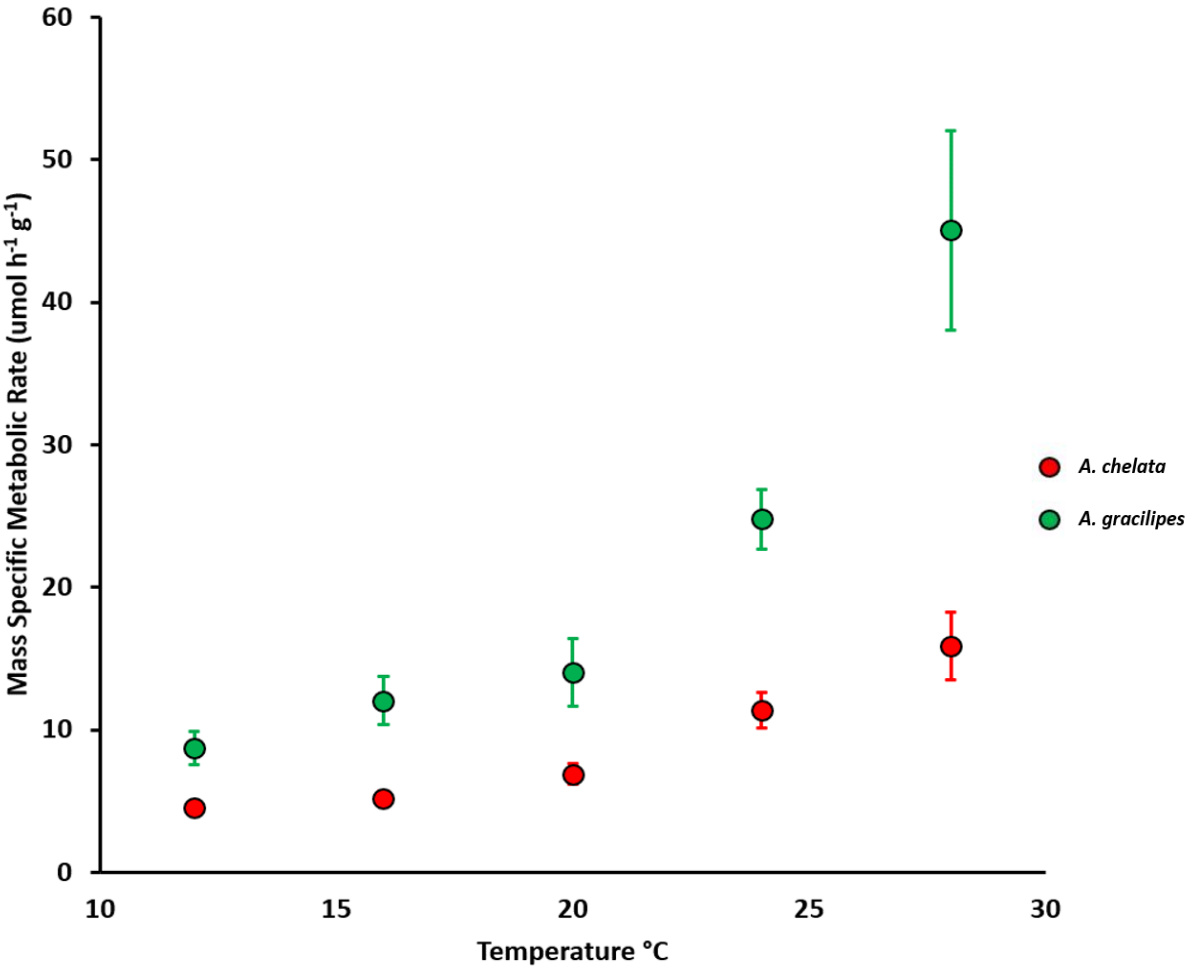

Figure 2. Mean mass specific metabolic rate (µmol $h^{-1}g^{-1}$) (mean ±SE) for all individuals measured at each temperature. (A) *Achelia chelata* (n=32 at 12°C, n=29 at 16°C, n=30 at 20°C, n=30 at 24°C, and n=7 at 28°C) (LMM, p<0.0001) and (B) *A. gracilipes* (n=20 at 12°C, n=19 at 16°C, n=18 at 20°C, n=19 at 24°C, and n=20 at 28°C) (LMM, p<0.0001) at ambient (12°C) and four elevated (16°, 20°, 24°, and 28°C) temperatures. treatments.

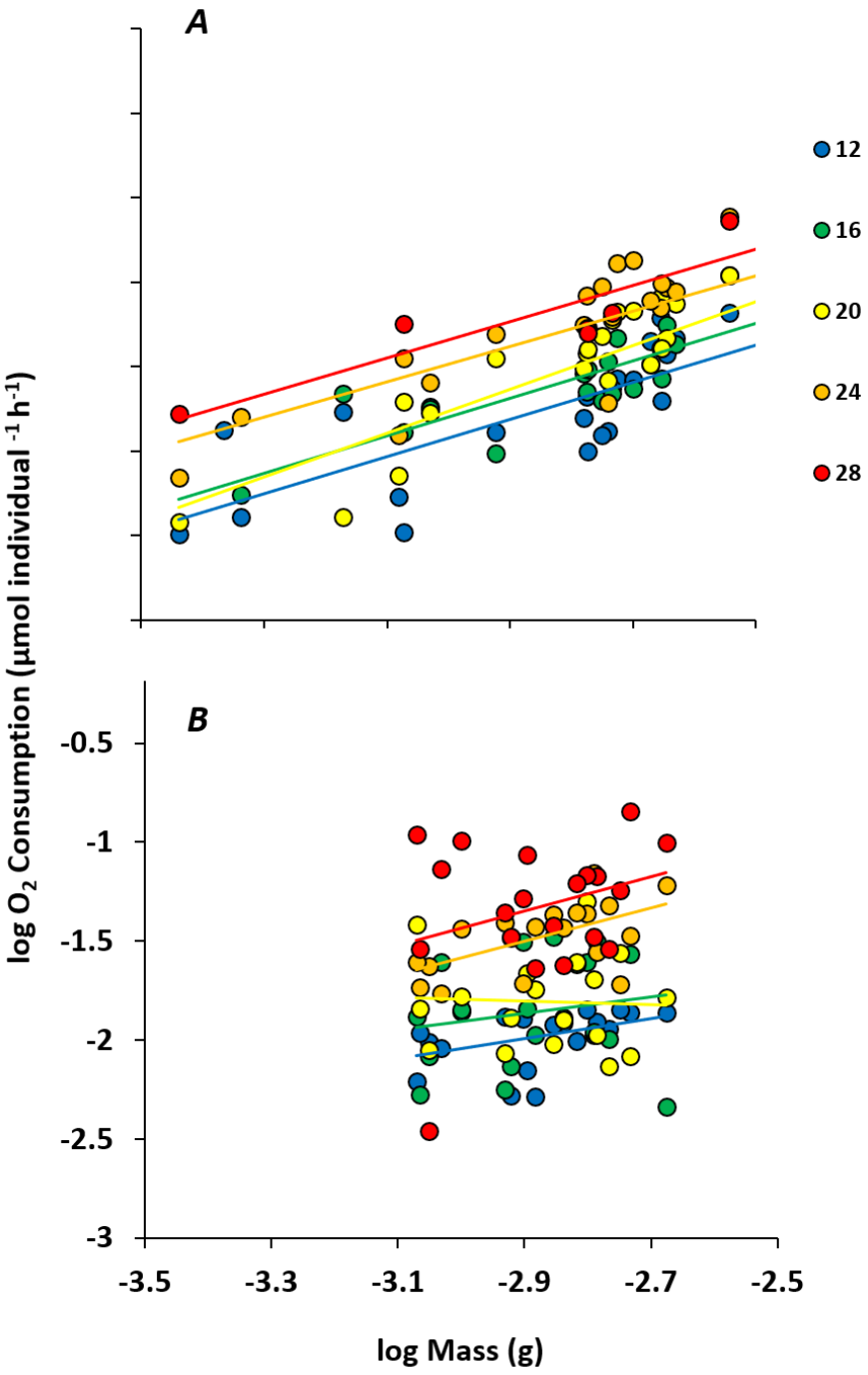

Figure 3. Metabolic rate (µmol individual $^{-1}$ h^{-1}) compared to body mass (g) of (a) *Achelia chelata* (n=32 at 12°C, n=29 at 16°C, n=30 at 20°C, n=30 at 24°C, and n=7 at 28°C) and (b) *Achelia gracilipes* (n=20 at 12°C, n=19 at 16°C, n=18 at 20°C, n=19 at 24°C, and n=20 at 28°C). See table 3 for regression results.

