

Evaluating the resilience of Antarctic echinoderms to Southern Ocean freshening: Short- and long-term responses



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September 2024

This thesis is submitted for the degree of *Doctor of Philosophy*

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Abstract

Climate change is impacting marine ecosystems worldwide, presenting a significant threat to biodiversity. In the Southern Ocean, organisms are facing increasing challenges due to warming, ocean acidification, reduced sea-ice cover, and freshening—the reduction in salinity caused by freshwater input. Salinity is a crucial environmental factor that affects the development, growth, reproduction, and survival of aquatic organisms. While the effects of warming, acidification and loss of sea ice cover on Antarctic marine life have been widely studied, the impact of low salinity has been under-researched. Understanding the physiological impact of environmental stressors, such as freshening, is crucial for identifying species particularly vulnerable to change. This may be particularly important for animals which are considered to have poor osmoregulatory abilities, such as echinoderms which are strictly marine with no known freshwater species. In Antarctica, echinoderms are highly abundant and conspicuous, making up around 10‰ of benthic fauna. Many are endemic to Antarctica, having adapted to the low, stable temperature environment over millions of years. Climate change will likely increase the rate and frequency of hyposalinity events in Antarctica, where vast amounts of freshwater from melting glaciers and liquid precipitation rapidly enters coastal waters, diluting seawater. Over the longer-term, climate change is also expected to cause widespread net freshening over the whole Southern Ocean. For a group of animals adapted to a thermally stable and predictable environment, and with limited abilities to function in low-salinity conditions, echinoderms are potentially vulnerable to climate-change-induced freshening in Antarctica. However, little is known about their ability to tolerate acute, short-term reductions in salinity, and even less is understood about their capacity to acclimate to sustained low-salinity conditions.

This thesis set out to assess the physiological and behavioural responses of common Antarctic echinoderms to low salinity exposure over both short- and long-term timeframes, in order to evaluate their vulnerability to present and future climate-change-induced freshening. An initial global literature review of echinoderms revealed inconsistencies in experimental approaches and descriptions of short- and long-term low salinity tolerance, making comparisons between species and regional groups challenging. To address this, a methodology was developed using the temperate echinoid *Echinus esculentus*, which showed distinct short- and long-term metabolic responses to low salinity. Experimental data

demonstrated that *E. esculentus* could acclimate to moderate salinity reductions, with thresholds identified between 21‰ and 26‰. This approach was then applied to Antarctic echinoderms. Acute tolerance experiments revealed species-specific responses, with unexpected variations across habitats and class. Despite this variability, phylum-wide similarities were observed in oxygen consumption, activity rates, and osmotic strategy responses. The brittle star *Ophionotus victoriae* showed the lowest tolerance to salinity reductions, highlighting its vulnerability to freshening, while holothurians demonstrated remarkable tolerance, in particular *Echinopsolus charcoti* and *Cucumaria georgiana*.

Long-term exposure studies on the echinoid *Sterechinus neumayeri* and asteroid *Odontaster validus* indicated successful acclimation to a mid-range salinity level (29‰) within their short-term tolerance range. However, at lower salinity (24‰), although survival rates remained high, physiological and behavioural responses failed to stabilise. Significant reductions in animal mass suggested that high catabolic tissue breakdown was necessary to maintain core homeostatic functions, with prolonged exposure likely leading to mortality.

To understand the mechanistic basis of low salinity acclimation, a metabolomic approach was applied to tissue samples of *S. neumayeri* and *O. validus* from the long-term experiments. These analyses provided insights into the micromolecular adaptations of cold-temperature-adapted echinoderms and their strategies for low salinity acclimation. In particular, the osmolyte profile in both species differs from temperate echinoderms and other marine invertebrates, with branched-chain amino acids (valine, leucine, and isoleucine) potentially acting as both compatible osmolytes, cryoprotectants and even an energy reserve.

Overall, this thesis demonstrates that Antarctic echinoderms are capable of short-term resilience to moderate salinity reductions, with certain species showing remarkable tolerance and varying levels of acclimation capacity. This acclimation potential may provide partial resilience to predicted climate change-induced freshening. However, their long-term acclimation potential is limited, and species-specific vulnerabilities highlight the need for further research. The study also reveals that unique adaptations, such as specific osmolyte profiles, may influence their survival and ability to cope with future environmental changes. These insights highlight the need for ongoing research into Antarctic echinoderms and other marine organisms to understand their physiological limitations, which is crucial for developing targeted conservation strategies to mitigate the impacts of future freshening and environmental changes on their survival.

For Sophie

Acknowledgements

Firstly, I would like to thank both my supervisors Lloyd Peck and Elizabeth Harper for their constant support, encouragement and guidance. Lloyd, you've been an inspiration and mentor throughout my PhD, and I've genuinely enjoyed our almost weekly one-on-one science discussions. Those conversations were the kind that make a PhD truly special.

Liz, thank you for your invaluable, balanced advice and wisdom throughout these four years. You were always a voice of reason, offering clarity when it was most needed, and I truly appreciate your constant availability, prompt responses and the time you dedicated to providing thoughtful and constructive feedback. Together, your insights and critiques have very much shaped my growth as a scientist, thank you.

I have also been fortunate enough to work with many inspirational scientists that have offered crucial support and advice throughout my PhD; in particular Melody Clark, Simon Morley, and Rod Wilson. Thanks also to Nadia Frontier and Katrin Linse. I also want to acknowledge and thank other members of the British Antarctic Survey (BAS) for their expertise and support, in particular Rebecca Smith, Guy Hillyard and Paul Geissler.

I am very grateful for the support I have had from my friends in the Adaptations student group at BAS; Kudzai, Bethany, Ainsley and Anne-Pia - what a great team! Thank you to past Adaptation members, Dennis and Sam. Special thank you to Rebecca De Leij for all your help with training during the pandemic, and of course Arlie, you have been both a great mentor and friend. I also want to acknowledge the support and comradery of my fellow BAS students.

A big thank you to my Pembroke friends and flatmates, Pablo, Alice, Greta and Callum. I thoroughly enjoyed the last two year living in Grantchester Meadows. Also, to my original S staircase friends, Emily, Mats, Fredrick and Sophie.

Support from good friends counts for a lot when you are away from home, so a big thank you to all of you, in particular Tom, Jono, Dave, James and all Snarls, for your patience, advice and friendship.

To Mike, I'm lucky to have a best friend as brother, thank you. To my parents, I will be forever grateful for your unwavering support, constant encouragement and for nurturing my curiosity about nature. I have you to thank for guiding me towards studying Biology as an

undergraduate—something that, funnily enough, has proven incredibly useful. To my stepdad Steve, thank you for motivating me to make a change and for your generous enthusiasm throughout. And to Sandra and Georgia, thank you for all your support and encouragement.

And finally, to Sophie, who I owe unlimited gratitude for allowing me to leave the life we built in London and move to Cambridge for four years to better myself. You have had the biggest influence on me and have one of the sharpest minds I know. Thank you for your constant love and support, and for holding on.

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Chapter 1 – Introduction

1.1 Climate change and Antarctica

There is now unequivocal evidence that human-induced activity, primarily the release of greenhouse gases over the past two centuries, are responsible for recent global warming (IPCC, 2023). However, the rate of warming is not globally uniform. Polar regions are some of the most rapidly warming areas (González-Herrero et al., 2024; Vaughan et al., 2003). Surface temperature in parts of the Western Antarctic Peninsula (WAP) have warmed by over 3°C since the 1950s (Turner et al., 2020) with corresponding Southern Ocean temperatures rising at a rate of 0.1–0.3°C per decade since the 1980s (IPCC, 2022a; Schmidtke et al., 2014). This warming has led to substantial increases in glacial and ice shelf melting and dramatic reductions in the seasonal extent of sea ice cover (Cook et al., 2005; Eayrs et al., 2021). Antarctic mean annual sea ice cover dropped from a record high in 2014 to a record low in 2017, then returned to average levels, highlighting its susceptibility to rapid changes driven by atmospheric fluctuations and ocean warming (Eayrs et al., 2021; Meehl et al., 2019). In parallel to warming, the Southern Ocean has experienced ocean acidification, with surface pH dropping by ~0.1 units since the industrial revolution with predictions of a further decline by 0.71 units between 2020 and 2100 under a high emissions scenario. (Key et al., 2004; McNeil and Matear, 2008; Metzl et al., 2024a) The increased solubility of gases at colder, higher latitudes makes the Southern Ocean a natural sink for carbon dioxide. Consequently, acidification has lowered the saturation state of calcium carbonate (CaCO₃) minerals, leading to predictions that the Southern Ocean will be the first global region to become undersaturated for aragonite, the more soluble form of CaCO₃ (Fabry et al., 2009; Metzl et al., 2024a).

Warming, ocean acidification and changes in sea ice cover are considered the three major environmental threats to Antarctica's native marine animals and ecosystems (Peck, 2018), generating concern from biologists (Barnes, 2015; Hancock et al., 2020; Peck et al., 2014). However, a fourth, less frequently addressed threat, 'freshening'—the lowering of seawater salinity—also warrants concern in Antarctic ecosystems. As a major environmental stressor for marine organisms (Kinne, 1964; Rivera-Ingraham and Lignot, 2017), the potential

impact of reductions in salinity on Antarctic marine fauna has largely been overlooked in environmental research. Globally, climate change is driving an increase in coastal freshening through increased precipitation and terrestrial run off (Mackenzie et al., 2007; Röthig et al., 2023; Sejr et al., 2017). In polar regions, the vast quantities of freshwater locked up as ice present increased threats of coastal freshening as meltwater enters the ocean. Since the 1950s, the Southern Ocean has experienced broadscale freshening, driven by anthropogenic warming (Swart et al., 2018). Given that the Antarctic ice sheets contain approximately 70% of the world's freshwater (IPCC, 2022a; NSF, 2024), and with predictions indicating that they will lose mass at an accelerated rate throughout the rest of this century (IPCC, 2022a) releasing large volumes of meltwater into the Southern Ocean, it is now critical to address the impact of freshening on Antarctic marine animals and ecosystems.

1.1.1 Freshening in the Southern Ocean

Southern Ocean salinity varies over both large and small scales due to the input of three main sources of freshwater: glacial and shelf-ice meltwater, precipitation, and sea-ice melt (Haumann et al., 2016; Jacobs et al., 2002). Between 1950-2010, surface waters across the whole Southern Ocean have freshened (IPCC, 2022a; de Lavergne et al., 2014), a trend that has been mirrored in the denser and deeper water masses that form from them (i.e., Antarctic Intermediate Water and Antarctic Bottom Water) (Helm et al., 2010; Purkey and Johnson, 2013). On a regional scale, the western Weddell Sea (see Figure 1.1) has experienced a freshening rate of $-0.01 \pm 0.007 \text{ g kg}^{-1} \text{ decade}$ since the 1970s, while the Ross Sea has been freshening at a rate of $-0.27 \pm 0.012 \text{ g kg}^{-1} \text{ per decade}$ since the 1990s (Schmidtke et al., 2014). Driving the net freshening are a combination of factors, with increases in precipitation and the northward transport of freshwater via sea-ice (Haumann et al., 2016), considered to have a greater impact than ice-sheet melt (Swart et al., 2018).

Nearshore, the Antarctic coastal environment is the interface between terrestrial meltwater and the ocean. Dramatic variations in salinity gradients occur at coastal sites, impacting the structure and function of benthic communities (Moon et al., 2015).

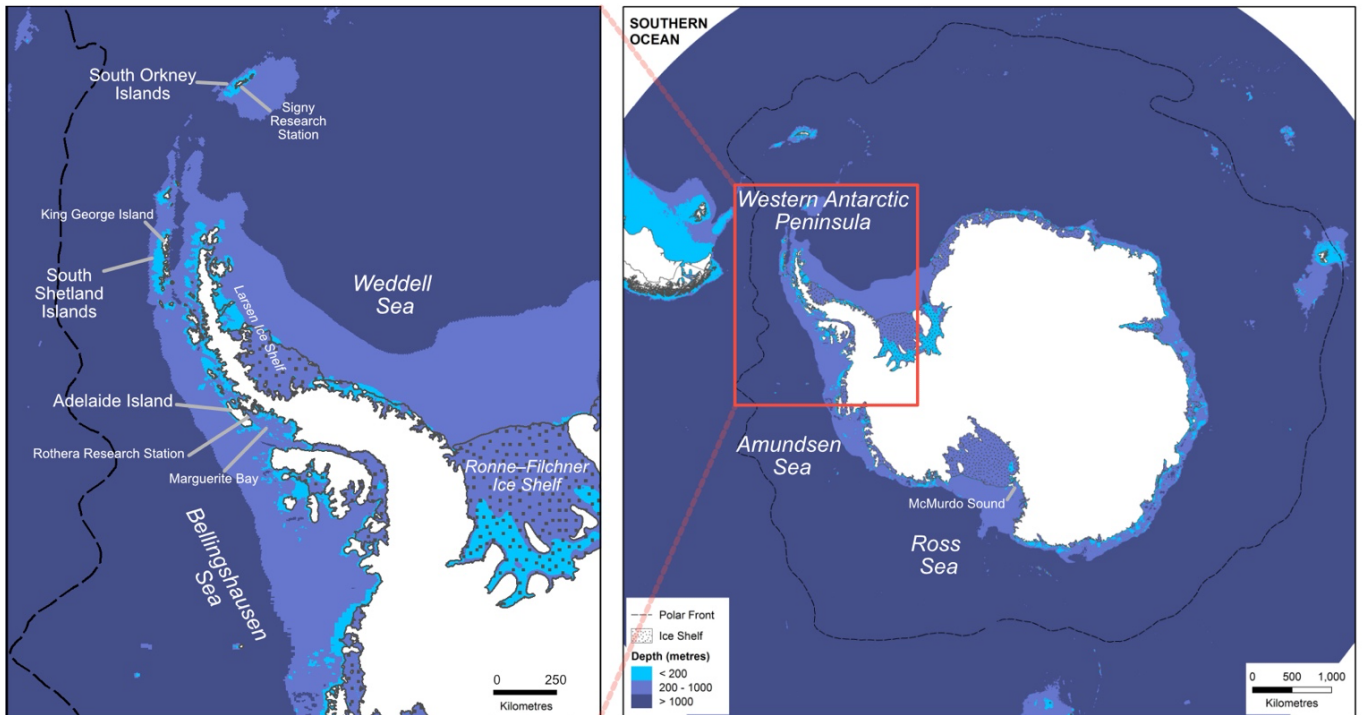


Figure 1.1 | Map of the Western Antarctic Peninsula (left) and map of the Antarctic continent and Southern Ocean (right) with the Western Antarctic Peninsula highlight by the red rectangle. Modified from Peck (2018), original image provided by P. Fretwell, British Antarctic Survey.

Nearshore surface salinities can show wide seasonal variations (Figure 1.2), due to the input of glacial meltwater. For example, surface salinities were shown to vary between 28.4‰ and 35.2‰ in Ryder Bay, Adelaide Island, Antarctic Peninsula between 2012 to 2021 (Clarke et al., 2022)]. Furthermore, glacial calving events can rapidly reduce surface salinity in enclosed bays [e.g., salinity dropped from 33‰ to 27‰ over a few days in 2017 in Fildes Bay, King George Island, South Shetland Islands, due to glacial calving (Navarro et al., 2019)].

The Antarctic intertidal is the most dynamic environment regarding salinity (Figure 1.2). Freezing on the seashore and seabed (known as the icefoot), can lead to localised increases in salinity, forming hypersaline pools. These pools have been documented to contain seawater with salinity levels exceeding three times that of the open ocean (Clarke and Beaumont, 2020). Conversely, freshwater run off from glacial, ice-shelf, and snowfields can reduce intertidal salinities to <12‰ (Waller et al., 2006a), while organisms caught in meltwater streams or a hyposaline lens can be exposed to near freshwater conditions (Davenport, 2001; Stockton, 1984).

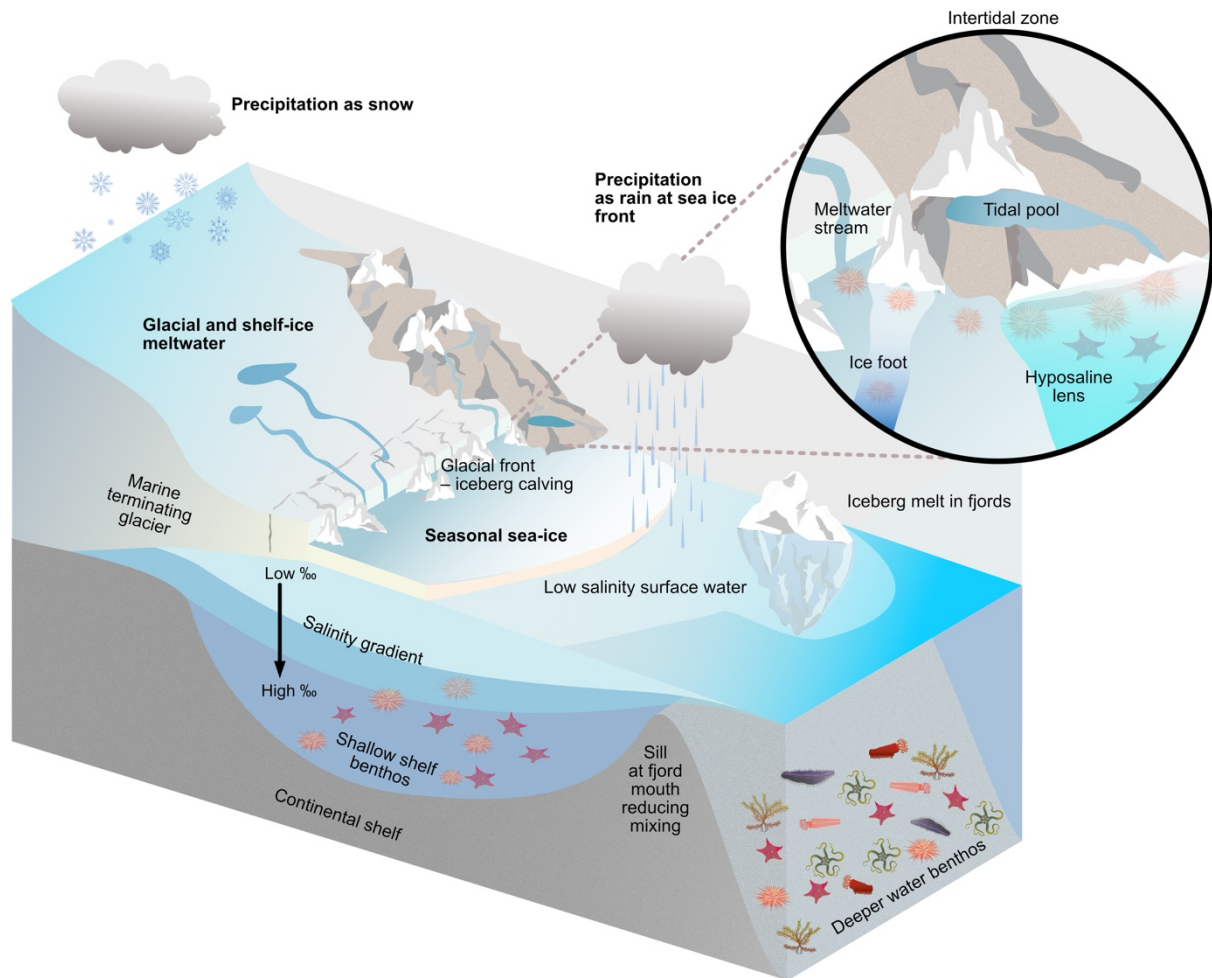


Figure 1.2 | Sources of salinity variation in an Antarctic marine fjord, with a marine terminating glacier. Main sources of freshwater are highlighted in bold.

Away from shore, the contribution of terrestrial run-off decreases with distance, however oxygen isotope data confirm its impact on lowering salinity hundreds of miles offshore (Meredith et al., 2013). Large scale ice shelf calving and subsequent iceberg melt can introduce vast amounts of terrestrial freshwater into the Southern Ocean. For example the calving of the Larson-C Ice Shelf in July 2017 released an estimated 152 giga-tonnes of freshwater into the seas around South Georgia island over a 3.5 year period (Braakmann-Folgmann et al., 2022). The influence of sea-ice on large scale salinity variation is more complex. Freezing surface seawater expels high salinity brine and stabilises the water column, reducing mixing; summer melting releases large quantities of freshwater, causing seawater dilution and increased mixing. Satellite data reveal seasonal sea surface salinity in the open Southern Ocean varies from 34.45‰ to 33.40‰ (2011-2017) (Garcia-Eidell et al., 2019),

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although these data lack the spatial resolution to identify local scale salinity variation due to sea-ice formation and melt. Low salinity is increasingly a year-round feature at the edges of the sea-ice, attributed to increased precipitation falling as rain and snow (Garcia-Eidell et al., 2019). Until recently, the annual extent of sea-ice cover was considered stable and was even shown to be increasing (Comiso et al., 2017) in stark contrast to the rapid decline of Arctic sea-ice (Serreze and Stroeve, 2015; Stroeve and Notz, 2018; Stroeve et al., 2012). However, from 2016 to 2019 there was a dramatic reduction in Antarctic sea-ice cover, 27% below mean annual coverage, matching the 30 year decline observed in the Arctic (Eayrs et al., 2021; Turner and Comiso, 2017). These declines have continued in recent years with the 4 lowest Antarctic minimum sea-ice extents all occurring in the last 6 years (NOAA, 2024). Reductions in sea-ice formation can add to overall net freshening (e.g. Jacobs et al., 2002) as less freshwater is held as sea-ice.

1.1.2 Vulnerable ecosystems

The impact of Antarctic freshening has been most acutely observed on the Western Antarctic Peninsula (see Figure 1.1 red rectangle). This region has warmed more rapidly than any other region of the continent (González-Herrero et al., 2024; Vaughan et al., 2003) with sea water temperatures rising over 1°C in the latter half of the twentieth century (Meredith and King, 2005; Schmidtke et al., 2014). Over the same period the region experienced the retreat of 87% of marine glacial fronts (Cook et al., 2005), while more recently the region has lost around 1339.68 km² of ice between 2001-2015 (Silva et al., 2020). There has also been an increase in the annual duration of melting conditions, signalling increases in meltwater run-off into the ocean under future warming predictions (González-Herrero et al., 2024; Vaughan, 2006). Glacial meltwater accounts for the dominant freshwater source in many regions of the WAP (Meredith et al., 2008), while there has also been an acceleration of snow accumulation on the Antarctic Peninsula and increased precipitation (Thomas et al., 2008; Wang et al., 2022). Although snow is the main form of precipitation in Antarctica, the WAP experiences liquid precipitation on more than 50 days per year, with more frequent and intense rain predicted by the end of the century (Silva et al., 2020). Sites at the northern end of the WAP, such as Eczurra Inlet on King George Island experience significantly warmer temperatures than higher Antarctic latitudes (Griffith and Anderson, 1989; Heinemann et al., 2019). Here, the impact of

freshening at glacier fronts has been considered as a factor in observed low polychaete diversity (Siciński, 2004; Pabis and Siciński, 2010). At lower trophic levels, coastal freshening along the WAP is implicated in alterations in phytoplankton community structure (Moline et al., 2004). The shift from diatoms to the more hyposaline-tolerant cryptophytes, instigates larger-scale food web shifts, seeing salp numbers dominate over krill, which in-turn can reduce krill availability for seals, penguins and whales (Mendes et al., 2013; Moline et al., 2004).

The marine topography of the WAP is characterised by extensive fjordic systems (Figure 1.2) carved out by many millennia of glacial erosion. Currently, Antarctic fjords are described as areas of high benthic abundance and are considered biodiversity hotspots (Grange and Smith, 2013). However, polar fjords with marine terminating glaciers can be highly sensitive to climate change (Kędra et al., 2009; Moon et al., 2015) as they serve as a major channel of freshwater into the ocean. As climate warming causes glaciers to melt and retreat, excess meltwater and sedimentation can be concentrated within the fjord basins, constrained by sill deposits at the fjord mouth (Renaud et al., 2007). This can result in distinct salinity stratification in the water column (Bianchi et al., 2020) (see Figure 1.2). In the Arctic, which is considered to be a later stage of climate warming than the Antarctic, many fjords are described as being low in species richness and functional diversity (Hop et al., 2002; Włodarska-Kowalczyk et al., 2012). High meltwater input reducing salinity is often cited as a contributing factor in the decline of biomass and diversity in Arctic fjords (e.g. Hop et al., 2002; Renaud et al., 2007) and there is concern that Antarctic fjords may follow this pattern in the future (Grange and Smith, 2013). Future salinity patterns in Antarctic fjords are difficult to estimate due to local topography and differential rates of melting, however current conditions in Arctic fjords may serve as a predictor. For example in Greenland, surface salinity in inner fjords can regularly drop to <15‰ (Meire et al., 2017; Sejr et al., 2021) while low salinity lenses of ~20‰ can persist down to depths of ~10 meters (Sejr et al., 2017; Sejr et al., 2022).

1.2 Marine animals and salinity

For all animals, the cell membrane functions as a barrier and interface to the extracellular environment. The necessity to obtain nutrients and eliminate waste products, along with many other cellular activities, requires cells to be permeable to both solutes and solvents (Kirschner, 1991). When extracellular solute concentration varies, there is a shift in the osmotic pressure on cells, which, if not regulated, drives the diffusion of solutes and water across the cell membrane, resulting in changes in cell volume and solute concentration (Schmidt-Nielsen, 1997). To function efficiently, it is vital for cells to maintain a stable intracellular environment in terms of solute composition and volume, regardless of external changes (Kirschner, 1991).

1.2.1 Osmoregulatory strategies

When considering how marine animals respond to changes in external salinity, it is important to distinguish between the different cell layers and compartments containing biological fluids (see Figure 1.3). Epithelial cells of the integument form an external barrier to seawater. Here, ion and water diffusion can take place either across the whole-body surface or in specific organs such as gills where they enter the extracellular space (Kirschner, 1991). Extracellular fluid surrounds the internal organs and tissue, and can be described as blood, haemolymph, intestinal or coelomic fluid dependant on the taxon in question (Gilles and Delpire, 1997). The fluid within cells and tissue, is referred to intracellular fluid or cytosol and is bounded by the phospholipid membrane. These two cellular interfaces provide two possible sites for osmoregulation: 1) the integument between the external medium and the extracellular fluid, and 2) the cell membrane of internal cells between the extracellular fluid and the intracellular fluid (Gilles and Delpire, 1997).