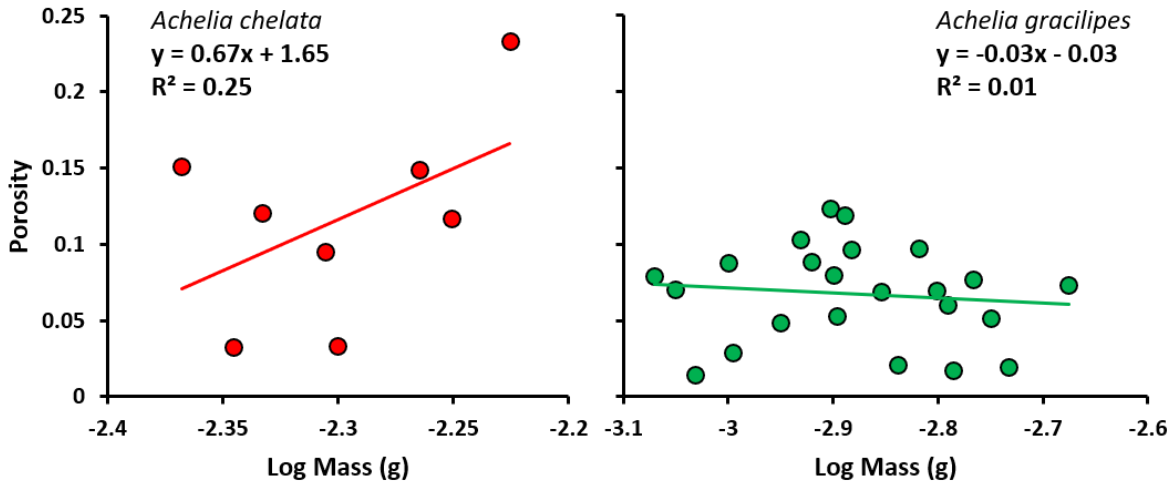

Figure 4. Regression lines for relationship between cuticle porosity and mass (g). Individuals of *Achelia chelata* (n=8, p=0.18) are represented by red circles and *A. gracilipes* (n=23, p=0.52) are represented by green circles.

CHAPTER 4 – EVALUATING THE THERMAL SENSITVITY OF THE ANTARCTIC PYCNOGONID, *Ammothea glacialis*, OVER ONTOGENY

Abstract

Antarctic ectotherms are thought to be highly stenothermal, meaning they can only function within a narrow temperature range. This idea, however comes primarily from work done on adults. Determining if thermal sensitivity changes over ontogeny is important, especially in order to make predictions about how a species may respond to a changing environment. We tested the thermal sensitivity of three distinct developmental stages (larvae at stages II, III, and IV, juveniles, and adults) of the Antarctic sea spider, *Ammothea glacialis* (Pycnogonida). We measured metabolic rate at a series of elevated temperatures: larvae (-1.8 and 1°C) and juveniles and adults (-1.8, 1, 4°C). We also calculated Q_{10} values as another measure of thermal sensitivity. Aerobic metabolism was increasingly sensitive to elevated temperatures over ontogeny; older larvae (stage IV), juveniles, and adults were more sensitive than early stage larvae. Adults were extremely sensitive to elevated temperatures which may be due to cold-adapted related evolutionary losses or tradeoffs which allow animals to function more efficiently at low temperatures but have consequences when exposed to increased temperatures. These large effects of temperature on Antarctic pycnogonids, highlight our need for a better understanding of these biological processes especially in light of climate warming.

Introduction

The Southern Ocean is one of the coldest and most thermally-stable marine environments on Earth (Barker and Thomas, 2006; Peck *et al.*, 2014). Temperatures in the Ross Sea, which is a deep bay of the Southern Ocean about 200 miles from the South Pole, vary by less than 1.5°C above the freezing point of water (-1.9°C) year-round (Clarke *et al.*, 1988; Hunt *et al.*, 2003). These cold, stable temperatures have persisted for at least 5-10 MY (Clarke and Crame, 1992) but are predicted to increase by 0.8- 1.4°C by 2200 (Timmermann and Hellmer, 2013). Although such increases may seem modest compared to daily or seasonal temperature fluctuations routinely experienced by temperate organisms (Richard *et al.*, 2012), appear with some exceptions to be highly stenothermal, meaning they can only function within a narrow temperature range; likewise, many aspects of organismal physiology and performance are sensitive to very small changes in absolute temperature (Somero and DeVries, 1967; Clarke and Johnston, 1996; Peck, 2002; Seebacher *et al.*, 2005; Pörtner *et al.*, 2007).

The effects of elevated temperatures on Antarctic species have been investigated using a variety of metrics (e.g. growth, survival, performance), and the nearly universal outcome is that these organisms have limited tolerance for even small increases in temperature. In short-term experiments, Antarctic marine ectotherms die when exposed to temperatures in the range of 5-10°C (Somero and DeVries, 1967; Peck and Conway, 2000; reviewed in Pörtner *et al.*, 2007, Peck *et al.*, 2008), even for a very brief period. Over longer terms, the brittle star *Ophionotus victoriae* cannot survive more than a few months at 2°C, a temperature less than 0.5°C above its summer maxima (Peck *et al.*, 2009), and the survivable temperature range of the Antarctic bivalve molluscs *Laternula elliptica* and *Limopsis marionensis* is two to six times smaller than the typical range of temperate or tropical bivalves (Peck and Conway, 2000). When looking at whole-organism performance instead of survivorship, temperature thresholds for negative effects are even lower (Pörtner *et al.*, 2007; Shishido *et al.*, 2019). For example, reburying ability of the bivalve *Laternula elliptica* (Peck *et al.*, 2007) and swimming ability of the scallop *Adamussium colbecki* (Peck *et al.*, 2004) are both negatively impacted (complete failure to rebury and swim, respectively) by temperatures only 1-2°C above the Antarctic summer maxima. These studies and others have led to predictions that Antarctic ectotherms will be particularly vulnerable to globally warming oceans (Peck, 2018).

In Antarctic ectotherms, temperature sensitivity of metabolic and whole-organism processes are often considered to reflect biochemical, physiological, and morphological cold adaptation (Peck, 2018), which results in tradeoffs between performance at low temperature and an inability to regulate function at higher temperatures (Flynn and Todgham, 2017). Another way that temperature affects performance is through its effects on oxygen consumption and transport. For ectotherms, as temperatures increase, oxygen consumption by metabolism increases more rapidly than the rate of oxygen supply and transport to internal tissues, leading to a mismatch of oxygen supply to demand and eventual loss of function and death (von Bertalanffy, 1960). Limits to organismal capacity to maintain oxygen supply to meet energetic demands are thought to determine how individuals, populations, and species cope with a warming environment (Pörtner and Knust, 2007; Flynn and Todgham, 2017).

While Antarctic organisms in general are sensitive to elevated temperatures, most studies have investigated only adults. Compared to adults however, early life history stages have very different morphologies, physiologies, and ecologies (Pechenik, 1999). Thus, determining whether thermal sensitivity changes over ontogeny is important especially in light of climate change; in order to make predictions about how a species may respond to a changing environment, it is important to identify the most vulnerable stages and define their physiological limits (Pörtner and Knust, 2007; Flynn and Todgham, 2017; Morley *et al.*, 2017). In general, early life history stages are thought to be more vulnerable to environmental stressors than adults (Vernon, 1899; Gosselin and Qian, 1997; Darling and Côté, 2008; Harvey *et al.*, 2013; Kroeker *et al.*, 2013; Przeslawski *et al.*, 2014; Karelitz *et al.*, 2017), for several reasons. Early life history stages generally have narrower thermal windows (thermal windows of early life stages in temperate fishes are generally half that of juvenile and adults) (Rombough, 1997; Pörtner and Peck, 2010; Schiffer *et al.*, 2014), fewer energetic resources and limited capacity to metabolically buffer increased temperature effects (Rombough, 1997; Hamdoun and Epel, 2007), and underdeveloped oxygen delivery systems (Peck *et al.*, 2013). Recent work by Peck *et al.* (2013) however, found that juveniles of four benthic Antarctic invertebrates survived to higher temperatures than adults and in 3 of the 4 species, performed better than adults at elevated temperatures. A handful of other studies also support greater resilience in juveniles and young adults (Peck *et al.*, 2009; Righton *et al.*, 2010; Clark *et al.*, 2013). For example, in a study comparing adult and juvenile Antarctic clams (*Laternula elliptica*), Clark *et al.* (2016) found that

compared to adults, juvenile *L. elliptica* showed significant up-regulation of chaperone and antioxidant transcripts that are associated with cryopreservation and thermal stress when exposed to acute elevated temperatures (12h at 3°C). Similarly, compared to adults, juvenile *L. elliptica* maintained higher respiration rates in response to increased sedimentation (Philipp *et al.*, 2011), reburied faster and survived better after an injury (Husmann *et al.*, 2014), and produced an enhanced immune response to upper lethal temperatures (Peck *et al.*, 2013). Likewise, in a study on the metabolic rates of Antarctic dragonfish (*Gymnodraco acuticeps*), thermal sensitivity decreased over larval development (Flynn and Todgham, 2017). These data emphasize the importance of considering the role of developmental stage in environmental stress responses, which will be key to understanding the vulnerability of Antarctic organisms to the impacts of climate change (Mintenbeck *et al.*, 2012; Flynn and Todgham, 2017).

To determine if thermal sensitivity of an important group of Antarctic organisms, the sea spiders (Arthropoda, Pycnogonida), changes over ontogeny, we evaluated the response of the Antarctic pycnogonids, *Ammothea glacialis* to acute temperature changes. On the Antarctic benthos, pycnogonids are abundant, diverse, and ecologically important predators and scavengers (Slattery and McClintock, 1995; Clarke and Johnston, 2003; Moran *et al.*, 2018). Sea spiders inhabit most marine environments (Arnaud and Bamber, 1987; Dunlop and Arango, 2005). In temperate and tropical waters, most species are small, with maximum leg spans of only a few centimeters. The Southern Ocean hosts a large proportion of the world's known pycnogonids; of the 1400 known species (Arnaud and Bamber, 1987; Child, 1998; Arango and Wheeler, 2007), about 190 species occur in Antarctic waters (Gusso and Gravina, 2001; Clarke and Johnston, 2003), and polar and abyssal species can have leg spans of over 70 cm (Arnaud and Bamber, 1987). Sea spiders lack specialized respiratory organs or pigments (Redmond and Swanson, 1968; Markl, 1986; Rehm *et al.*, 2012) but instead obtain oxygen through diffusion across the cuticle (Douglas *et al.*, 1969; Davenport *et al.*, 1987; Lane *et al.*, 2018). For convective internal transport pycnogonids rely on a weak dorsal heart and peristaltic contractions of the gut which extends into the legs (Woods *et al.*, 2017).