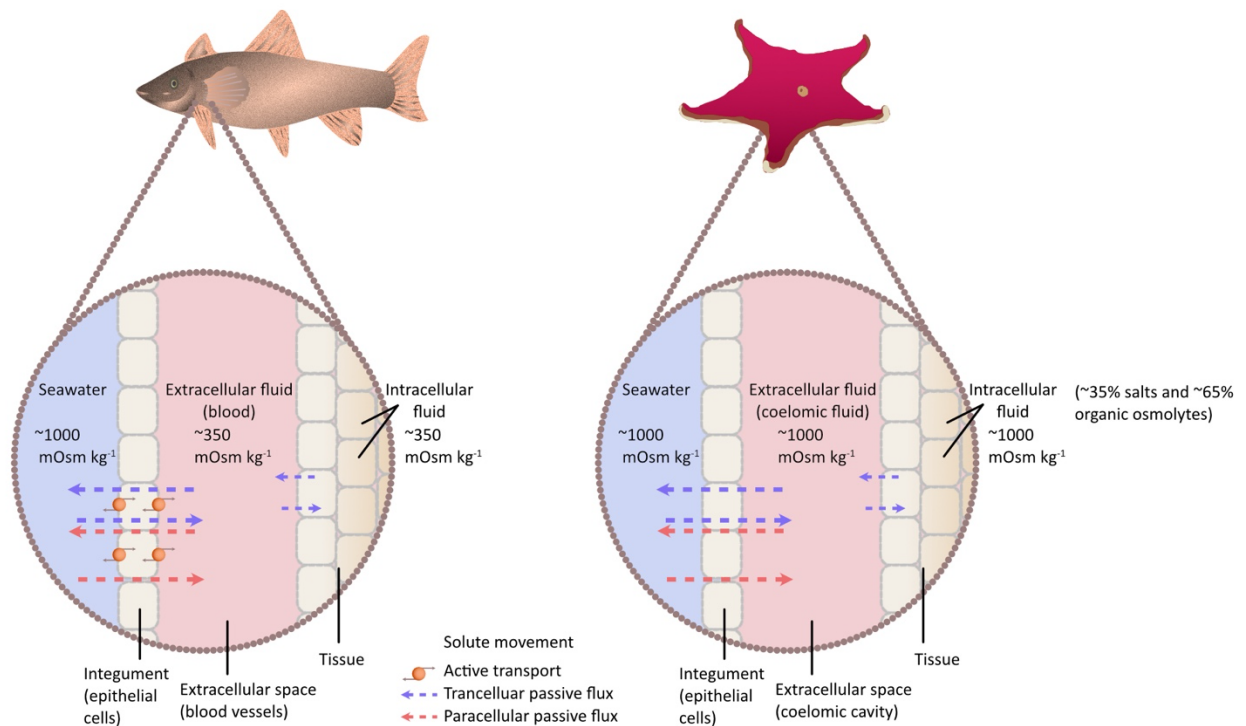


Figure 1.3| Comparison between a typical osmoregulator (a gill in a teleost fish–left diagram) and osmoconformer (integument of an asteroid echinoderm–right diagram), showing the cellular interfaces and body fluid compartments where osmotic and ionic exchange takes place. Note that active transport mechanisms are used in the osmoregulator to maintain the blood hypoosmotic relative to seawater, while the osmoconformer has a higher intracellular osmolality due to the presence of a high concentration of organic osmolytes.

Marine animals that have epithelia exposed to external seawater adopt one of two strategies to maintain osmotic homeostasis of the intracellular environments (Figure 1.3) (Kirschner, 1991; Schmidt-Nielsen, 1997). Osmoregulators, such as teleost fish and some crustaceans, implement energetically costly active transport mechanisms in their epithelial cells to regulate the ionic and osmotic concentrations of extracellular fluids (Rivera-Ingraham and Lignot, 2017; Schmidt-Nielsen, 1997), in addition to the use of specialised excretory organs (e.g., kidneys in fish; antennal and maxillary glands in crustaceans) to excrete water and solutes (Freire et al., 2008; Greenwell et al., 2003). Under this strategy, the blood or haemolymph—the extracellular fluid—which surrounds internal cells and tissue, remains at a relatively consistent concentration and thus the intracellular environment is unaffected by external changes in salinity (Kirschner, 1991). In diluted seawater or freshwater, these animals are hyper-regulating and maintaining extracellular fluids at higher osmolalities than the external medium. At average seawater salinity (~35‰) or higher salinities, hypo-

regulation of extracellular fluids is necessary, maintaining extracellular osmolality at lower osmolalities than seawater (Schmidt-Nielsen, 1997).

For the majority of marine invertebrates (including echinoderms), regulation of extracellular fluids at exposed epithelia is minimal. Instead of expending energy on maintaining osmotic gradients between the extracellular compartment and the external medium, these animals allow extracellular fluid to remain in equilibrium or near, with the seawater (Gilles and Delpire, 1997). Animals implementing this strategy are termed osmoconformers. When experiencing a change in salinity, the extracellular fluid of osmoconformers will eventually equilibrate with that of the external seawater, increasing or decreasing the osmotic pressure on internal cells and tissue (Gilles and Delpire, 1997). However, osmoconformers still need to maintain a stable intracellular environment. When environmental salinity is lowered, extracellular fluids are diluted causing the movement of water into cells, which in turn swell (Diehl and Lawrence, 1984). The opposite happens when external salinity increases, as water is drawn out of cells down the osmotic gradient, causing cell shrinkage (Diehl and Lawrence, 1984). To prevent excessive cell shrinkage or swelling, osmoconformers utilise a process of cellular volume regulation by adjusting solutes or 'osmolyte' concentration within cells (Gilles and Delpire, 1997). Osmolytes can be either inorganic such as salts (e.g., Na^+ , K^+ , Cl^-) or organic such as amino acids and their derivatives (e.g., alanine, proline, glycine, taurine and betaine) (Robertson, 1980; Shaw, 1958; Wiesenthal et al., 2019). While extracellular solute concentration mainly consists of inorganic osmolytes, intracellular fluid is instead made up of 60-70% organic osmolytes in osmoconformers (Somero and Yancey, 2011; Somero et al., 2017) (Figure 1.4). The reason for these differences is due to the perturbing effects on enzyme and protein folding if salts were in such high concentrations within the cell (Bowlus and Somero, 1979; Somero and Yancey, 2011; Von Hippel et al., 1973). Certain organic osmolytes, termed 'compatible' osmolytes are favoured for their non-perturbing impact on protein folding and function (Somero et al., 2017). Responding to salinity change, osmoconformers can accumulate or degrade their pool of intracellular organic osmolytes, and/or modify membrane-bound transporter proteins to regulate permeability to osmolytes (Gilles, 1987; Somero and Yancey, 2011). Ultimately, this strategy maintains the ionic balance at acceptable levels, while preventing an excessive influx or loss of cellular water (Figure 1.4B).

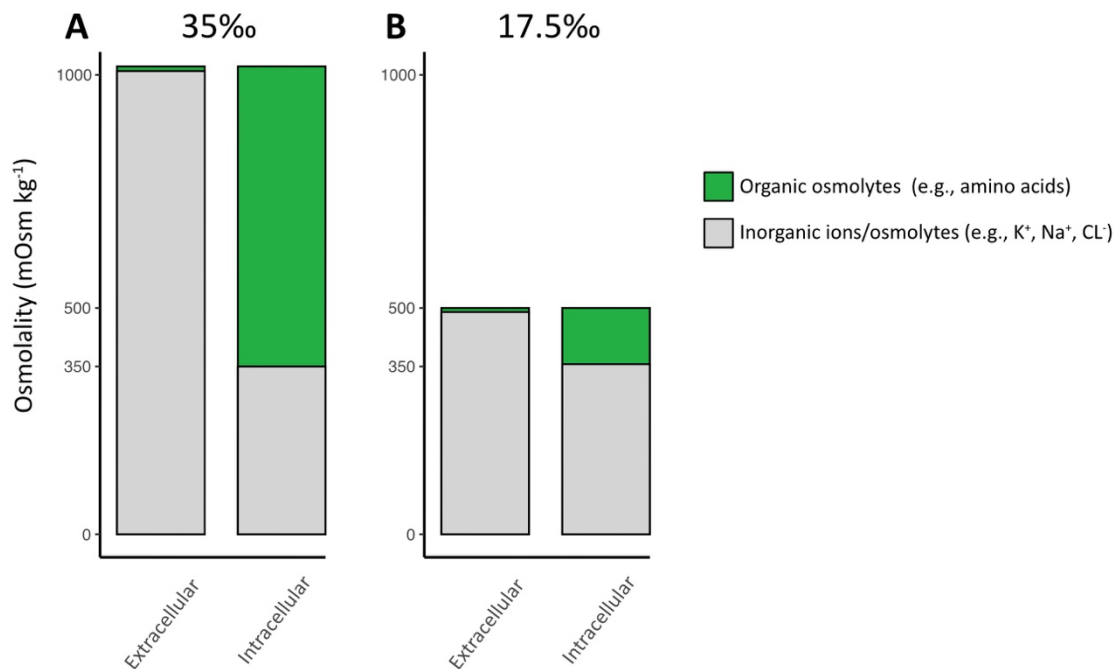


Figure 1.4 | Levels of organic and inorganic osmolytes (mOsm kg⁻¹) typically found in osmoconforming invertebrate extracellular and intracellular fluids. A) at full strength salinity (35‰), B) at reduced salinity (17.5‰) after acclimation. Note that under reduced salinity (B) the level of intracellular inorganic osmolytes remains relatively constant. Adapted from Gilles and Delpire (1997) and Somero et al. (2017).

Although metabolic costs associated with cellular volume regulation in osmoconformers are not considered to be anywhere near as high as those associated with active transport in osmoregulators, osmoconforming is far from passive (Kirschner, 1991; Rivera-Ingraham and Lignot, 2017). Metabolism of the osmolyte pool, adjustments to cell membranes and even cytoskeletal structural changes related to cell volume regulation (Gilles, 1987; Gilles and Delpire, 1997; Pedersen et al., 2001), mean that acclimatising to salinity changes bears metabolic costs. These costs are evident when examining osmoconforming invertebrates that enter low salinity environments or are exposed to reduced salinity. For example, in the brackish environment of the Baltic Sea, breeding in the sea star *Asterias rubens*, ceases when living at the limit of its salinity tolerance range [as referenced in Binyon (1972)], while Torres et al. (2011) demonstrated that larval stages of osmoconforming decapod crustaceans exhibit reduced growth or biomass loss when exposed to low salinities.

Marine organisms can also be classified by their ability to tolerate varying salinities, often reflecting their distribution. Organisms that are able to tolerate a broad range of salinities are

termed euryhaline, while stenohaline organisms are limited to a narrow range of salinities (Schmidt-Nielsen, 1997). Osmoregulators and osmoconformers can both display a capacity for euryhalinity. Even echinoderms which are traditionally considered strictly stenohaline osmoconformers (Brusca and Brusca, 2003; Hyman, 1955), demonstrate species-specific tolerance to low salinity which can occasionally be remarkable (e.g., Barker and Russell, 2008).

1.3 Echinoderms

Echinoderms are marine animals in the phylum Echinodermata, with around 7600 extant species described worldwide (WORMS, 2024), and estimates of around 13000 extinct species (Pechenik, 2005). The phylum is divided into five extant classes; Crinoidea (sea lilies and feather stars), which is an early branching and distinct class, and the more derived classes Echinoidea (sea urchins and sand dollars), Holothuroidea (sea cucumbers), Asteroidea (sea stars and sea daisies) and Ophiuroidea (brittle stars and basket stars) (Reich et al., 2015) (Figure 1.5). As deuterostomes closely related to chordates, they have been extensively studied as developmental model organisms for well over a century (e.g., Boveri, 1895; Hyman, 1955) playing a pivotal role in advancing the understanding of cell biology, fertilisation, and heredity (Ernst, 1997). Their diverse and often remarkable regenerative capabilities—ranging from organ and body part, to full organism regeneration after fission in some holothurians—have furthered their prominence as models for regenerative studies (Medina-Feliciano and García-Arrarás, 2021).

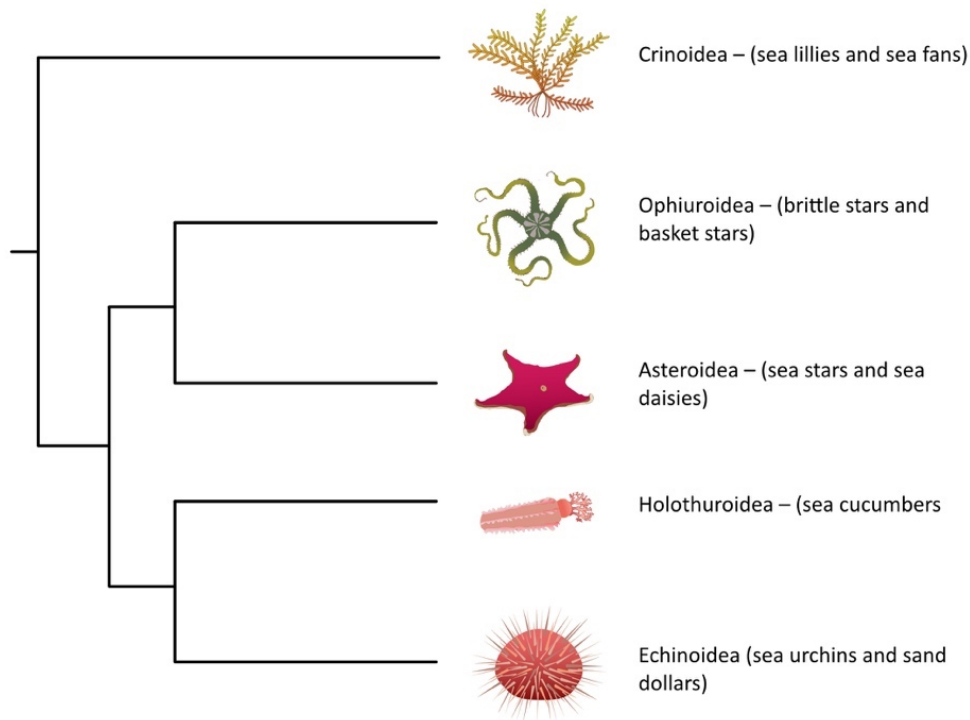


Figure 1.5| Current accepted hypothesis for the phylogenetic relationships within the phylum Echinodermata based on molecular systematics (Reich et al., 2015; Telford et al., 2014).

1.3.1 Ecology

Echinoderms have a global distribution and contribute to critical ecological roles across diverse ecosystems (Iken et al., 2010). For example, brittle stars are considered key contributors to carbon remineralisation on Arctic shelves due to their high biomass (Ambrose et al., 2001). In temperate coastal regions, sea urchin species such as *Strongylocentrotus droebachiensis* and *Echinus esculentus* exert significant control over macroalgal growth through intense grazing pressure (Breen and Mann, 1976; Chapman, 1981; Jones and Kain, 1967). Similarly, in the Indo-Pacific tropics, species such as the crown-of-thorns sea star, *Acanthaster* spp., a dominant predator of stony corals, can be responsible for extensive and widespread coral loss when at high densities (Foo et al., 2024). In other tropical ecosystems, feather star crinoids (order Comatulida) sustain high reef biodiversity and support intricate trophic networks by establishing symbiotic relationships with a highly diverse range of fauna, due to their complex morphology (Terrana et al., 2024). In deep-sea ecosystems, sea cucumbers can often be among the most abundant megafauna. Here, they contribute

substantial biogenic sediment mixing and can improve water quality through their burrowing and feeding activities (Iken et al., 2001; Mohsen and Yang, 2021).

1.3.2 Anatomy

Anatomically, extant echinoderms are unique among bilaterians for their pronounced radial, fivefold symmetry in adults (in the majority of species), while their larval forms retain bilateral symmetry (Hyman, 1955). Almost all echinoderms have a calcium carbonate internal skeleton forming intracellularly from specialised cells (sclerocytes) within the dermis. These can form inter-locking plates, such as the test on echinoids, or remain as individual microscopic ossicles embedded in the body wall in holothurians. Internally, most echinoderms have a large fluid-filled coelomic cavity (with the exception of crinoids, which instead have a series of web-like smaller coelomic cavities made up of connective tissue), lined by an epithelium of mesodermal origin, containing digestive and reproductive tissue (Hyman, 1955).

Extracellular body fluids in echinoderms are called coelomic fluids and they are named according to the body compartments from which they originate (Figure 1.6). Fluid in the main coelomic cavity is referred to as perivisceral coelomic fluid. Being osmoconformers, echinoderm perivisceral coelomic fluid is isosmotic with seawater, however, depending on the species, ions can be differentially accumulated above or below the levels found in seawater (Binyon, 1972; Vidolin et al., 2007). In this thesis, when referring to coelomic fluid, it is in reference to perivisceral coelomic fluid.

A second coelomic fluid is referred to as ambulacral fluid, which fills the water vascular system, a feature unique to echinoderms (Binyon, 1972; Hyman, 1955). The water vascular system is a series of fluid filled canals that lead to tubular extensions projecting outside the body wall and endoskeleton (Figure 1.6). These extensions, called tube feet or podia, work by hydraulic action controlled by muscular contraction, and have multiple functions including locomotion, feeding and respiration (Hyman, 1955; Pechenik, 2005; Weber, 1969). In the majority of echinoderms, the water vascular system features a calcareous sieve-like structure called the madreporite, which extends to the outside of the animal (although it

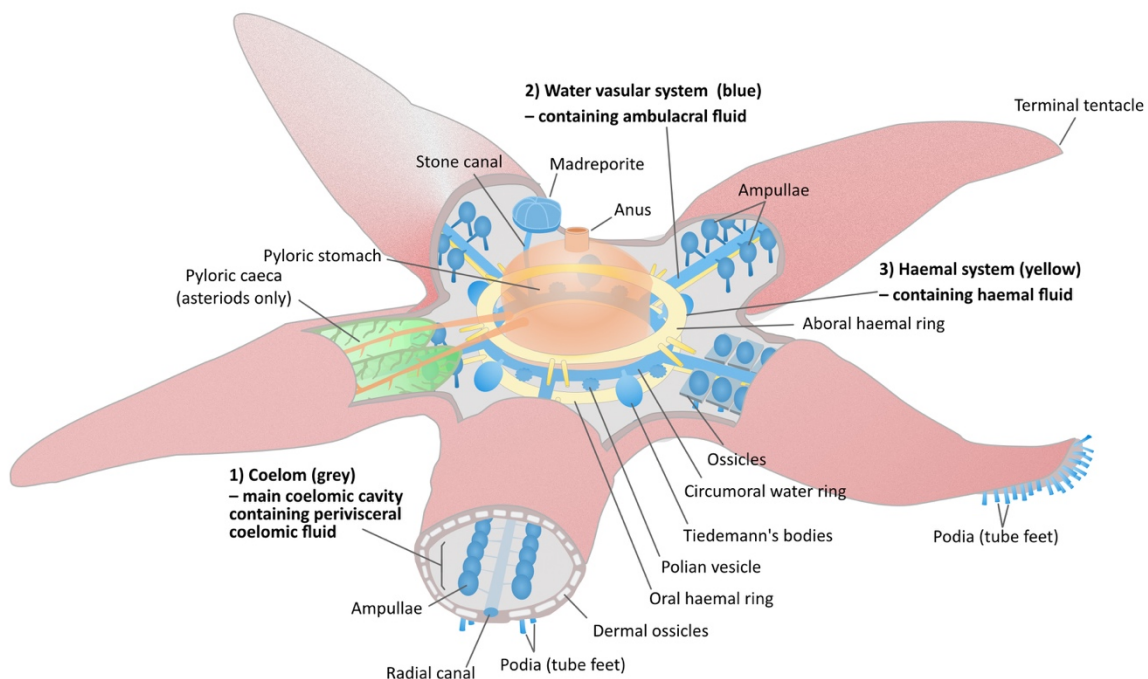


Figure 1.6| Diagrammatic representation of an asteroid's internal anatomy, highlighting the various fluid-filled coelomic compartments typical of most echinoderms. Redrawn and modified after Binyon (1972) and Pechenik (2005).

can remain internal, as in most holothurians) (Binyon, 1972). It may function as an entry point for external sea water to top up internal water-vascular system volume, however this is much debated in the literature and it is likely to have different roles across echinoderm classes (Ferguson, 1996; Tamori et al., 1997). Indeed, direct connections between the main perivisceral coelomic compartment and the water vascular system appear minimal in echinoids, while in asteroids they are clearly linked and likely used to keep sea stars inflated (Ferguson, 1996). Again, although osmotic pressure in the ambulacral fluid is isosmotic with external seawater, some species appear able to increase the concentration of specific ions above that of the external water. For example, Binyon, (1962) demonstrated that K^+ levels increased ~60% above that of external seawater upon encountering reduced salinity in *A. rubens* which may be the result of active transport processes.

A third coelomic body compartment called the haemal system contains a fluid that may have a role in nutrient transport but is not directly connected to the water vascular system (1.6). The significance of the haemal system is widely debated and there is a lack of agreement on its true function (Binyon, 1972; Hyman, 1955; Pechenik, 2005).

1.3.3 Antarctic echinoderms

Echinoderms in Antarctica play dominant roles in the benthic ecosystems of both shallow and deep-water Southern Ocean ecosystems. For example, the echinoid *Sterechinus neumayeri* and the asteroid *Odontaster validus*, are considered keystone species (McClintock et al., 1988; Ortiz et al., 2017) integral to food web dynamics and energy transfer within the nearshore shallow marine ecosystem (McClintock, 1994; Sporta Caputi et al., 2020; 2024). Antarctic echinoderms are often considered generalist feeders, scavenging on dead animals or detritus (McClintock, 1994). However, many are active predators of benthic fauna (e.g., *O. validus* Pearse, 2013) with spongivory being particularly common in Antarctic asteroids (Dayton et al., 1974; McClintock, 1994). Similarly, many species of Antarctic ophiuroids are active predators of other macroinvertebrates including cnidarians, annelids, crustaceans, bivalves and other echinoderms (Dearborn, 1977; McClintock, 1994). In the deeper sea (>200m), ophiuroids and crinoids can form dense assemblages in an otherwise sparse environments (Ambroso et al., 2016; Boschen et al., 2013; Bowden et al., 2011), while the high abundance of suspension-feeding echinoderms such as stalked crinoids (order Isocrinida) and deposit feeding echinoderms such as sea cucumbers and brittle stars, likely play an important role in nutrient cycling in deep-sea ecosystems (Rossi and Elias-Piera, 2018).

Antarctica is home to a remarkable diversity of echinoderms, representing around 10% of the recorded fauna [~4100 species: Clarke and Johnston (2003) as referenced by O'Loughlin et al. (2011)]. Compared to temperate and tropical regions, echinoderms make up a much higher proportion of the benthic fauna. For example it is estimated that echinoderms in Antarctica represent around 10% of all marine fauna compared to only 3.5% in the tropics (Mariana Islands) and 2.2% of European species (as referenced in O'Loughlin et al., 2011), while Antarctic echinoderm species richness and diversity is considered to be significantly higher than the Arctic (Clarke 2008). Numerous favourable conditions appear to have driven the success and high diversity of echinoderms in Antarctica, with the extreme conditions of the physical environment appearing not to be a barrier for echinoderms (O'Loughlin et al., 2011). Tolerance to unfavourable winter conditions is evident with species such as *S. neumayeri* being able to go without feeding for up to six months (Brockington, 2001), while holothurians are known for their ability to enter a state of torpor when food abundance is

low and, in some species reabsorb and regenerate organs on a seasonal basis as an expression of seasonal diapause (Fankboner and Cameron, 1985). Secondly, geographical barriers, such as the Antarctic Convergence are considered to have had less impact on echinoderms. The Antarctic Convergence or Polar Front, is the oceanographic boundary marking the transition between the warmer, northward flowing waters and the colder waters of the Southern Ocean, often acting as a biogeographic barrier to species dispersal (Moore et al., 2018). However, the Convergence has a lesser impact on deeper water species and cold-tolerant shallow water species, and does not appear to have been a barrier to the dispersal of some echinoderms into and out of the region (Brandt et al., 2007; Moore et al., 2018; O’Loughlin et al., 2011). Dispersal across the Convergence was likely supported by the rich biodiversity of adjacent areas, such as Australia providing a diverse foundation of colonising species which could then diversify in the Antarctic region (O’Loughlin et al., 2011). High dispersal by the Antarctic current is likely responsible for the circum-polar distribution of many echinoderm species observed today, however, the numerous islands and isolated habitats may have previously facilitated high allopatric speciation by providing refugia during glacial cycles and restricting connections between populations (Clarke and Crame, 1989; O’Loughlin et al., 2011). Thirdly, climatic cooling in the late Eocene led to dramatic declines in decapods, elasmobranchs and teleost fish in shallow Antarctic seas (Aronson and Blake, 2001). The post-Eocene absence of skeleton-breaking predation in shallow nearshore Antarctic habitats is thought to have favoured the rise to dominance of slow moving-invertebrate predators, exemplified by the echinoderm-dominated benthic communities observed today (Aronson and Blake, 2001; Aronson et al., 2007).

1.3.4 Echinoderms and salinity

Like most marine invertebrates, echinoderms are osmoconformers with extracellular coelomic fluid which is near isosmotic with the external seawater. The absence of excretory organs in addition to having highly permeable epithelial membranes, have likely restricted echinoderms to marine habitats (Binyon, 1972), and consequently, there are no examples of freshwater species (Russell, 2013). However, there are a number of species (>40; Russell, 2013) that inhabit hyposaline and brackish water [e.g., *Ophiophragmus filograneus* (Turner

and Meyer, 1980); *Asteria rubens* (Wolff, 1968)], and many others have been experimentally demonstrated to tolerate low salinity (Russell, 2013; Stickle and Diehl, 1987).

1.3.5 Short term responses to low salinity

The rate of salinity change in the environment is an important consideration regarding the capacity of echinoderms to tolerate low salinity. Sharp reductions in salinity, such as acute exposure to glacial meltwater, may last only a few hours before ambient conditions return. Therefore, a strategy of short-term tolerance to a lower salinity or evasive behaviour can prevent the need for significant physiological changes before ambient conditions return. Evasive behaviours in response to reduced salinity have been observed in several echinoderms, particularly those living in environments where low salinity is common (Castellano et al., 2018; Mercier et al., 1999). For example, in response to salinity reductions, the holothurian *Holothuria grisea* retracts ambulacral feet and tentacles (Castellano et al., 2018), while burrowing was induced in *Holothuria scabra* (Mercier et al., 1999). The echinoid *Strongylocentrotus droebachiensis* has been observed to hide in crevices or move to deeper water upon heavy rainfall (Vadas et al., 1986).

In addition to behavioural responses to reductions in salinity, some echinoderms have physiological mechanisms that can be interpreted to minimise the impact of cell and tissue swelling/shrinkage upon acute osmotic stress. The formation of steep osmotic and ionic gradients between the coelomic fluid and the external media upon a change in salinity have been observed in numerous echinoderm taxa (Diehl and Lawrence, 1984; Vidolin et al., 2007). These transient gradients can persist for 12-24 hours (Diehl, 1986) (sometimes shorter e.g., Madrid et al., 1976), implying that echinoderms have some control over epithelial membranes (Santos et al., 2013). By slowing the rate at which extracellular fluids conform to the external seawater, cells and tissues are shielded from the full osmotic gradient, thereby preventing rapid swelling or shrinking. (Stickle and Ahokas, 1974; Vidolin et al., 2007). From an ecological perspective, the duration of these mechanisms correlates with a diurnal tidal cycle, allowing some echinoderms to inhabit intertidal zones and tolerate acute hypoosmotic stress.