We previously explored the effects of temperature on *Ammothea glacialis* using righting ability as a measure of physiological performance (Shishido *et al.*, 2019; chapter 2). In that study, locomotory performance of *A. glacialis* was highly sensitive to elevated temperatures and

decreased by almost half at 4°C (~ 6°C above ambient). In the current study, we measured oxygen consumption at a range of elevated water temperatures of three ontogenetic stages: larvae, juveniles, and adults. We measured oxygen consumption because unlike locomotory performance, it can be directly compared among different ontogenetic stages which do not share many morphological or behavioral traits. Likewise, while performance gave us an indication of adult pycnogonids' response to thermal stress under maximum oxygen demand (via exercise), oxygen consumption is a measure of routine metabolic rate and provides a window on the immediate energy requirement under fully aerobic conditions (Clarke, 1987). We hypothesized that like locomotory performance, metabolic rate (measured as rate of oxygen consumption) of adult *A. glacialis* would be strongly affected by elevated temperatures. We also assessed the thermal sensitivity of oxygen consumption at different life history stages to shed preliminary light on how warming temperatures will affect metabolic rates and energy dynamics across the life cycle.

Methods

Collection and Maintenance

Pycnogonids were collected on SCUBA near McMurdo Station, Antarctica (77°51´S, 166°40´E) between 10 and 40 m depth, in October and November 2015 and 2016. Animals were transported to McMurdo Station in coolers filled with chilled seawater (-1.8°C) and were kept in flow-through seawater tables maintained at 1-2°C above ambient temperatures (-1.8°C) until used for experiments.

Our focal species was *Ammothea glacialis* (Ammotheidae). This species attains very large body sizes as adults (leg spans greater than 10cm) (Figure 1), and all life history stages can be found close to McMurdo Station. Adult individuals of *A. glacialis* were identified to species using the keys of Child (1995), and larval stages were collected from egg-carrying males that had been identified to species. Juveniles, which are free-living, were identified using genetic barcoding (cytochrome c oxidase subunit 1) (methods in Lane *et al.*, 2017). In total, our dataset contained five ontogenetic stages: larval stages II, III, IV (staging after Sánchez and López-González, 2013) juveniles (free-living individuals >0.002g and <0.1g), and adults (Figure 1).

Temperature experiments

Larvae

Respiration (O_2 consumption) of larvae was measured at three developmental stages and two temperatures using the μ-BOD endpoint determination method (Marsh and Manahan, 1999). Male pycnogonids carry eggs and developing larvae (Cano and López-González, 2009). Therefore, to obtain larvae, adults were collected in the field as above, and egg masses were gently removed from egg-carrying individuals and staged under a dissecting microscope. Egg masses and larvae were kept cold (-1.8 to 0°C) during handling by immersing their containers in a salt-ice slurry bath. Our observations of development matched those of Sánchez and López-González (2013) for the same species, and we utilized their terminology for staging. Six egg masses were chosen that contained larvae at a mix of developmental stages from Stage II (first hatching stage) to Stage IV (2 pr walking legs fully formed, 1 pr limb buds; oldest stage found on any mass). Individual larvae were gently separated from each mass using fine forceps and larvae from the six masses were combined and pooled by stage (II, III, and IV). Then, for each stage and temperature, groups of 2-35 individuals were placed in 5-6 replicate small (500-800 μl) individual gas-tight vials of known volume in temperature-equilibrated, oxygen-saturated sea water filtered to 0.2 μm. Vials were incubated at -1.8°C or 4°C for approximately 2-7 h (depending on temperature and the number of larvae in a vial, but in no case did the partial pressure of oxygen drop below 85% of saturation), after which a 300-μl sample was taken from each vial using a temperature-equilibrated gas-tight syringe. Oxygen partial pressure in each sample was measured with a polarographic oxygen sensor which was calibrated before each run at full oxygen saturation and at zero oxygen saturation (Model 1302, Strathkelvin). Respiration rate was calculated as the slope of the regression line of oxygen consumed (μmol O_2 $vial^{-1}$ h^{-1}) plotted against number of larvae in the vial. Some of the regressions had a nonzero Y-intercept (see Marsh and Manahan, 1999); these were corrected by the Y-intercept in order to allow for comparison of oxygen consumption rates between runs via an ANOVA approach (after Walther *et al.*, 2013). We calculated the mass of an individual larvae from each stage using samples that were previously frozen. Larval mass was measured by blotting the larvae dry to remove any excess water and then weighing a known number of 10-15 larvae per stage on an electronic balance (Model MS104S, Mettler Toldeo). Mean mass was calculated as total weight divided by number of larvae per sample. This process was repeated three times for each stage, and mass of

each stage was calculated as the mean of the three per-individual masses calculated for that stage.

Juveniles

To measure the metabolic rate of juvenile pycnogonids (n=22) we used the same end-point determination method setup as for larvae, except that because juveniles were much larger, we were able to measure respiration of single individuals. For juveniles, we measured the oxygen consumption of individual sea spiders per vial at three temperatures (-1.8, 1, and 4°C). Temperatures were controlled by immersing respiration vials in a water bath set to the experimental temperature, and incubation times were 12 hours at -1.8°C, 6 hours at 1°C, and 3 hours at 4°C. After incubation, oxygen tension was measured (as for larval vials) to determine the rate of oxygen consumption by each animal. Oxygen consumption of each animal was measured sequentially at -1.8, 1, and 4°C. Between measurements, juveniles were returned to ambient conditions for at least 24 h before being exposed to the next higher temperature. Each juvenile was assessed daily for normal appearance, movement, and behavior over the course of the experiment. After all metabolic measurements were complete, each animal was blotted dry and weighed on an electronic balance (Model AE163, Mettler Toledo) to obtain wet mass and then preserved in 100% ethanol for later genetic analysis.

Adults

O_2 consumption of adults of *A. glacialis* (n=9) was measured on individuals using a closed system respirometry oxygen optode system (NeoFox Sport, Ocean Optics) with continuous oxygen measurements. Measurements were made in 0.27-L glass dishes containing a magnetic stir bar shielded by a small housing made of plastic mesh. During experiments, chambers were held in a 5-L bath of water set on top of a magnetic stir plate. The sensor spot of the optode system was affixed to the lid of the chamber, which was made of a flat and thin (less than 2 mm) piece of glass; the gas-tight seal was created by a thin rubber gasket between the lid and the edge of the chamber, and the lid was held in place with a 1 lb rubber coated lead weight placed on top of the glass disc.

Experiments were performed in a cold room set at such a temperature that the bath of water with the chamber was at (or close to) the target temperature. Target temperatures of -1.8, 1, and 4 to

match the temperatures of the experiments done with larvae and juveniles; realized temperatures, which we measured throughout the experiments, averaged -1.6°C ±0.06 ("-1.8°C"), -0.4°C ±0.05 ("1°C"), and 3.33°C ±0.1 ("4°C") (supplementary material, S1). The optode was calibrated in temperature-equilibrated, air-saturated seawater at the beginning of every run. For each run, the chamber was then filled by submersing it a 5-L water bath containing 0.2-um filtered, air-saturated seawater at the experimental temperature. The pycnogonid was then added to the chamber and the chamber was sealed and inspected for trapped air bubbles which were removed prior to oxygen measurements. A chamber with identical setup but no sea spider was run once for every three animal trials as a blank, and values from animal chambers were corrected by subtracting the slope calculated from the blank from the slope calculated from the measured metabolic rate with the sea spider. Experiments were run in the dark to minimize the potential photosynthetic oxygen production. Oxygen levels in the chambers and temperature of the water bath were recorded with NeoFox recording software. Incubations were ended when individual sea spiders had drawn down oxygen to ~20% of air saturation. Each animal was sequentially exposed to each progressively higher temperature for ~12 hours at -1.8°C, 3 hours at 1°C, and 3 hours at 4°C. Between each measurement, animals were returned to flowing sea water at ambient temperature for at least 24 h before being exposed to the next higher temperature. Rate of oxygen consumption was calculated by subtracting the slope of oxygen decline of the blank chamber from the slop of the experimental chamber, then multiplying by the experimental chamber volume (270 ml). After measurements at all three temperatures, each animal was blotted dry and weighed on an electronic balance (Model PE1600, Mettler Toledo).

Statistical analysis

We compared the effect of temperature on the metabolic rate of the larvae of *A. glacialis* (stages II-IV) using a linear mixed effects model with larval stage and temperature as independent variables and individual pycnogonids as a random effect. Metabolic rate data were log-transformed to meet the assumptions of normality and to give reasonable distributions of the residuals.

Metabolic rates of juveniles and adults were analyzed separately because they included an intermediate temperature. We used a linear mixed effects model with metabolic rate as the dependent variable and developmental stage and temperature as independent variables. Individual pycnogonids were included as a random effect, and metabolic rates were log-

transformed to meet the assumptions of normality. Individuals that did not measurably draw down oxygen at a particular temperature were excluded from the analysis for that temperature trial (juvenile n= 2, adult n= 3).