The relatively short duration needed to equilibrate coelomic fluid with the external medium, means echinoderm cells and tissue are still exposed to an osmotic challenge and

must either volume regulate or tolerate tissue swelling upon hypoosmotic shock (Diehl, 1986; Santos et al., 2013). Early cell volume regulation during hypoosmotic shock in echinoderms may involve the immediate transport of inorganic ions via ion transporters (e.g., Na⁺/K⁺-ATPase pump and sodium-potassium chloride cotransporters) (Castellano et al., 2016). This causes water to follow, preventing cells from excess swelling. Overtime, the inorganic cellular concentration may be restored and instead, organic osmolytes are reduced to balance cell osmolality (Smith and Pierce, 1987).

Both cell volume regulation and the maintenance of gradients are important mechanisms for echinoderms inhabiting challenging environments such as shallow subtidal or intertidal zones. Indeed, strong osmoconformers appear to have greater capacity for rapid cell volume regulation than species that can maintain a larger osmotic coelomic fluid gradient (Santos et al., 2013). For Antarctic echinoderms, the ability to mount short-term behavioural and physiological responses will be crucial if the intensity and frequency of acute freshening events continue to accelerate, as observed globally (Röthig et al., 2023).

1.3.6 Long term responses to low salinity

For chronic exposure to lower salinity, phenotypic plasticity exemplified as the capacity to acclimatise to changed conditions, is necessary to enable efficient functioning without expending energy on extended stress responses. Acclimatisation refers to the ability for an organism to modify physiological processes from one stable state to another in response to environmental change, distinct from acclimation, which refers to the same physiological process but under experimental manipulation and conditions (Peck et al., 2014; Prosser, 1991). Phenotypic adjustments to low salinity include genetic, biochemical, and structural changes (Gilles and Delpire, 1997; Khlebovich, 2017; Podbielski et al., 2022b), and are considered to take between one to four weeks in temperate invertebrates (i.e., bivalve and gastropod molluscs, amphipods and jellyfish) (Baginski and Pierce, 1977; Khlebovich, 2017). To achieve low salinity acclimation, echinoderms must reduce their organic osmolyte pool, by direct efflux, catabolism or through the synthesis of proteins or other macromolecules (Gilles, 1987). Over time, the intracellular environment may be modified by selecting more compatible osmolytes, which are favoured for optimal stabilisation and functioning of macromolecules (Somero and Yancey, 2011).

The threshold for low salinity acclimation or critical salinity limit (S_{crit}) is proposed to result from the complete depletion of the organic osmolyte pool (Podbielski et al., 2016). This likely impairs the animal's ability to synthesise new proteins due to the reduced availability of free amino acids, ultimately diminishing overall fitness. However, it appears that this is not universal in echinoderms (Podbielski et al., 2022b), suggesting there are species-specific responses regarding the capacity for low salinity acclimation. The composition of organic osmolytes used by echinoderms in volume regulation appears to be similar across species, with glycine being the main osmolyte favoured (Podbielski et al., 2022a; Podbielski et al., 2022b). The preference for glycine as an osmolyte is likely due to it being the smallest and simplest stable amino acid, with its lack of hydrophobic properties contributing to optimisation of enzyme and other macromolecule stability in the cytoplasm (Taneja and Ahmad, 1994).

In order to acclimate to lower salinity, energy expenditure must be increased to enable adjustments that enable cells to maintain a stable intracellular environment (Sokolova et al., 2012a). For Antarctic echinoderms likely operating near the limits of their physiological performance in extreme environments (De Leij et al., 2022; Peck et al., 2014) the allocation of precious metabolic resources to fuel low salinity acclimation may have long-term fitness consequences, with impacts on reproduction and growth.

1.3.7 Freezing and salinity

As the freezing point of seawater is a function of salinity, average Antarctic salinity surface seawater (~34‰) is still in a liquid state until ~-1.9°C (Davenport, 1992). As osmoconformers, echinoderm coelomic fluid is isosmotic with external seawater; therefore, by remaining in the liquid portion of seawater, they are unlikely to require specific survival strategies to resist freezing, even when salinity decreases. However, in the intertidal zone, the combination of low salinity from meltwater intrusion and emersion at low tide exposes animals to air temperatures significantly below the freezing point of seawater, increasing their vulnerability to freezing. Additionally, during the Antarctic summer, sea-ice melt and terrestrial meltwater run-off can form a hyposaline lens, a freshwater layer sandwiched between the sea-ice and denser saline seawater below (Barnes and Conlan, 2007). This has the potential to cause mass mortalities in osmoconforming invertebrates. For example in McMurdo Sound (see Figure

1.1), Stockton (1984) reported high mortality in the epifaunal scallop, *Adamussium colbecki*. Dead scallops were observed suspended in the water column, encased in a meshwork of ice crystals. This was attributed to the following scenario: scallops trapped in a warmer hyposaline lens experienced a reduction in extracellular haemolymph osmolality. Upon the rising tide, the sudden exposure to colder seawater triggered extracellular ice crystal formation before osmotic rebalancing in the haemolymph could occur.

For many intertidal organisms, toleration of ice formation in the extracellular space is widely established as a primary cold-hardiness strategy (Ansart and Vernon, 2003; Davenport and Macalister, 1996; Murphy, 1983). For Antarctic intertidal species, freeze tolerance to ice formation has been demonstrated in the limpet *Nacella concinna*, which can survive after all osmotically active water has frozen (Davenport and Macalister, 1996; Waller et al., 2006b). However, Waller et al. (2006) suggest that the main cold-tolerant strategy across a wide range of Antarctic marine invertebrates initially involves freeze-avoidance through supercooling. Supercooling (also called undercooling) describes a process where a solution remains liquid below its thermodynamic freezing point (Somero et al., 2017). Biological mechanisms to achieve supercooling can include, use of antifreeze proteins (AFPs) to inhibit ice crystal growth and the accumulation of low molecular weight compounds (i.e., osmolytes) which act in a colligative manner to lowering the freezing point (Ansart and Vernon, 2003). Although Waller et al. (2006) did not demonstrate physiological mechanisms for supercooling, both echinoderms studied (*S. neumayeri* and *O. validus*) were capable of tolerance down to a median air temperature of $\sim -7^{\circ}\text{C}$. Currently there is little evidence of AFPs in intertidal invertebrates—Antarctic or otherwise—with the possible exception of the Antarctic nemertean *Antarctonemertes validum* (see Waller et al., 2006b). This suggests that mechanisms enabling supercooling likely involve the colligative action of organic osmolytes, as is the common strategy in insects (Murphy and Pierce, 1975; Sømme, 1982; Zachariassen and Kristiansen, 2000). For example taurine, strombine and alanine have all been proposed as cryoprotectants in the haemolymph of the blue mussel *Mytilus edulis* (Loomis et al., 1988; Williams, 1970), however their role as osmolytes regulating osmoregulation (Williams, 1970) suggests that they may have dual roles.

For echinoderms adapted to Antarctic environments where osmotic stress and freezing are common threats, the specificity of the osmolyte profile of a species may result from overlapping functions and strong selective pressure from multiple stressors. Considering

common species such as *S. neumayeri* and *O. validus* are very occasionally found in the intertidal, and many others in the shallow subtidal, they may be vulnerable to freezing if salinity levels are substantially lowered.

1.4 Antarctic marine animals under low salinity

Antarctica's long geographic isolation at the polar south, and historically stable physical conditions (Arntz et al., 1994), have led its marine species to adapt to an extreme yet predictable environment, resulting in high levels of endemism (Hogg et al., 2011; Peck, 2018). This has led to the evolution of spectacular adaptations, including the loss of red blood cells and pigment in the channichthyid icefish (Ruud, 1954), the presence of antifreeze glycoproteins in Antarctic notothenioid fish (Devries, 1971), gigantism (Chapelle and Peck, 1999), and the loss or modification of molecular stress responses across multiple species (Clark and Peck, 2009; Hofmann et al., 2000). However, there is concern that the stability of the environment has reduced the selective pressure to maintain mechanisms that enable tolerance to environmental change, as expected under climate change. Antarctic marine ectotherms are considered to have poor abilities to acclimate to increased temperatures, while those that can take considerably longer than temperate or tropical species (Bilyk and DeVries, 2011; Peck, 2018; Peck et al., 2014). For example, the Antarctic brittle star, *Ophiotus victoriae* is incapable of acclimating to +2°C which is only <0.5°C above current maximum summer sea temperatures in the WAP (Peck et al., 2009a). The capacity for Antarctic marine animals to tolerate low salinity and acclimate to long-term freshening are less clear.

1.4.1 Experimental evidence

Currently, only 16 studies (Table 1.1 and 1.2) have measured the physiological responses to manipulated salinity levels on Antarctic marine animals. These approaches can be separated into short-term (≤ 96 hours) acute responses to salinity change (Table 1.1), and longer-term (≥ 5 days) acclimation or habituation (Table 1.2). Several recent studies on Antarctic fish have concentrated on the notothenioid, *Harpagifer antarcticus*. This species shows a tolerance for moderate reductions in salinity; however, at lower salinity levels, it experiences osmotic imbalances and struggles to maintain homeostasis (Vargas-Chacoff et

al., 2021a). These challenges are significantly intensified when combined with warming (Vargas-Chacoff et al., 2021b). For invertebrates, there are several studies on the limpet *Nacella concinna* which is one of the most dominant macroinvertebrates of the inter and shallow subtidal zones of the WAP (Hoffman et al., 2010). Despite significant populations inhabiting the challenging intertidal zones, *N. concinna* exhibits limited short-term tolerance to low salinity (Davenport, 2001) and shows stenohaline traits when acclimating to reduced salinity levels (Navarro et al., 2020). Very small reductions in salinity (34 to 30‰) impair behaviour functions critical to long term survival in the isopod *Serolis polita* (Janecki et al., 2010). Numerous studies on Antarctic fauna have shown that low salinity can have significant negative impacts on fitness when combined with increased temperature or reduced pH (Martínez et al., 2021; Navarro et al., 2020; Park et al., 2020).

There are currently only two studies that have investigated the impact of low salinity on Antarctic echinoderms (Table 1.1). The sea star *O. validus* was observed to maintain stable body volume under low salinity conditions, suggesting it can control the permeability of its external epithelia or regulate intracellular volume (Pearse, 1967). This mechanism is believed to help withstand summer freshwater runoff in the shallow Antarctic environment. A more recent study looking at the response of reduced salinity on embryonic development in the Antarctic sea urchin, *S. neumayeri* suggests that echinoderm early life stages are much less tolerant to salinity change. Cowart et al. (2009) observed that a decrease in salinity from 34 to 30‰ led to a dramatic reduction in viable *S. neumayeri* embryos reaching the morula stage after 36 hours post-fertilization, with only ~1% surviving. The implications of these results are significant considering the small salinity decreases investigated, which are within salinity ranges observed at near-shore Antarctic coastlines (Clarke et al., 2022).

More broadly, reviews on low salinity tolerance across global echinoderms fail to distinguish between short- and long-term tolerance, with comparisons of physiological and behavioural responses evaluated without accounting for the duration of exposure or dilution methods (e.g., Russell 2013). Since the mechanisms underlying tolerance to acute salinity reduction and longer-term low salinity acclimation are likely different—such as behavioural evasion and transient coelomic fluid gradients versus modifications to the osmolyte profile—it is crucial to differentiate between short- and long-term responses to low salinity as a first step in assessing Antarctic echinoderm resilience.

Chapter 1

Table 1.1 | Studies assessing short-term salinity responses in Antarctic marine organisms. Bold text in 'Salinities' column indicates control groups.

Phylum: Class: Order	Species	Life stage	Salinities	Dilution Methods	Exposure time	Additional interactions	Measurements	Significant salinity response	Significant interactive response	Study
Arthropoda: Hexanauplia (subclass: Copepoda): Harpacticoida	<i>Tigriopus kingsejongensis</i>	Juvenile and Adult	0‰, 5‰, 10‰, 25‰, 34‰ , 45‰, 60‰, 75‰	Instant immersion	96 hours	None	Survival	Different stages of the copepods (i.e., nauplius, male, and female) generally showed tolerance to hypo- and hypersalinity.	None	Kim et al., 2022
Arthropoda: Malacostraca: Isopoda	<i>Paraserolis polita</i> (reported as <i>Serolis polita</i>)	Adult	30‰, 31‰, 32‰, 33‰, 34‰	Instant immersion	2 hours	Temperature (0, 1, 2, 3, 4, 5°C)	% righting & re-burying, % swimming, swimming time in response to food odour.	Salinity and Temp both impaired performance functions.	Righting, reburying (most significant) and time spent swimming.	Janecki et al., 2010
Echinodermata: Asteroidea: Valvatida	<i>Odontaster validus</i>	Adult	~17.5 ‰, ~ 35‰	Instant immersion	Repeat wet weighing's over 60 hours	None	Wet weight change	Able to control coelomic volume and withstand hypoosmotic conditions.	N/A	Pearse, 1967
Echinodermata: Echinoidea: Camarodonta	<i>Sterechinus neumayeri</i>	Embryonic	30‰, 32‰, 34‰	Instant immersion	36 hours	None	Rate of development (cell divisions/hr), % viable embryos, % reaching morula	Slower development rates at lower salinity. 1% developmental survival at 30‰ salinity.	N/A	Cowart et al., 2009
Mollusca: Gastropod: Patellogastropoda (subclass)	<i>Nacella concinna</i>	Adult	0‰, 7‰, 13‰, 18‰, 19‰, 24‰, 26‰, 28‰, 33‰	Instant immersion	96 hours. 5 hours for fresh water.	None	Haemolymph osmolality, median lower lethal limit (LLL)	LLL= 20.9 ‰ After 1hr at 0 14% increase in body volume, 27% loss of ions.	N/A	Davenport, 2001
Mollusca: Gastropod: Patellogastropoda (subclass)	<i>Nacella concinna</i>	Adult	0.0 ‰, 3.4‰, 6.8‰, 13.6‰, 20.4‰, 27.2‰, 34.0‰, 40.8‰, 47.6‰, 54.4‰, 61.2‰, 68.0 ‰	Instant immersion	96 hours	Temperature tolerance, desiccation and shore height	Mortality	Stenohaline, could not survive at salinities 20.4 ‰ or lower after 96 hours exposure, or higher salinities.	Salinity tolerance linked to shore height, <i>N concinna</i> is a lower shore species and had low salinity tolerance.	Davenport and Macalister, 1996

Table 1.2 | Studies assessing long-term salinity acclimation responses in Antarctic marine organisms. Bold text in ‘Salinities’ column indicates control groups. Arrow symbols (↑/↓) indicate a respective increase or decrease in response.