We also calculated mass specific metabolic rates (MSMR) for all stages. Juvenile and adult MSMRs were calculated by dividing the rate of oxygen consumption of an individual by the mass of that individual. We then compared the effect of temperature on the metabolic rate of juvenile and adult *A. glacialis* using a linear mixed effects model, developmental stage and temperature and independent variables and individual pycnogonids as a random effect. Mass specific metabolic rates were log transformed to meet assumptions of normality. For larvae, since we could not measure the mass of individuals, we calculated MSMR by dividing the rate of oxygen consumption per individual (calculated for each vial) and dividing that number by the mean mass of individual larvae at that particular stage. We then compared the effect of temperature on the metabolic rate of between the three larval stages using a different linear mixed effects model with MSMR as the dependent variable, and larval stage and temperature as independent variables. Individual vials were included as a random effect and MSMRs were log transformed to meet assumptions of normality. Larvae were not included in quantitative analysis with the juveniles and adults because the different methods for mass calculation and absence of data for 1°C made statistical comparisons with adults and juveniles difficult.

Temperature sensitivity was also assessed using the temperature coefficient Q_{10}, a dimensionless index that can be used to compare the thermal sensitivity of organisms with vastly different masses and across different temperature intervals (Hochachka and Somero 2002). We calculated Q_{10} with the standard formula:

$$Q_{10} = \frac{R_2^{\left(\frac{10}{T_2 - T_1}\right)}}{R_1},$$

where R_1 and R_2 are oxygen consumption rates at temperature T_1 and T_2, respectively. For larvae, Q_{10} values were calculated for each vial between -1.8 and +4°C (in each vial, the same individuals were used at the two temperatures); for juveniles, Q_{10} was calculated for each individual for both the overall temperature range (-1.8 to 4°C) and between each temperature step (-1.8 to 1 and 1 to 4°C). For adults, because temperature for each run varied slightly from

the target, Q_{10} values were calculated using the actual temperatures measured during each incubation.

Q_{10} values were compared among all stages between the highest and lowest temperatures using linear mixed effects model with Q_{10} as the dependent variable and developmental stage as the independent variable. Vials were included as a random effect and Q_{10} values were log-transformed to meet the assumptions of normality. A Tukey HSD post hoc test was performed to determine which stages were significantly different from each other. To compare juveniles and adults across three temperatures, we used a linear mixed effects model with Q_{10} as the dependent variable and developmental stage as the independent variable. Individual pycnogonids were included as a random effect and Q_{10} values were log-transformed to meet assumptions of normality. A Tukey HSD post hoc test was performed to determine which stages and temperatures were significantly different from each other. All statistical analyses were performed with JMP Pro (version 14, SAS institute Inc., Cary, North Carolina).

Results

Our dataset contained five ontogenetic stages with the following average masses: larval stage II (0.00011g ±0.00017), stage III (0.00021g ±0.00005), stage IV (0.00084g ±0.00038), juvenile (0.0109g ±0.001), and adult (1.04g ±0.12) (mean ±SE) (Table 1).

Oxygen consumption

The oxygen consumption rate of larvae increased both over ontogeny (stage II < III < IV) and as temperature increased (LMM, larval stage $p<0.0001$*, temperature $p<0.0001$*, larval stage*temperature $p=0.081$; Figure 2). Similarly, oxygen consumption of both juveniles and adults increased with temperature (LMM, Temperature, $p<0.0001$; Figure 2). Adult metabolic rates were also significantly higher than juveniles (LMM, Tukey HSD, $p<0.05$, Figure 2). There was no significant interaction between developmental stage and temperature (LMM, $p=0.29$).

Mass-specific metabolic rate

There was no significant difference in MSMR between larval stages (LMM, Larval Stage, $p=0.19$) but the MSMR did significantly increase with temperature (LMM, Temperature, $p<0.0001$, Table 1). There was no significant interaction between development stage and

temperature for larval stages (LMM, p=0.39). MSMR of both juveniles and adults increased with temperature (LMM, Temperature, p<0.0001) but MSMR of juveniles was significantly lower than of adults (LMM, Developmental Stage, p<0.0001, Table 1). There was no significant interaction between developmental stage and temperature (LMM, p= 0.39).

Thermal sensitivity of oxygen consumption

Overall, developmental stage explained a significant amount of the variance in Q_{10}, and this was largely attributable to lower thermal sensitivity at the larval stage and very high sensitivity at the adult stage (LMM, p=0.0007*; Table 2, Figure 3). The two youngest stages, II and III, had significantly lower Q_{10} values than stage IV larvae, juveniles, and adults (LMM, Tukey HSD, p<0.05; Table 2, Figure 3).

We also compared Q_{10}s between temperature steps in adults and juveniles and found that both stage (LMM, p=0.0025*) and temperature (-1.8 to 1°C, 1 to 4°C; LMM, p=0.0006*) had significant effects (Table 2). There was also a significant interaction between developmental stage and temperature step (LMM, p=0.0004*) such that the Q_{10} values of adult *A. glacialis* between -1.8 to 1°C were significantly higher than any of the other stage*temperature combinations (Tukey HSD, p<0.05; Table 2).

Discussion

Overall, we found that oxygen consumption rates of all developmental stages of the pycnogonid *Ammothea glacialis*, from larvae to adults, increased with temperature. However, thermal sensitivity of oxygen consumption, estimated with Q_{10}, increased over ontogeny: older larvae (stage IV), juveniles, and adults were more sensitive to elevated temperatures than early stage (stage II and III) larvae (Figure 3). While interpreting metabolic rates is complex (Peck, 2018), these results contradict the prediction that early life history stages are more sensitive to environmental changes than later life history stages (Rombough, 1997; Peck *et al.*, 2009; Righton *et al.*, 2010; Flynn and Todgham, 2017; Messmer *et al.*, 2017). Q_{10} values of metabolism have only been measured for a handful of other species, but our data for larvae of *A. glacialis* are comparable; Q_{10} values for larval metabolic rates of the Antarctic seastar, *Odontaster validus*, were 4.4 (Peck and Thomas 2002), and for larvae of the Antarctic

nudibranch *Tritonia challengeriana*, were 9.6-30 (depending on stage) (Moran and Woods, 2008). All of these values were high compared to what is expected of normal biological systems (2-3) (Clarke, 1983) and to Q_{10}s measured for comparable stages of related, temperate species (e.g. *Tritonia diomedea* embryos, Q_{10}= 2.1 (early stage), Q_{10}=2.5 (mid stage), Q_{10}=2.6 (late stage) (Moran and Woods, 2007). Increases in metabolic rate affect all aspects of an organism's energy budget, and are likely to have profound effects on developing systems that rely on endogenous yolk reserves (Mueller *et al.*, 2011; Zuo *et al.*, 2011). *A. glacialis* have yolk-feeding (lecithotrophic) larvae (Cano and López-González, 2009), so temperature-driven increases in metabolism will result in more rapid yolk depletion over development unless the rate of development is sped up by the same amount. For most organisms, increases in temperature tends to speed up metabolism more than development rate (Kamler, 2008; Mueller *et al.*, 2011). While we do not know the effects of temperature on developmental rate of pycnogonids, higher metabolic rates, if not matched by equivalently faster development, may compromise juvenile quality, or even the ability of the larvae to reach the juvenile stage (Blaxter, 1992; Kamler, 2008; Mueller *et al.*, 2011).

While larvae of *A. glacialis* were metabolically quite sensitive to increases in temperature, juveniles and especially adults were even more so. This is clearly not a feature of pycnogonids in general; adult and juvenile *A. glacialis* had much higher Q_{10}s than temperate confamilial species *Achelia chelata* and *Achelia gracilipes*, which were in the range of 2-3 (Chapter 3; Shishido et al., in prep.). Unlike larvae, juveniles and adults could potentially offset higher metabolic costs by increasing consumption of exogenous food. However, in previous work we showed that the locomotor ability of adult *A. glacialis* was reduced by ~50% at 4°C (relative to ambient) (Shishido *et al.*, 2019), suggesting that feeding abilities would be greatly compromised. Therefore, in the absence of temperature acclimatization, at warmer temperatures Antarctic pycnogonids are likely to experience substantial energy deficits from increased metabolic demands accompanied by reduced ability to obtain food. Even if they do not starve to death, temperature effects would still limit the amount of energy that animals could put towards for reproduction, which can have population-level effects (Petes *et al.*, 2007; Rogers-Bennett *et al.*, 2010; Lemoine and Burkepile, 2012).

Another pattern we observed was that when comparing between the two lower temperature increments, -1.8 and -0.4°C (realized), adult *A. glacialis* had a Q_{10} value of 7.05×10^5, higher than Q_{10}s reported for any other Antarctic organism (or for any organism, to our knowledge). Other studies have also found very high Q_{10} values for different rate processes in Antarctic organisms over comparable temperature increments, such as Ashton *et al.* (2017) who found that in an Antarctic bryozoan, growth rate had a Q_{10} of ~1000 with 1°C in warming over ambient. A compelling conceptual framework for understanding these very high thermal sensitivities is still lacking, but possibilities include enzyme and related biochemical processes that are associated with cold adaptation (e.g. problems associated with protein synthesis and folding in cold temperatures (Peck, 2016), an increase in the energy required to complete processes such as growth (Heilmayer *et al.*, 2005) or limited functions of essential pathways due to critical enzymes that are temperature sensitive (Clark *et al.*, 2016). These very large effects of temperature on biological processes in Antarctic animals highlight our need to better understand these processes, especially in light of ocean warming.

Our experiments were based on acute temperature changes, and we did not test for acclimatory ability. Temperate acclimation is very common and may be the component of phenotypic plasticity that confers the most resistance to environmental change (e.g. Somero, 2010, 2012; Stillman and Somero, 2015; Messmer *et al.*, 2017). In Antarctic ectotherms, however, temperature acclimation appears to be very slow and limited in range (reviewed in Peck et al., 2014). For example, Audzijonyte *et al.* (2019) found that when Arctic sculpin, *Myoxocephalus scorpius*, were acclimated to elevated temperatures for 8 weeks, Q_{10} values for metabolic rate decreased from 2.4 to 1; experiments with other organisms have yielded similar results (e.g. Peck *et al.*, 2009; Pörtner and Peck, 2010; Bilyk and DeVries, 2011; Morley *et al.*, 2011; Suckling *et al.*, 2015; Enzor *et al.*, 2017). Peck (2018) suggested that for Antarctic ectotherms, acclimation could take much longer to complete because of the lack of ability to modulate intracellular membrane-related processes when the animals are warmed. Therefore, the thermal sensitivity we measured for *A. glacialis* would be unlikely to be greatly mitigated by experimental acclimation of animals, even if long-term (months) acclimation were experimentally practical. This is particularly the case for early life history stages such as larvae, which may lack complex physiological mechanisms for acclimation.

Our data on mass-specific metabolic rates were also in agreement with previous data from Antarctic ectotherms. We found that the MSMRs of adult *A. glacialis* were an order of magnitude lower than MSMRs of relatives from temperate regions (Chapter 3); this a common pattern that helps to create another linkage to the temperature sensitivity of Antarctic organisms (Clarke, 1980; Johnston *et al.*, 1991; Peck *et al.*, 2002). The MSMR of adult *A. glacialis* at ambient (-1.8°C) was 0.28 ± 0.06 μmol h^{-1} g^{-1} (mean ± SE), compared to two temperate species from the same family (Ammotheidae), *Achelia chelata* (4.55 ± 0.38 μmol $h^{-1}g^{-1}$) and *Achelia gracilipes* (8.69 ± 1.16 μmol h^{-1} g^{-1}) (Chapter 3). According to Pörtner *et al.*, (2007), low MSMR is feature of cold-adapted organisms that is linked to the requirement for tight energetic efficiency to support higher organismic functions such as growth in such a cold environment. Studies involving cold-adapted species have shown that many Antarctic animals demonstrate at least partial compensation for low temperatures through elevated mitochondrial ATP synthesis capacity (Sommer and Pörtner, 2004), increased enzyme activities (Crockett and Sidell, 1990), and/or greater mitochondrial density (Guderley, 2004; Lurman *et al.*, 2010; Lurman *et al.*, 2010). However, cold adaptation is also associated with evolutionary losses of a number of traits that are no longer adaptive under the thermally stable, cold conditions of the Southern Ocean (Pörtner *et al.*, 2007). One of these losses is the ability to rapidly response to environmental change such as elevated temperatures (Pörtner *et al.*, 2007). For example, Hofmann *et al.* (2000) found that Antarctic nototheniod fish lack a molecular heat-shock response, the only such example among vertebrates. There are also cold adapted related tradeoffs that may aid an animal's performance at low temperatures but can disrupt function at higher temperatures (Pörtner *et al.*, 2007). For example, the notothenioid fish, *Pagothenia borchgrevinki*, has elevated levels of a neurotransmitter related enzyme to aid in swimming control at cold temperatures; but this enzyme is also very temperature sensitive and could result in the loss of swimming ability within minutes of being exposed to elevated temperatures (Baldwin and Hochachka, 1970; Macdonald *et al.*, 1988; Pörtner *et al.*, 2007). Although we did not examine these tradeoffs in this study, the low metabolic rates and high degree of thermal sensitivity point to *A. glacialis* as being a cold adapted species that may have lost the ability to rapidly respond to elevated temperatures.

Our results demonstrate that the aerobic metabolism of all stages of *A. glacialis* is highly sensitive to elevated temperatures, and that this sensitivity increases over ontogeny. Higher metabolic rates occurred at temperatures that have been shown to strongly reduce locomotory

ability of adults (Shishido *et al.*, 2019), suggesting that for adults, temperature-driven increases in metabolic energy expenditure cannot be offset by greater feeding and assimilation. Aerobic metabolism of larvae was also highly sensitive to temperature (although less so than juveniles or adults), suggesting that small changes in temperature will strongly impact the energetics of development. Assessing the sensitivity of ectotherms to stressors such as elevated temperature provides important baseline information, especially in the face of a rapidly warming ocean (Morley *et al.*, 2017). While the results of acute laboratory experiments can shed valuable light on the 'winners and losers' under global warming (Schram *et al.*, 2015; González *et al.*, 2016; Tarling *et al.*, 2016; Clark *et al.*, 2017), the challenge now is to extrapolate these results toward annual and decadal scales which are the most appropriate for assessing impacts of climate change (Messmer *et al.*, 2017).

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Tables

Table 1. Mass and Mass Specific Metabolic Rates of *Ammothea glacialis*

Developmental Stage	Mass (g) (mean ± SE)	Mass specific metabolic rate (µmol h^{-1} g^{-1}) (mean ± SE) -1.8 °C	Mass specific metabolic rate (µmol h^{-1} g^{-1}) (mean ± SE) 1 °C	Mass specific metabolic rate (µmol h^{-1} g^{-1}) (mean ± SE) 4 °C
Larvae – Stage II	0.00011g ±0.00017	0.93 ±0.1	-	2.20 ±0.16
Larvae – Stage III	0.00021g ±0.00005	1.15 ±0.18	-	2.40 ±0.33
Larvae – Stage IV	0.00084g ±0.00038	0.81 ±0.22	-	2.37 ±0.33
Juvenile	0.0109g ±0.001	0.04 ±0.004	0.11 ±0.08	0.22 ±0.03
Adult	1.04g ±0.12	0.28 ±0.06	0.81 ±0.1	1.16 ±0.05

Table 2. Average Q_{10} values at each developmental stage

Developmental Stage	-1.8 to 1°C	1 to 4°C	-1.8 to 4°C
Larvae – Stage II	-	-	5.14 ± 1.10
Larvae – Stage III	-	-	3.53 ± 0.55
Larvae – Stage IV	-	-	7.72 ±1.96
Juvenile	54.17 ±21.22	49.41 ±20.74	22.58 ±5.19
Adult	704578.99 ±547275	8.92 ±5.71	81.78 ±40.16

Figures

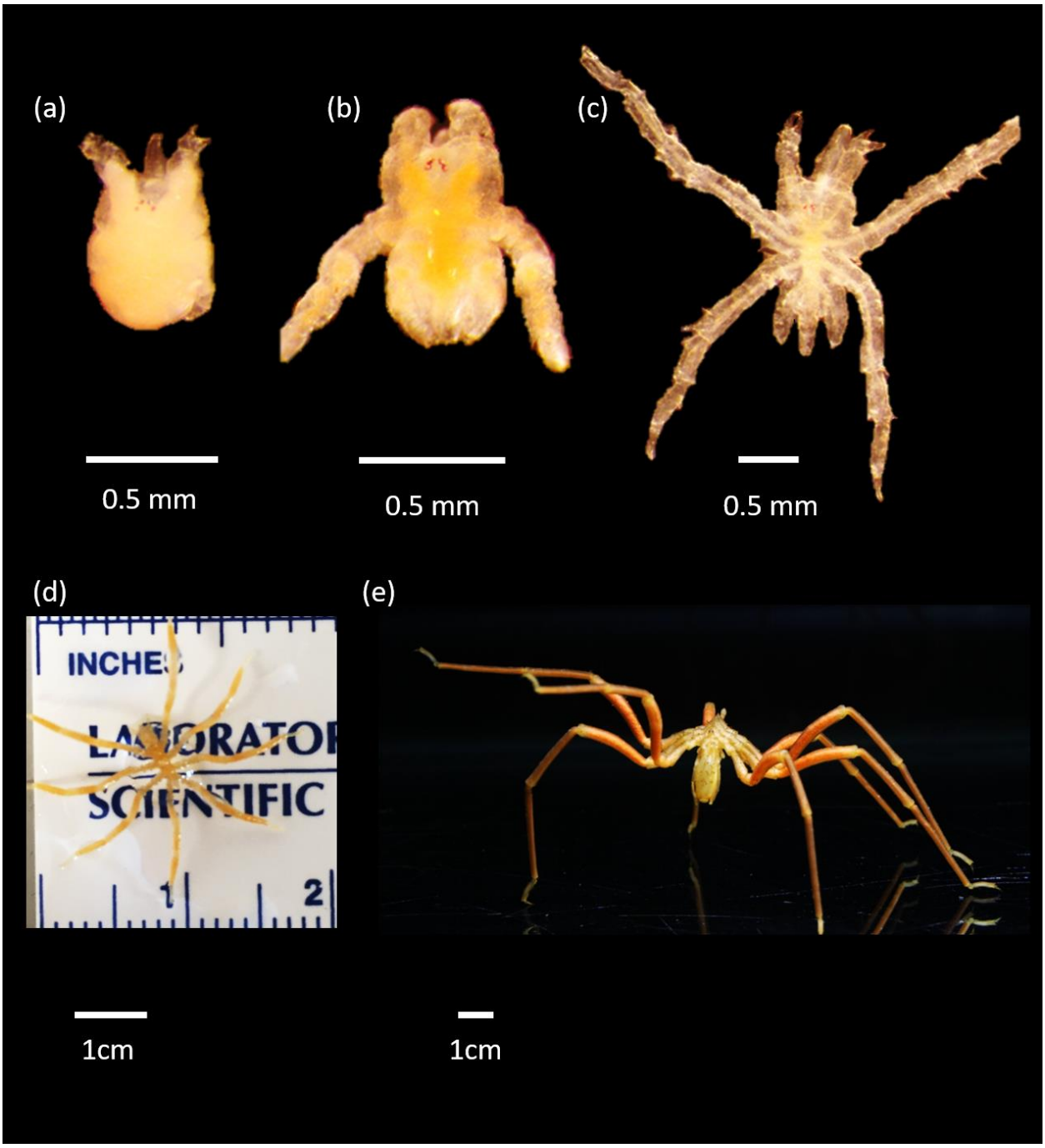

Figure 1. Developmental stages of *Ammothea glacialis* used in this study. (a) larval stage II; Photo: A. Moran, (b) larval stage III; Photo: A. Moran, (c) larval stage IV; Photo: A. Moran, (d) juvenile, (e) adult; Photo: Timothy R. Dwyer, PolarTREC 2016/courtesy of ARCUS