Phylum: Class: Order	Species	Life stage	Salinities	Dilution Methods	Acclimation time	Additional interactions	Measurements	Significant salinity response	Significant interactive response	Study
Arthropoda: Hexanauplia (subclass: Copepoda): Harpacticoida	<i>Tigriopus kingsejongensis</i>	Adult	5‰, 10‰, 25‰, 34‰ , 45‰, 60‰, 75‰	Dilution at 1‰ per 10 minutes; Salinity increase at 1‰ per 15 minutes.	Up to 206 days	N/A	Survival/Lifespan	Lifespan was significantly shortened by hypo- and hypersalinity compared to control salinity	None	Kim et al., 2022
Arthropoda: Amphipoda	<i>Gondogeneia antarctica</i>	Adult	27‰ 34‰	Instant immersion.	26 days. Measurements taken throughout.	pH (7.6 and 8.0)	Mortality/cannibalism, swimming activity, shelter use	Increased cannibalism, swimming activity during day, reduced at night	None	Park et al., 2020
Arthropoda: Malacostraca: Amphipoda	<i>Gondogeneia antarctica</i>	Adult	25‰, 30‰, 35‰	24-hour dilution (& temp change)	5 days	Temperature (0, 2.5, 5°C)	Oxygen consumption, Total nitrogenous ammonia excretion	Oxygen consumption and Ammonia excretion increased with lower salinity	Oxygen consumption and ammonia excretion significantly higher at high temp (5°C) and lowering salinities.	Gomes et al., 2013
Chordata: Actinopterygii: Perciformes (suborder: Notothenioidei)	<i>Harpagifer antarcticus</i>	Adult	5‰, 10‰, 20‰, 33‰	Instant immersion.	7 days	N/A	Plasma variables (osmolality and calcium, muscle water content), NKA activity, plasma cortisol levels, mRNA expression of osmoregulatory-related proteins via qPCR in gill, kidney, and intestine, PRLR, CFTR, NKCC2.	Low salinity: osmolality and calcium; ↓, plasma cortisol and muscle water content ↑; Na+K+-ATPase (NKA) activities: Gill ↓, intestine ↑. mRNA expression differed in different tissue.	N/A	Vargas-Chacoff et al. 2021
Chordata: Actinopterygii: Perciformes (suborder: Notothenioidei)	<i>Harpagifer antarcticus</i>	Adult	23 ‰, 28 ‰, 33‰	Instant immersion.	10 days	Temperature (2, 5, 8, 11°C)	Plasma osmolality (mOsm/kg); NKA activity in the gills, posterior kidney, and foregut;	gill-kidney-intestine NKA activity, gene expression of NKA α, NKCC, CFTR, Aquaporins 1, and 8	gill-kidney-intestine NKA activity, gene expression of NKA α, NKCC, CFTR, Aquaporins 1, and 8	Vargas-Chacoff et al. 2021
Chordata: Actinopterygii: Perciformes (suborder: Notothenioidei)	<i>Harpagifer antarcticus</i>	Adult	23 ‰, 28 ‰, 33‰	Instant immersion.	10 days	Temperature (2, 5, 8, 11, 14°C)	Cellular stress response - transcription of genes using qPCR from liver and gill tissue	Liver: ↑All genes except E2, PSMA2, PSMB7. Gill: ↑ HSP70, GRP78, E3-herc1, CHIP, SMAC/Diablo, BAX, PSMC1	Temperature had a larger effect than salinity in the gill, while in the liver, both modulated expression and was much higher than in the gills: ↑All genes except PSMB7. Gill: ↑ HSP70, GRP78, E3-herc1, CHIP, SMAC/Diablo, BAX, PSMC1	Martínez et al. 2021
Chordata: Actinopterygii: Perciformes (suborder: Notothenioidei)	<i>Harpagifer antarcticus</i>	Adult	23 ‰, 28 ‰, 33‰	Instant immersion.	10 days	Temperature (2, 5, 8, 11, 14°C)	Fish mortality, ingestion rate mg/day-1, absorption efficiency and rate, Oxygen uptake ml/day-1, Scope for growth J/day-1	Low salinity (23 and 28 ‰) significantly decreased both the digestion and absorption rate of <i>H. antarcticus</i> compared to the control (33 ‰)	Oxygen consumption is reduced at high temperature (11 °C) and lower salinities.	Navarro et al., 2019
Chordata: Actinopterygii: Perciformes (suborder: Notothenioidei)	<i>Harpagifer antarcticus</i>	Adult	23 ‰, 28 ‰, 33‰	Instant immersion.	10 days.	Temperature (2, 5, 8 and 11°C)	Neuroendocrine responses in brain, stomach and gut tissues, measuring Serotonin (5HT), 5HIAA, Dopamine (DA), and	Low salinity (23 ‰) significantly increased brain dopamine.	Lowest salinity (23 ‰) and higher temperatures (8 and 11 °C) caused significant changes in neurotransmitter levels	Vargas-Chacoff et al., 2019
Mollusca: Gastropod: Patellogastropoda (subclass)	<i>Nacella concinna</i>	Adult	27 ‰ 34 ‰	Instant immersion.	35 days. Measurements taken	pH (7.55 and 8.00)	behavioural response (shell righting), mortality, condition factor	Shell dissolution and condition factor	Shell dissolution	Sin et al 2020
Mollusca: Gastropod: Patellogastropoda (subclass)	<i>Nacella concinna</i>	Adult	20‰, 30‰	Instant immersion.	60 days. Measurements every 10 days	Temperature (1,4, 8, 11 and 14°C)	Mortality, The absorption efficiency (AE), organic ingestion rate (OIR), oxygen uptake (VO2), scope for growth (SFG), Upregulated genes (transcriptomics)	Low salinity: 50% mortality, ↓SFG, Cell remodelling genes ↑	Cellular stress response (CSR) genes ↓ (inhibition of CSR); Glycolysis genes ↓; plasma membrane ATPase ↓	Navarro et al., 2020

1.5 Aims and objectives

This thesis aims to inform our understanding of the ability of Antarctic echinoderms to tolerate both short- and long-term reductions in environmental salinity, using laboratory-based experiments. The following aims are addressed:

1. Establish a methodology that could identify and differentiate between short- and long-term responses to low salinity.
2. Produce experimental data from non-Antarctic echinoderms that would serve as a template for later Antarctic species comparisons.
3. Assess species-specific responses to acute low salinity in Antarctic echinoderms.
4. Assess the acclimation capacities to low salinity of Antarctic echinoderms.
5. Explore the mechanistic underpinnings of low salinity acclimation in Antarctic echinoderms using cellular and 'omic approaches.
6. Identify knowledge gaps and suggest future research approaches.

1.6 Thesis structure

Chapter 1 sets out the background context for climate changed induced freshening in the Southern Ocean and the impact on Antarctica's endemic echinoderms. It presents a review of the physiological and behavioural responses of echinoderms to low salinity, contextualised within the Antarctic environment. Chapters 2 to 5 are written as research papers for publication each with individual introduction and methods sections.

Chapter 2 presents a temperate echinoderm study that establishes an experimental framework for comparison with Antarctic species. The behavioural and physiological responses of an ecologically important and near-threatened species of temperate sea urchin, *Echinus esculentus* are analysed under acute hypoosmotic shock and its acclimation capacity to low salinity investigated. The results advance knowledge of the acclimation process by decoupling the responses to hypoosmotic shock, from those of chronic exposure. This has been published as Barrett et al. (2024a). Chapter 3 presents an assessment of short-term responses to hypoosmotic shock in six common Antarctic echinoderms. By understanding

species-specific responses to hypoosmotic shock, vulnerable species can be highlighted and the focus for future research and conservation efforts identified. This chapter also provides an initial assessment of tolerance thresholds amongst Antarctic echinoderms, establishing suitable salinity levels for later acclimation experiments and providing comparative metrics. This has been published as Barrett et al. (2024b). Chapter 4 evaluates the acclimation capacity of two of the most conspicuous and ecologically important Antarctic echinoderms, *O. validus* and *S. neumayeri*, to altered salinity, revealing successful acclimation to mid-range salinity levels within their short-term tolerance range (29‰), but limitations at lower salinities (24‰). Chapter 5 is an exploration of mechanisms underpinning acclimation to low salinity in echinoderms using a metabolomics approach. This chapter uses GC-MS metabolomic profiling to explore changes in osmolyte profiles after low salinity acclimation. It reveals novel micromolecular adaptations that may result from overlapping osmolyte functions, addressing the challenges of protein synthesis at low temperatures alongside osmoregulation and freeze tolerance strategies. This provides data for a future comparative transcriptome analysis. Chapter 6 provides an overall synthesis of the main findings in the thesis and identifies pathways for future research.

Chapter 2 – Behavioural and physiological impacts of low salinity on the sea urchin *Echinus esculentus*

A version of this chapter has been published in the *Journal of Experimental Biology* as:

Barrett, N. J., Harper, E. M., Last, K. S., Reinardy, H. C., and Peck, L. S. (2024). Behavioural and physiological impacts of low salinity on the sea urchin *Echinus esculentus*. *J. Exp. Biol.* 227. <https://doi.org/10.1242/jeb.246707>

This aim of this chapter was to develop an experimental approach that would distinguish between short- and long-term responses to low salinity while also providing data from a temperate echinoderm that could later be compared with Antarctic echinoderms. I was the primary investigator and contributor to all aspects of this research, including conceptualisation, investigation, formal analysis, writing the original draft and reviewing and editing. All above authors provided supervision and contributed to editing and reviewing.

The data from this chapter is publicly available here: https://github.com/njb202/Chapter_2

2.1 Abstract

Reduced seawater salinity as a result of freshwater input can exert a major influence on the ecophysiology of benthic marine invertebrates, such as echinoderms. While numerous experimental studies have explored the physiological and behavioural effects of short-term, acute exposure to low salinity in echinoids, surprisingly few have investigated the consequences of chronic exposure, or compared the two. In this study, the European sea urchin, *Echinus esculentus* was exposed to low salinity over the short-term (11, 16, 21, 26, and 31‰ for 24 hours) and longer-term (21, 26, and 31‰ for 25 days). Over the short-term, oxygen consumption, activity coefficient and coelomic fluid osmolality were directly correlated with reduced salinity, with 100% survival at $\geq 21‰$ and 0% at $\leq 16‰$. Over the longer-term at 21‰ (25 days), oxygen consumption was significantly higher, feeding was significantly reduced, and activity coefficient values were significantly lower than at control

salinity (31‰). At 26‰, all metrics were comparable to the control by the end of the experiment, suggesting acclimation. Furthermore, beneficial functional resistance (righting ability and metabolic capacity) to acute low salinity was observed at 26‰. Osmolality values were slightly hyperosmotic to the external seawater at all acclimation salinities, while coelomocyte composition and concentration were unaffected by chronic low salinity. Overall, *E. esculentus* demonstrate phenotypic plasticity that enables acclimation to reduced salinity around 26‰; however, 21‰ represents a lower acclimation threshold, likely limiting its distribution in coastal areas prone to high freshwater input.

2.2 Introduction

Salinity is one of the main abiotic factors impacting the physiology and ecology of marine and brackish water organisms (Kinne, 1964) and is key to determining species distributions, reproductive success, growth and survival (Gunter, 1961; McNamara and Faria, 2012). Freshwater input in coastal and estuarine environments can reduce seawater salinity (coastal freshening) and exert hypoosmotic stress on marine organisms by increasing the cost of osmoregulation (Sokolova et al., 2012a) and even lead to cellular damage (Jäckle et al., 2001; Lee et al., 2022). Climate change is predicted to cause increased coastal freshening in many areas, primarily driven by increased rainfall and glacial melting, thus contributing to increases in freshwater land runoff (Jacobs et al., 2002; IPCC, 2022a). Benthic marine invertebrates are considered particularly vulnerable to coastal freshening (Moon et al., 2015; Vuorinen et al., 2015) due to their characteristically limited mobility (Snelgrove, 1999) making it a challenge to evade rapid changes in salinity.