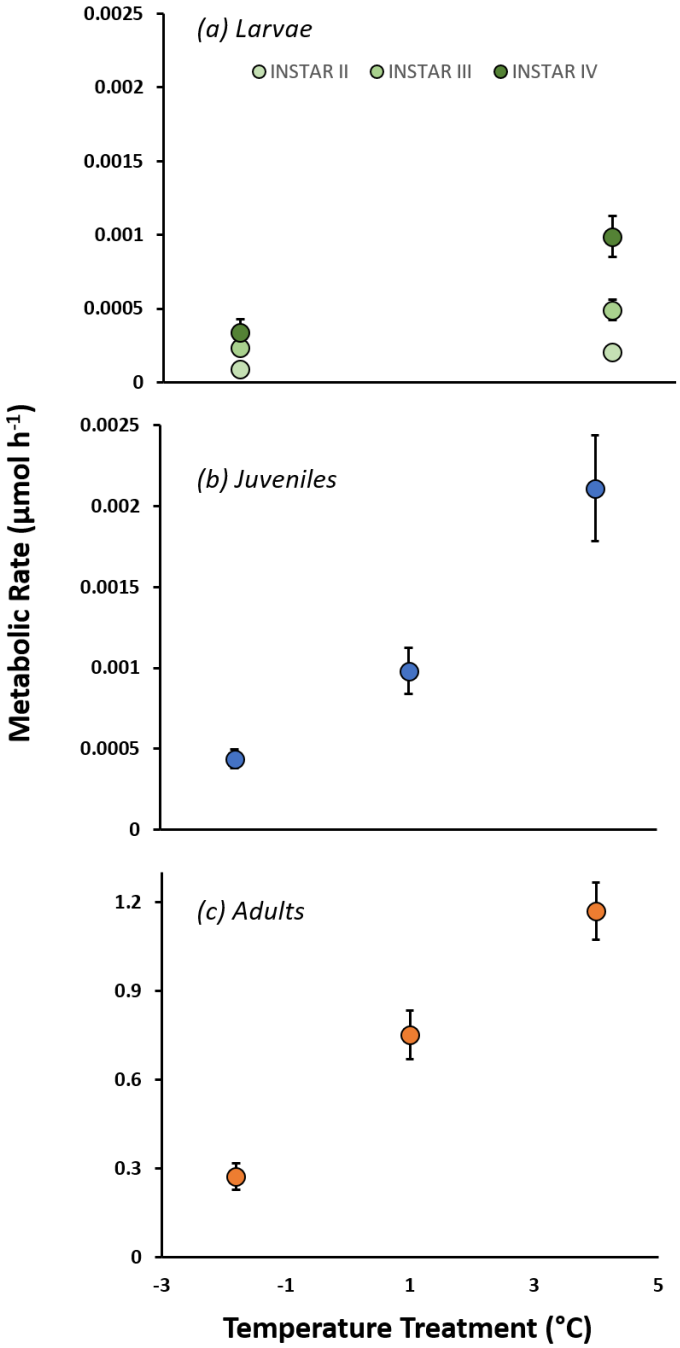

Figure 2. Metabolic rate (mean ±SE) for *Ammothea glacialis* over ontogeny. (a) Larvae at stage II, III, IV at -1.8 and 4°C (LMM, larval stage p<0.0001*, temperature treatment p<0.0001*, stage*temperature p=0.0812), (b) juvenile at -1.8, 1, and 4°C and (C) adult pycnogonids at -1.6, -0.4, and 3.33°C (realized temperatures, S1) (LMM, larval stage p<0.0001*, temperature treatment p<0.0001*, stage*temperature p=0.2907). Note that y axis is different for (c) and for (a) and (b). Note that for analysis, adult pycnogonids were grouped into target temperature groups of -1.8, 1, and 4°C.

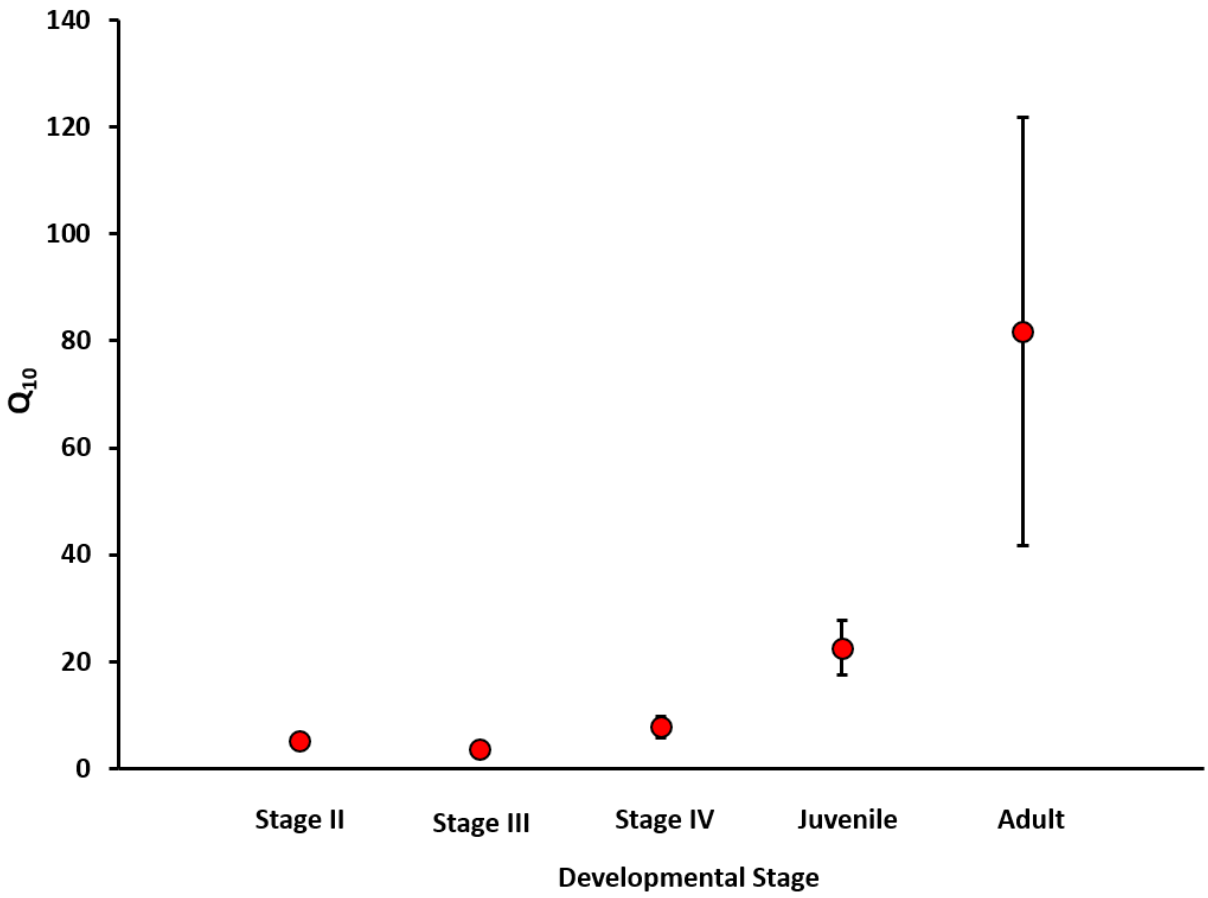

Figure 3. Q_{10} of *A. glacialis* across ontogeny (LMM, p=0.0007*, Tukey HSD). Q_{10} values of adults were calculated with actual temperature ranges (see methods and S1) but, for visual purposes we grouped them together here with -1.8, 1 and 4°C.

CHAPTER 5- CONCLUSIONS AND FUTURE DIRECTIONS

Oxygen limitation has been proposed as one of the central factors limiting body sizes in ectotherms at high temperatures (the 'oxygen-temperature hypothesis') (von Bertalanffy, 1960; Atkinson *et al.*, 2006; Audzijonyte *et al.*, 2019). In recent years, mismatches between oxygen supply and demand have become a leading hypothesis for why body sizes of adults of many aquatic and marine environments appear to be shrinking as the environment warms (Sheridan and Bickford, 2011; Cheung *et al.*, 2012; Verberk *et al.*, 2016). Using pycnogonids, I evaluated how body size is limited by oxygen-temperature interactions to shed light on how large-bodied ectotherms may respond toclimate warming.

In Chapter 2, I tested the oxygen-temperature hypothesis by exposing species from two genera of giant Antarctic pycnogonids, *Colossendeis* and *Ammothea*, from a range of body sizes to four elevated temperatures (-1.8, 4, 7, and 9°C) and testing their righting ability as a metric of performance. Here I found that both *Colossendeis* and *Ammothea* were both highly sensitive to elevated temperature, consistent with the idea that Antarctic organisms are highly stenothermal (Somero and DeVries, 1967; Peck and Conway, 2000; Peck *et al.*, 2008). We also found that the two species differed in their sensitivity; *C. megalonyx* was able to maintain performance up to 7°C where *A. glacialis* showed reductions of ~50% in righting rate at 4°C. While I found significant effects of temperature on performance, I found no evidence that large bodied sea spiders were more affected by elevated temperatures than small individuals, which did not support the oxygen-temperature hypothesis. One potential explanation came from my examination of the microstructure of the cuticle of both species, which showed that as pycnogonid body size increased, porosity increased as well. This pattern occurred in both species although the magnitude of the increase in porosity with body size was larger in in *C. megalonyx*. This increase in porosity likely increases cuticular permeability for diffusive oxygen supply, potentially offsetting the greater diffusion distances and higher metabolic demand of larger bodies. These data suggest that polar giants are able to attain large body sizes not only because their metabolic rates are limited by cold temperatures, but also because the porosity of the cuticle increases as they get larger. In the future, it would be interesting to look for this relationship between performance and porosity in other species of Antarctic sea spiders, and to determine

whether there are tradeoffs between cuticle porosity and cuticle strength that may create new, biomechanically-driven limits to body size.

In Chapter 3, I tested the oxygen-temperature hypothesis using two species of temperate pycnogonids (*Achelia chelata* and *Achelia gracilipes*). In warmer environments, the constraints on body size limits are more stringent than cold environments like the Southern Ocean (warmer temperatures increase the rate of diffusive supply to tissues, but organismal oxygen demand also increases as metabolic rates increase with temperature) (Gillooly *et al.*, 2001; Jacobsen *et al.*, 2003; Makarieva *et al.*, 2005; Verberk *et al.*, 2011). I measured oxygen consumption at five temperatures from 12 to 28°C using a range of body sizes and found that the metabolic rates of both species were significantly affected by temperature but the effect was size-independent; thus, there was no evidence to support the oxygen-temperature hypothesis. However, unlike Antarctic pycnogonids (Chapter 2), cuticular porosity did not change with body size. We hypothesized that the small body size exhibited by these temperate sea spiders then may be attributable to other selective factors besides oxygen limitation; these factors could include, predation, competition pressure or drag forces from wave action or high current velocities. It is also possible that these pycnogonids may use other pathways for increasing oxygen supply such as increasing the rate of gut peristalsis, which has been shown to increase as temperature increases (Woods *et al.*, 2017). Porosity was also highly variable among individuals, although there was no relationship between metabolic rate and porosity for individuals (i.e. porous individuals did not have higher metabolic rates). Previous work on cuticular structure suggested that pores may have secretory functions which may aid in desiccation, and could be useful for *A. gracilipes* as it is an intertidal species, or provide protection from nematocysts of hydroid hosts (King, 1973; Tomaschko and Brückmann, 1990; Tomaschko, 1992). However, in general, little is known about the how these pores function. All of these ideas would also be interesting to test in tropical pycnogonids where temperatures are warmer so constraints on body size should be even stronger.

In Chapter 4, I examined the thermal sensitivity of the Antarctic *Ammothea glacialis* over ontogeny by exposing three stages of larvae, juveniles, and adults to three temperatures (-1.8, 1, 4°C) and measured oxygen consumption. I found that metabolic rate of all stages increases with temperature, suggesting high temperature sensitivity. The increase in metabolic rate is not likely to reflect high aerobic scope, difference between minimum and maximum oxygen consumption

rate, because as in since we found in Chapter 2, exposure to 4°C reduced performance in adult *A. glacialis*. Sensitivity to temperature, expressed as the Q_{10} of oxygen consumption, also increased over ontogeny, with adults being the most sensitive stage. Adult *A. glacialis* exhibited extremely high Q_{10} values between -1.8 and 1°C which is a range that is ecologically relevant to near future ocean warming temperatures. Determining the pathways that lead to such high thermal sensitivity would be interesting and would be important in considering how these organisms will respond to ocean warming. Often certain tradeoffs are associated with cold adapted physiology, one of which is the loss of heat shock response; thus it could be useful to examine if this functionality is also lost in pycnogonids. Further interesting steps would test the thermal sensitivity other species of pycnogonids including *Colossendies megalonyx* and compare that with our results from *A. glacialis*. In Chapter 2, I found that *C. megalonyx* was able to maintain performance at a much higher temperature than *A. glacialis*, but they were also considerably more porous. It is possible that because *C. megalonyx* are more porous they may have a greater capacity for gas exchange and therefore be capable of higher levels of aerobic activity than *A. glacialis*. Thus, I would expect that *C. megalonyx* would be less temperature sensitive than *A. glacialis*. However, expectations of thermal sensitivity over ontogeny for *C. megalonyx* are difficult to predict because so little is known about its early life history stages. Finally, investigations into the acclimation potential and ultimately capacity of Antarctic pygnogonids should be examined as acclimatory capacity has been suggested as the component of phenotypic plasticity that will confer the most resistance to environmental change.

Overall, in this dissertation I established that both temperate (*Achelia* spp.) and Antarctic pycnogonids (*Colossendeis* and *Ammothea*) are affected by elevated temperatures. I also found no support for the oxygen-temperature hypothesis in either temperate or Antarctic sea spiders, indicating that the large bodied polar giants may not be as vulnerable to climate warming as predicted. However, as stated at the end of Chapter 2, predicting the ‘winners’ or ‘losers’ of climate change is complicated and will require a more nuanced, whole-organism approach that integrates across many levels of a species’ ecology, life history, and physiology. Thus, these studies are part of a larger puzzle that will help better inform how different species may respond to changes in our climate.

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APPENDIX

Appendix A: Supplementary Material for Chapter 2

S1: Cuticle Morphology

To measure cuticle thickness (CT, cm) and areal porosity (AP, %), we used a scalpel to cut three thin cross sections from the femur of the second right leg of each individual (Figure 1); we standardized the leg and leg segment to control for any potential morphological variation in cuticle thickness across the body of the animal. Each cross section was mounted under a compound microscope (Olympus BX41TF, OPtical ELements COrportation, Dulles, Virginia, USA) with an attached camera (Q Color 3, Olympus America, Pennsylvania, USA) and imaged using the Qcapture program (version 2.9.13, Quantitative Imaging Corporation, Surrey, B.C., Canada). Within each image, cuticle thickness was measured at three haphazardly chosen points as the distance from the outer to the inner surface. Measurements were not taken where the cuticle appeared anomalously thick or thin, for example near apodemes. Cuticle thickness was calculated for each individual by averaging the three CT measurements from three pictures of each cross section from three cross sections of the femur (27 measurements per animal). These measurements were multiplied by 1.05 for *C. megalonyx* and 1.06 for *A. glacialis* to correct for our observation that femur CT was slightly thinner, on average, than the cuticle of other leg segments. Because the cuticle of the femur was slightly thinner than the cuticle of some of the other leg segments, we derived a correction factor for each species based on the difference between cuticle thickness of the femur vs. the cuticle thickness of the rest of the legs, and the relative lengths of each leg segment. First, we measured the cuticle thickness of each segment of all eight legs of two individuals from each species as above (3 measurements from 3 thin slices of each leg segment). We then measured the length of each segment of each leg ($L_{segment}$) and calculated the proportional length of each segment relative to the entire leg (= $L_{segment}/L_{leg}$), multiplied this proportion by the CT for that segment (=$CT_{segment}$ x ($L_{segment}/L_{leg}$), and calculated an average CT of the entire leg by summing the products across each leg ($CT_{leg} = \Sigma\ CT_{segment}$ x ($L_{segment}/L_{leg}$). This sum was then divided by the measured CT of the femur of that leg to get our correction factor ($CF = CT_{average}/CT_{femur}$). The correction factors from individuals were then averaged to get a correction factor for each species. This correction factor was then applied to the

original measurements of the femur and used for all subsequent calculations ($CT_{corrected} = CT_{femur}$ x CF).

To calculate areal porosity (AP) (the total cross-sectional area that is void space divided by the total area of the cuticle, *sensu* Nimmo 2004), we measured the total area of pores within each cross section and then divided that area by the total area of the cross section, and calculated an average AP for each individual as above for CT.

Surface area (cm^2) was estimated by summing the area inside the projected outline of each spider and multiplying by two to account for the dorsal and ventral sides of the animal, then assumed the body was a long cylinder and multiplied this number by a correction factor of 1.57 to account for the three-dimensionality of the body. Then, to take into account the three-dimensionality of the animal's body, we assumed the body to be a long open cylinder with a surface area equal to $SA_{\text{open cylinder}} = 2\pi r_1 h_{segment}$, where r_1 is the radius and h is the length of a leg segment. To convert from two to three dimensions, we multiplied the two-dimensional estimate from each animal by a correction factor of 1.57, which was derived from the relationship between the surface area of a two-dimensional rectangle ($SA_{rectangle}$=2 x 2rh) of the same diameter and length and the surface area of an open cylinder: $\frac{SA_{open\ cylinder}}{SA_{rectangle}} = \frac{\pi}{2}$. Thus, SA_{3D}=1.57 x SA_{2D}, where SA_{2D} was the surface area found using ImageJ (as in Lane et al. 2018).

To obtain the total volume of pores in each individual, we first calculated the cuticle volume as a hollow cylinder $Vcuticle = \pi h_{total}(r_1^2 - r_2^2)$ where h_{total} was the total summed length across all legs, r_1 was the radius of the entire leg, and r_2 was the radius of the inner hollow part of the leg. Pore volume was then calculated as the product of cuticle volume and areal porosity (PV = CV * AP).

S2:

Table 1. Zero-inflated generalized linear mixed-effect model (ZIGLMM) of pycnogonid righting performance.