The phylum Echinodermata, as with the majority of marine invertebrates, are osmoconformers and are poorly equipped to regulate extracellular body fluid concentrations in response to changes in external salinity (Gilles, 1987; Hyman, 1955; Stickle and Diehl, 1987). However, many echinoderm species are frequently found in the intertidal and even brackish environments, demonstrating a degree of euryhalinity (Binyon, 1972; Russell, 2013; Stickle and Diehl, 1987). For tolerant species, short-term abrupt reductions in salinity appear to be countered by the establishment of transient ionic and osmotic gradients between the internal coelomic fluid and the external seawater, suggesting a degree of epithelial regulation (Freire

et al., 2011a; Santos et al., 2013). This is considered a buffering mechanism to protect internal cellular tissue from swelling on encountering fresher water, for example over a tidal cycle, enabling time for cellular volume regulation (Vidolin et al., 2007). Cell volume regulation in osmoconformers involves adjusting concentrations of compatible organic and inorganic ions, known as osmolytes, to balance cell osmotic pressure (Gilles and Delpire, 1997; Podbielski et al., 2022b). With abrupt changes in salinity, intracellular inorganic osmolytes can be rapidly released (e.g., K^+ and Cl^-), followed later by organic osmolytes (e.g. taurine) (Smith and Pierce, 1987), preventing structural cell damage. However, with chronic exposure to reduced salinity, organic osmolyte composition and concentration is gradually modified, favouring selection for osmolytes with the best osmoprotecting qualities (Somero et al., 2017). This may involve cellular reorganisation with modifications to membrane-bound transporters (Gilles, 1987; Meng et al., 2013) and even cell cytoskeleton structural changes (Barrett et al., 2022; Pedersen et al., 2001). These energy dependent, long-term physiological adjustments occur during the process of acclimation to reduced salinity, which is considered to take between one to four weeks in temperate invertebrates i.e., bivalve and gastropod molluscs, amphipods and jellyfish (Khlebovich, 2017). The extent to which organisms can acclimate to changing environmental conditions is considered key to defining their future success under climate change scenarios (Peck, 2011; Somero, 2012). Experimental approaches investigating salinity tolerance in echinoids, have mainly focused on behavioural and metabolic responses to short-term acute exposure, and only occasionally longer-term acclimation (see; Binyon, 1972; Stickle and Diehl, 1987; Russell, 2013). Rarely, has short- and long-term tolerance been compared; indeed, reviews often compare different species lower tolerance limits without distinguishing the duration of the experiment (e.g., Russell, 2013).

The European sea urchin (hereafter 'urchin'), *Echinus esculentus* Linnaeus, 1758, is a widespread member of the benthic north-east Atlantic temperate ecosystem, commonly associated with the giant kelp forests of *Laminaria hyperborea* (Comely and Ansell, 1988). Although occasionally exposed at low tide, *E. esculentus* is most abundant in the sub-littoral zone from ~15 to 40 metres, where densities of 4 per m² have been recorded (Comely and Ansell, 1988), and is rarely found at depths > 40m (Comely and Ansell, 1988; Mortensen, 1927). *E. esculentus* plays a prominent role in the ecology of the sublittoral zone (Forster, 1959), exerting control on the distribution of *Laminaria* spp. and numerous epiphytic macroalgae through grazing pressures (Jones and Kain, 1967). Indeed, their grazing impact

may be detrimental to kelp forest recovery in a dysfunctional ecosystem (Bekkby et al., 2015). However, *E. esculentus* is listed on the IUCN Red List as ‘near threatened’, citing severe population fragmentation and declines in numbers of mature individuals (IUCN, 2023). As a subtidal inhabitant, they are less likely to experience dramatic reductions in surface salinity than urchins more commonly found in the intertidal [e.g., *Psammechinus miliaris* (Kelly and Cook, 2001)] and therefore may be predicted to have lower tolerance to osmotic stress. Previous investigations of *E. esculentus* have been concerned with the impacts of commercial exploitation around the British Isles for both consumption of urchin roe and the collection of urchin tests for ornamental purposes (Comely and Ansell, 1988; Comely and Ansell, 1989; Kelly et al., 2001). However, there is little to no knowledge on the salinity tolerance of *E. esculentus* and its capacity to acclimatise to a fresher coastal environment. In this study, experiments were conducted on populations of *E. esculentus* originally collected from Loch Linnhe, west coast of Scotland. Salinity in Scottish coastal waters has been dramatically decreasing since 2014 (Dye et al., 2020). In future, winter rainfall on the west coast of Scotland is expected to rise on average by 19% under a high emission scenario by 2080 (Adaptation Scotland, 2021). Understanding the degree to which *E. esculentus* can tolerate reductions in salinity, may allow predictions about its future distribution under increased coastal freshening (i.e., migration to deeper, higher salinity water), and how this may impact local ecosystem functioning (i.e., by reducing macroalgal grazing pressure in shallower areas).

Through investigating physiological and behavioural responses to reduced salinity over different time frames, this study aims to decouple the effects of acclimation from short-term, immediate responses to osmotic shock. Three hypotheses are addressed: 1. That chronic exposure to salinity levels at the lowest acutely tolerated level will have a detrimental impact on routine physiological functions (e.g., respiration, feeding and righting abilities) and in addition, may impact innate immune cell (coelomocytes) composition and concentration, as has previously been shown (Honorato et al., 2017); 2. Urchins exposed to a mid-level salinity within their tolerance range will acclimate, evidenced by a return of physiological functions to levels that are indistinguishable from those at ambient salinity; 3. If acclimation is achieved, future hypoosmotic stress will be reduced due to the cellular adjustments established during the acclimation process.

2.3 Methods

2.3.1 Experimental animals

Individual *E. esculentus* ($n=140$) with a test diameter ranging from 21-57 mm were selected from stock originally collected from Loch Linnhe, then maintained in flow-through aquaria. The aquarium utilises filtered natural seawater directly from the waters surrounding the Dunstaffnage Peninsula at the Scottish Association of Marine Science (SAMS) aquarium, Scotland. Urchins were transferred to a temperature-controlled room ($13.7^{\circ}\text{C} \pm 0.3$, s.e.m., $n=7$) and held in replicate 50-L closed-circuit aquaria (maximum $n=20$ per tank) for seven days prior to each experimental procedure to allow a period of habituation after handling and changing aquaria. Animals were not fed over this period to ensure that when feeding trials commenced, animals were at comparable points in their digestive cycle. Each tank was fitted with a nano protein skimmer (REEF-Skim Nano 100AC, TMC Ltd) and bio-filter (ZB-150, Ziss) with an attached airline. Water was changed daily using UV sterilized filtered seawater which had a mean salinity of $31\text{‰} \pm 0.05$ (s.e.m., $n=7$). A photoperiod of 16 hours light and 8 hours dark was maintained throughout all experiments to represent local summer photoperiods. Urchins were maintained and handled in accordance with UK animal welfare regulations.

2.3.2 Experiment 1. Acute salinity tolerance

Five salinity treatments were used to assess urchin salinity tolerance to an acute challenge over a 24-hour period: 31‰ (control), 26‰, 21‰, 16‰ and 11‰. Due to logistical constraints each treatment was conducted in a single 50-L tank with a set-up as previously described, with tank water averaging $13.75^{\circ}\text{C} \pm 0.17$ (s.e.m., $n=10$). The impact of tank effects was considered minimal due to the short duration of the experiment (24 hours) in clean water, which was monitored for temperature, salinity, and water chemistry, while each urchin was isolated in a floating tray. In addition, the impact of abrupt reductions in salinity on urchin functions was considered to be substantially greater than any potential variations due to tank effects, and therefore each urchin was considered a biological replicate. Reverse osmosis water was mixed with filtered seawater until experimental salinity levels were achieved.

Salinity was measured using a conductivity probe (CDC40101, Hach). Ten urchins were selected at random for each experimental salinity treatment ($n=50$ in total). For each treatment oxygen consumption, righting ability, coelomic fluid osmolality and mortality were assessed. After 24 hours, tank water was immediately replaced with ambient seawater (31‰).

2.3.3 Experiment 2. Low salinity acclimation

Three salinity treatments were selected to assess acclimation over a 25 day period based on salinity data from sites of wild collection (e.g., minimum surface salinity was 28‰ and 31‰ in May, and 15‰ and 23‰ in October 2011 and 2012 respectively) (Rabe and Hindson, 2017) and the results of the acute trial: control (31‰), medium (26‰) and low (21‰) salinity. For each salinity treatment, 30 urchins were randomly distributed between three, 50-L tanks (set up as previously described), with a total of 10 urchins per tank (total $n=90$ for the acclimation experiment). This design allowed an evaluation of tank effect variation within salinity treatments to assess whether each individual urchin could be considered a biological replicate.

After a seven-day habituation period, the medium and low salinity tanks were diluted by adding reverse osmosis (RO) freshwater to each tank and adjusting for tank volume in a stepwise manner, reducing salinity by $\sim 2\%$ per day until experimental treatment levels were reached (as per Shirley and Stickle, 1982a). Within each salinity treatment, 10 urchins were placed in numbered floating trays (split between the three tanks in each treatment in a ratio of 3:3:4) which facilitated repeated measurements for oxygen consumption, feeding and mass. Water quality was assessed twice a week (Saltwater Master Test Kit, API, USA). Ammonium (NH_4^+), nitrates (NO_3^-) and nitrites (NO_2^-) were kept below 0.05, 1 and 0.025 mgL^{-1} respectively, by refreshing tank water at least twice a week by at least 30-L per tank. Tank water temperature was checked daily ($13.76^\circ\text{C} \pm 0.03$, mean $\pm$ s.e.m., $n=25$) over the experimental period. Tank water pH was monitored twice weekly using a pH probe (Hach, Germany) and remained stable over the experimental period ($\text{pH } 7.93 \pm 0.014$, mean $\pm$ s.e.m, $n=8$). On days 1, 5, 9, 17 and 25 after the target experimental salinity was reached (hereafter termed ‘timepoints’) oxygen consumption, feeding rate (additionally on day 13), righting

ability and coelomic fluid osmolality were assessed. Coelomocyte analysis, wet weight change and mortality were assessed on day 25.

2.3.4 Experiment 3. Hypoosmotic shock on acclimated urchins

At the end of the acclimation experiment (day 25), all urchins in each treatment were transferred to 18‰ salinity to assess the response to an acute osmotic shock for a 6-hour duration. A salinity of 18‰ was chosen as this was 2‰ above the lethal salinity identified in the acute experiment and near the predicted LC_{50} value (18.5‰). Oxygen consumption ($n=8$ per treatment), righting ability ($n=11$ per treatment) and coelomic fluid osmolality ($n=6$ per treatment) were measured. All urchins were then transferred back to their prior acclimated salinity and assessed for mortality over the next 72 hours.

Oxygen consumption – Oxygen consumption was assessed using closed chamber techniques following Suckling et al. (2015) and Morley et al. (2016). Individual urchins ($n=10$ per treatment in experiment 1 and 2, $n=8$ per treatment in experiment 3) were immersed directly into experimental salinity tanks and placed within open respirometry chambers (experiment 1 and 3) or transferred underwater to open respirometry chambers (experiment 2). As handling is likely to affect oxygen consumption due to stress, animals were left for two hours prior to commencing measurements (pre-trial testing in ambient salinity showed that two hours was sufficient for a return to pre-handling metabolic rate). Once the respirometry chambers were sealed, oxygen concentration was measured using a Fibox-4 fibre optic oxygen sensor (PreSens, Germany). After 3 hours in the closed chambers a second measurement was taken. Three hours was sufficient to allow a drop in oxygen levels from full saturation to 90-70%. Three control chambers without animals were measured to account for background oxygen changes, which were used to correct the calculated animal oxygen consumption rates. Chamber volumes were adjusted for animal volume (obtained by displacement) to produce a 'respired water volume' used in calculating oxygen consumption. In experiment 2, oxygen consumption was measured in the same 10 urchins within each treatment at each timepoint. Oxygen consumption was measured at approximately the same time of day (mid-morning) to minimise any circadian rhythm effects. Each timepoint measurement was scheduled four days after the last feeding assessment to minimise the

impact of raised metabolism associated with feeding (the specific dynamic action of feeding (SDA)). Temperate marine invertebrates typically require 2-5 days to return to basal oxygen consumption levels after feeding (Secor, 2009). Animal wet mass ($g \pm 0.01$), volume ($g \pm 0.01$) and test diameter ($mm \pm 0.5$) were recorded for each urchin. To obtain ash-free dry mass (AFDM; $g \pm 0.01$), all animals were euthanised by freezing at -20°C and then dried in a convection oven at 60°C to constant mass ($g \pm 0.01$). Dried urchins were then transferred to a muffle furnace, which was heated to 475°C for 6 hours. AFDM was obtained by subtraction and is used to estimate the mass of respiring organic tissue in the animal.

Righting ability – Urchins orientate oral side down; inverting them elicits a righting response. The time taken for urchins to fully right themselves was recorded and converted to an activity coefficient ($AC = 1000/\text{righting time in seconds}$) after Lawrence and Cowell (1996). The maximum time allocated for a righting response was 30 minutes, which equals a minimum AC value of 0.55 ($1000/1800$ seconds). Care was taken when handling urchins to minimise damage to tube feet that were attached to the tank surface, by gently applying pressure and waiting for tube feet to detach. In experiment 1 ($n=5$ per treatment) and 3 ($n=11$ per treatment), AC was assessed after five hours in the experimental salinity. In experiment 2, AC assessments were performed on 7-10 animals per treatment at each timepoint.

Coelomic fluid osmolality – Coelomic fluid samples were taken by sub-lethal extraction to assess osmolality ($n=5$ per treatment in experiment 1 after 24 hours; $n=5$ per treatment in experiment 2; $n=6$ per treatment in experiment 3 after 6 hours). A syringe with a 21-gauge needle was inserted through the peristomial membrane at a 45° angle away from the oral area and into the coelomic cavity (after Reinardy and Bodnar, 2015). Approximately 1ml of coelomic fluid was extracted per urchin. Coelomic fluid was centrifuged (800 g for 4 minutes) in sterile microcentrifuge tubes to separate the coelomocyte pellet from the coelomic fluid supernatant. The supernatant was pipetted into a separate tube and stored at -80°C for later analysis. Triplicate tank water samples were collected at each sample point and a mean seawater osmolality obtained for use in one-way t-test comparisons to coelomic fluid. The osmolality of the coelomic fluid and seawater was measured on a Vapro 5600 vapour pressure osmometer (ELITech Group).