Species	n	Estimate ±SE	z	p
Colossendeis sp.				
Temperature	42	-0.128 ±0.007	-18.99	<0.0001*
Mass		0.198 ±0.256	0.77	0.44
Temperature*Mass		-0.002 ±0.012	-0.17	0.86
Colossendeis megalonyx				
Temperature	20	-0.116 ±0.015	-7.92	<0.0001*
Mass		1.510 ±0.489	3.09	0.002*
Temperature*Mass		0.126 ±0.045	2.81	0.005*
Ammothea glacialis				
Temperature	26	-0.182 ±0.179	-14.11	<0.0001*
Mass		-0.245 ±0.155	-1.58	0.11
Temperature*Mass		0.016 ±0.012	1.35	0.18

S3:

Table 2. Range and mean (±SE) number of rightings per hour (RPH) at each temperature treatment.

Species	Mass range (g)	Temperature °C	RPH range	Mean RPH (±SE)
Colossendeis sp.	0.21 -21.80	-1.8	1 – 70	30.12 ±2.93
		4	0 – 101	26.26 ±22.45
		7	0 – 40	11.75 ±14.44
		9	0 – 23	3.57 ±5.21
Colossendeis megalonyx	0.21 – 2.26	-1.8	1 – 63	20.20 ±3.92
		4	0 – 54	14.45 ±3.67
		7	0 – 34	16.0 ±6.51
		9	0 – 11	1.90 ±0.70
Ammothea glacialis	0.18 – 2.22	-1.8	14 – 75	48.19 ±3.14
		4	0 – 49	25.39 ±2.75
		7	0 – 46	8.27 ±1.98
		9	0 – 12	2.19 ±0.74

S4:

Table 2. Range and mean (±SE) number of rightings per hour (RPH) at each temperature treatment.

Species	Temperature °C	Mean RPH (±SE)	95% CI
Colossendeis sp.	-1.8	30.12 ±2.93	(24.21, 36.03)
	4	26.26 ±22.45	(19.27, 33.26)
	7	11.75 ±14.44	(2.58, 20.92)
	9	3.57 ±5.21	(1.95, 5.20)
Colossendeis megalonyx	-1.8	20.20 ±3.92	(11.99, 28.41)
	4	14.45 ±3.67	(6.76, 22.14)
	7	16.0 ±6.51	(-2.08, 34.08)
	9	1.90 ±0.70	(0.44, 3.36)
Ammothea glacialis	-1.8	48.19 ±3.14	(41.72, 54.67)
	4	25.39 ±2.75	(19.71, 31.06)
	7	8.27 ±1.98	(4.18, 12.36)
	9	2.19 ±0.74	(0.66, 3.73)

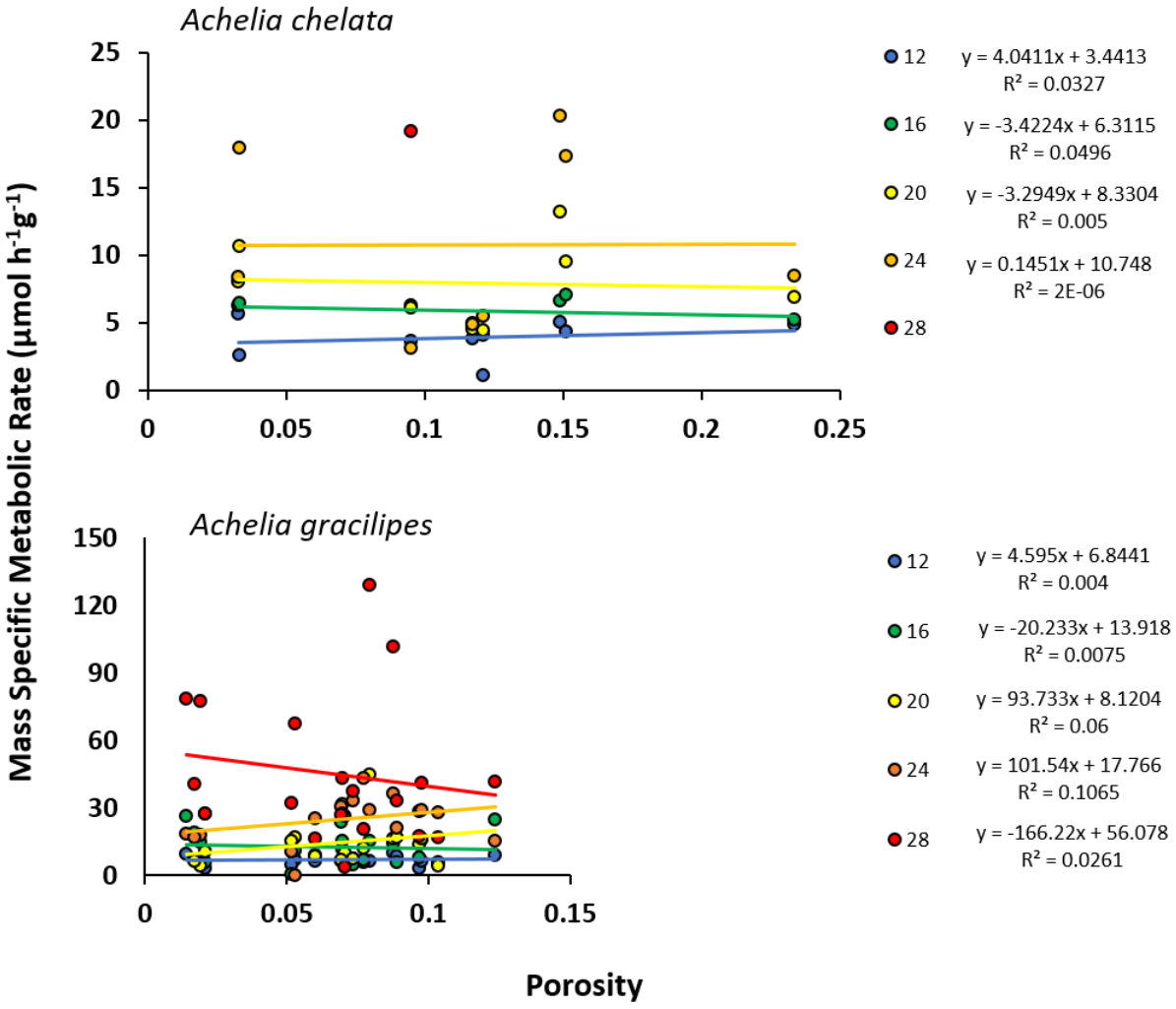
S5:

Table 3. Scaling of cuticular morphology in pygnogonids.

Models	n	a	95 % CI	P	b (actual)	b (isometry)	95 % CI	P	R^2
log(CT) vs log(Mass)									
Colossendeis megalonyx	24	-2.26	(-2.35,-2.17)	< 0.001	0.51	0.33	(0.35,0.67)	<0.001	0.67
Ammothea glacialis	27	-2.21	(-2.25,-2.17)	< 0.001	0.58	0.33	(0.46,0.70)	<0.001	0.80
log(SA) vs log (Mass)									
Colossendeis megalonyx	24	1.45	(1.43,1.48)	<0.001	0.62	0.67	(0.58,0.66)	<0.114	0.98
Ammothea glacialis	27	1.31	(1.29,1.33)	<0.001	0.78	0.67	(0.73,0.83)	<0.001	0.98
AP vs log(Mass)									
Colossendeis megalonyx	24	0.18	(0.16,0.21)	<0.001	0.11	-	(0.07,0.15)	<0.001	0.63
Ammothea glacialis	27	0.10	(0.09,0.11)	<0.001	0.05	-	(0.03,0.07)	<0.001	0.52
log(PV) vs log(Mass)									
Colossendeis megalonyx	24	-1.62	(-1.76,-1.48)	< 0.001	1.50	1	(1.25,1.75)	<0.001	0.87
Ammothea glacialis	27	-1.93	(-1.98,-1.87)	< 0.001	1.61	1	(1.45,1.77)	<0.001	0.94

"n": Number of individuals used in each analysis. Regression coefficients: intercept ("a"), scaling exponent calculated from the slope of the regression line (“b actual”), expected scaling exponent derived from geometric isometry (“b isometry). Expected scaling exponents for AP are unknown.

Appendix B: Supplementary Material for Chapter 3



Supplementary Figure 1. Porosity vs Mass Specific Metabolic Rate

Appendix C: Supplementary Material for Chapter 4

S1: Realized temperatures for adult *A. glacialis* respiration trials. Temperatures were calculated by taking the mean (±SD) temperature over the entire trial period.

Adult ID	Target Temp		
	-1.8°C	**1°C**	**4°C**
1	-1.53 ± 0.02	-0.27 ± 0.02	2.97 ± 0.03
2	-1.45 ± 0.07	-0.34 ± 0.05	3.40 ± 0.15
3	-1.69 ± 0.03	-0.65 ± 0.03	3.38 ± 0.14
4	-1.69 ± 0.008	-0.40 ± 0.05	3.28 ± 0.12
5	-1.48 ± 0.06	-0.41 ± 0.03	3.7 ± 0.1
6	-1.59 ± 0.04	-0.4 ± 0.04	3.24 ± 0.1
7	-1.64 ± 0.11	-0.39 ± 0.02	3.21 ± 0.06
8	-1.63 ± 0.05	-0.17 ± 0.09	3.35 ± 0.08
9	-1.58 ± 0.14	-0.34 ± 0.08	3.54 ± 0.07

S2: Q_{10} values calculated from mean metabolic rates at each developmental stage. Adult Q10 value was calculated using the mean realized temperature range of -1.6 and 3.3°C.

Developmental Stage	**Q_{10}**
Larvae – Stage II	4.09
Larvae – Stage III	3.52
Larvae – Stage IV	6.34
Juvenile	15.08
Adult	24.34