Chapter 2

Mortality – After experiments 1 and 3, animals were examined every 24 hours (up to 5 days) for signs of life (observed movement of spines and tube feet) and re-tested for their righting response. Mortality rates were assessed on animals that had not been subjected to coelomic fluid extraction. In experiment 2, urchin mortality was monitored daily with urchins that appeared dead (i.e., inability to right after 24 hours and lack of movement in spines and tube feet) removed.

Feeding rate (experiment 2 only) – Feeding trials began when urchins had reached their experimental salinities and was repeated every four days. A diet of brown kelp (*Laminaria* spp.) was offered as it had been previously used to feed urchins at SAMS, is locally abundant and known to be a main source of food in the wild (Bekkby et al., 2015; Jones and Kain, 1967). A pre-weighed amount of fresh kelp was offered to each urchin isolated in a tray and left for 24 hours (based on initial feeding trials to assess amount). After 24 hours, remaining kelp was removed and re-weighed, with the difference taken as the amount eaten. Buoyant weight was selected as a weighing metric (adapted from Jokiel et al., 1978), as this is insensitive to water contained in the fresh porous kelp tissue. For the remaining urchins which were not being assessed for their feeding rate, excess un-weighed quantities of kelp were provided at each feeding point.

Coelomocyte collection and analysis (experiment 2 only) – Coelomocytes were collected at the last sample point (day 25) from 7 urchins per treatment. Coelomic fluid was collected as previously described, with the addition of using syringes prefilled with 0.5ml ice-cold calcium/magnesium-free seawater (460 mM NaCl, 10 mM KCl, 7 mM Na₂SO₄, 2.4 mM NaHCO₃), containing 30 mM ethylenediaminetetraacetic acid (EDTA) anticoagulant [pH 7.4] to prevent cell clumping (Liu et al., 2022). A volume of ~0.5ml coelomic fluid was extracted per urchin. Cell concentration, differential cell counts (red spherulocytes and clear cells) and cell viability were assessed on a Neubauer haemocytometer. To assess viable cells, coelomic fluid was mixed with 0.5% trypan blue (ratio 1:1) and the number of dead blue cells and clear live cells counted (Liu et al., 2022).

Wet weight change and percentage AFDM (experiment 2 only) – Wet weight (g) was recorded before and after the 25-day exposure in the isolated urchins ($n=10$ per treatment) with the

difference noted, after Podbielski et al. (2022). On both occasions, there was a gap of seven days between the last feeding point and weighing, to ensure that animals were at similar points in their digestive cycle. The relationship between AFDM (organic mass) and ash mass (inorganic mass) was used to determine changes in organic mass over the trial, expressed as:

$$\% AFDM = \frac{AFDM}{(AFDM + ash\ mass)} \times 100$$

2.3.5 Statistics

One-way analysis of variance (ANOVA) tests (including Welch's ANOVA for data with unequal variances) were performed at each timepoint to compare responses between salinity treatments. Two-way ANOVAs were conducted to compare response variables between treatments, timepoints and their interactions. Where repeat measurements were conducted on the same animal across different timepoints, the addition of individual animal as a random factor was included in the model. Within the acclimation experiment, tank effects were assessed on oxygen consumption, feeding rates and righting data utilising a two-way ANOVA with tank and timepoints as fixed variables, and individual as a random factor when repeat measures were taken. Assumptions of normality and equal variance were formally tested using the Shapiro-Wilk test and Levene's test respectively. When assumptions of normality were violated, data were either log or square root transformed. With continued violations of normality, non-parametric Kruskal-Wallis tests were implemented. For significant ANOVA/Kruskal-Wallis test results, post-hoc testing was carried out to determine differences between treatments. Tukey tests were used for normally distributed data, Dunn's test for non-parametric data and Games-Howell tests for data with unequal variance. When assumptions of normality were violated in two-way models, an aligned rank transformation ANOVA (ART ANOVA) was implemented as a non-parametric alternative. One sample t-tests were used when comparing coelomic fluid values to mean tank water osmolality. Two sample t-tests were used when comparing two group means. All percentage data were arcsine transformed prior to statistical analysis. Statistical tests were considered significant if $P < 0.05$. All analysis were carried out in R (version 4.0.5).

2.4 Results

2.4.1 Experiment 1. Acute salinity tolerance

After 24 hours, mortality was 100% in the 11‰ and 16‰ salinity treatments. All urchins in the 21‰, 26‰ and the control (31‰) treatments survived, showing no significant differences in mean righting times between treatments after five days recovery at 31‰ (Table A1). Mortality was 100% at 16‰ and 0% at 21‰, therefore if the relationship is linear the LC_{50} value (salinity which is lethal to 50% of exposed urchins) for *E. esculentus* is estimated to be 18.5‰.

Oxygen consumption differed significantly between treatments (Kruskal-Wallis: $\chi^2_{(4)}=22.68$, $P<0.001$), with the following treatment comparison showing significant changes: 11‰ and 26‰, 11‰ and 31‰, 16‰ and 26‰ (Dunn: all $P<0.03$) (Figure 2.1; Table A1).

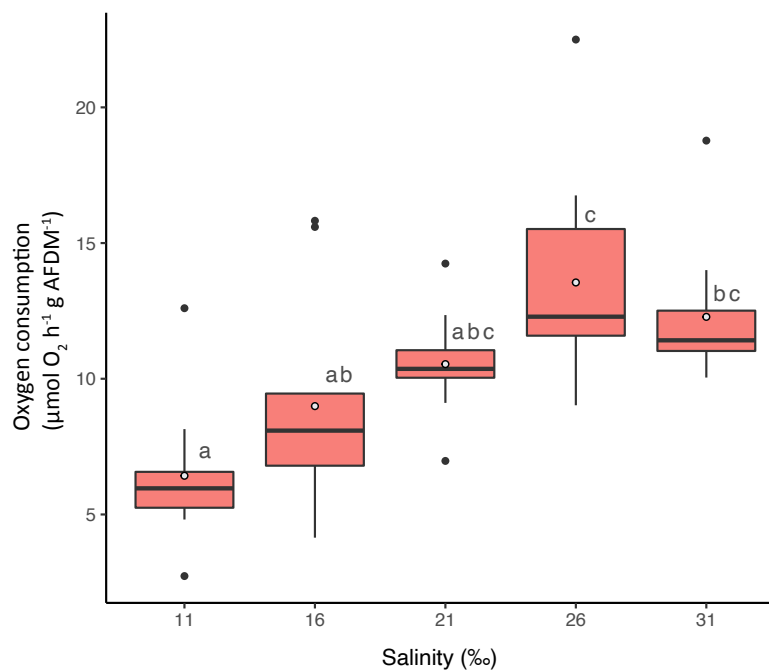


Figure 2.1| Oxygen consumption of *E. esculentus* two hours after transfer from ambient salinity (31‰). Results are shown for Kruskal–Wallis and post-hoc Dunn test with different letters indicating significant differences ($P<0.05$; $n=10$ biological replicates for each treatment) between treatments. Boxplots show medians (black line), upper and lower quartiles, and maximum and minimum (whiskers). Means are represented by open points and outliers by black points within corresponding box-whisker plots.

After five hours of immersion, mean AC values were significantly different between the control treatment and all other salinities (Dunn: all $P < 0.001$), except 26‰ (Figure 2.2; Table A1). There were also significantly different AC values between 26‰ paired with 21‰, 16‰ and 11‰ (Dunn: all $P < 0.03$) (Figure 2.2; Table A1). In the 16‰ and 11‰ treatments no urchins righted within 30 minutes.

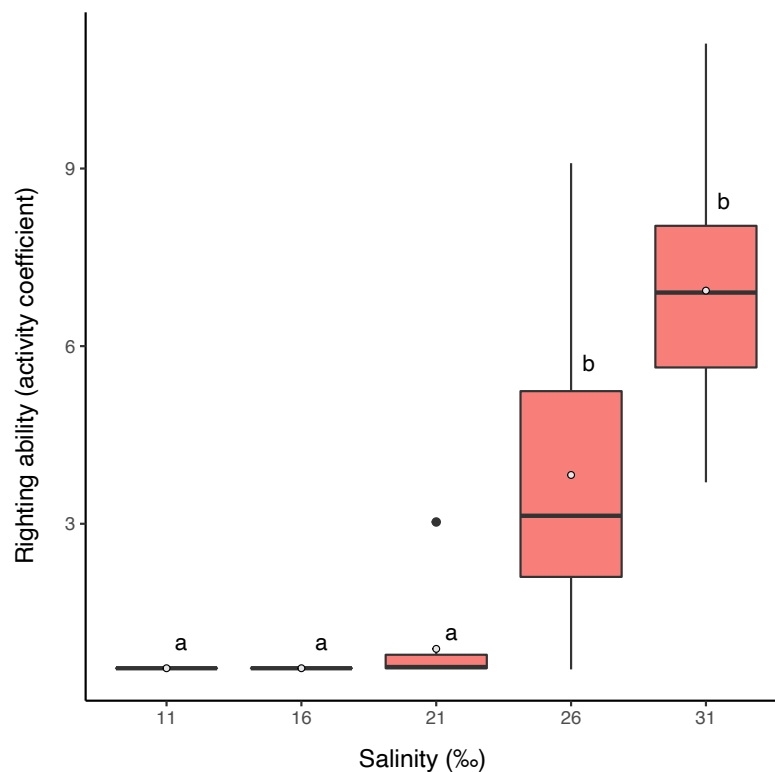


Figure 2.2 | Activity coefficient (AC= 1000/righting time in seconds) values of adult *E. esculentus* five hours after transfer from ambient salinity (31‰). Note: a smaller AC value indicates longer righting times. Results are shown for Kruskal–Wallis and post-hoc Dunn test with different letters indicating significant differences ($P < 0.05$; $n=10$ biological replicates for each treatment) between treatments. Boxplots show medians (black line), upper and lower quartiles, and maximum and minimum (whiskers). Means are represented by open points and outliers by black points within corresponding box-whisker plots.

Mean coelomic fluid osmolality was significantly different between all salinity treatments after 24 hours (Welch's ANOVA: $F_{(4)}=8666$, $P < 0.00001$; Games-Howell, all $P < 0.0001$) (Figure 2.3; Table A1). Mean coelomic fluid osmolality was slightly but significantly hyperosmotic to tank seawater in each salinity treatment (One sample t-test; all $P < 0.05$) (Figure 2.3; Table 1).

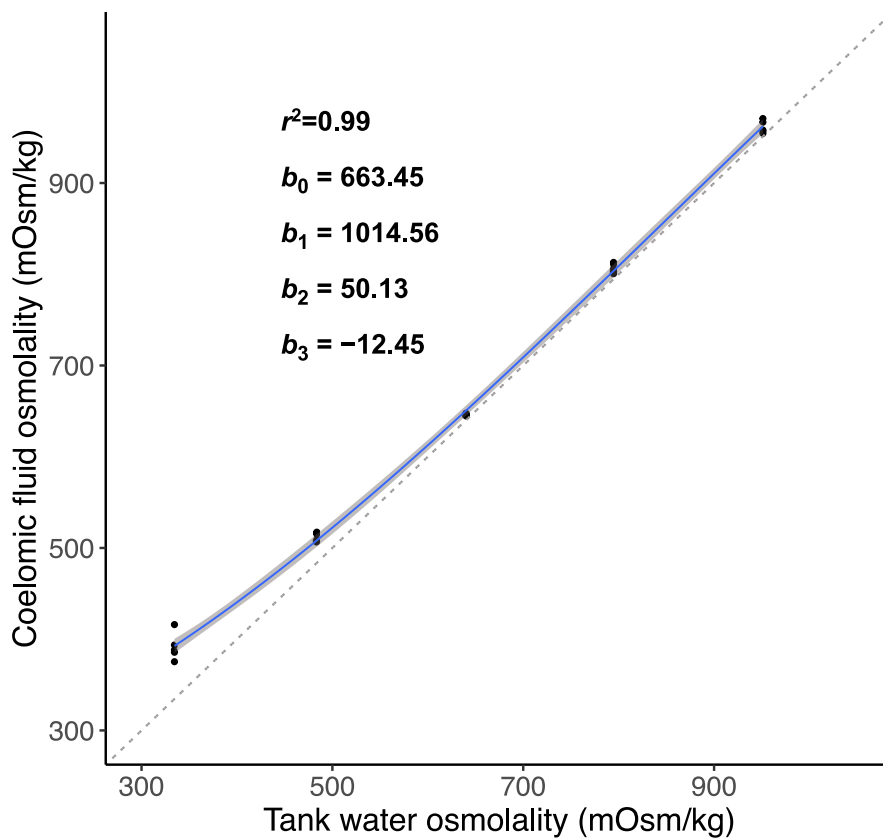


Figure 2.3| Coelomic fluid osmolality of adult *E. esculentus* 24 hours after transfer from ambient salinity (31‰) and the corresponding tank water. All coelomic fluid values are significantly different from their corresponding tank water (one sample t-test: all $P < 0.05$; $n=5$ biological replicates at each salinity; 31, 26, 21, 16, and 11‰ corresponds to 951, 794, 640, 484 and 329 mOsm/kg tank water osmolality respectively). A regression line (blue line) and the 95% confidence intervals (shaded grey area) were fitted to the data. The iso-osmotic line is represented by the dashed grey line. Linear regression coefficients and R-squared value are included to the left of the regression line.

2.4.2 Experiment 2. Low salinity acclimation

Tank effects – There was no significant variation between treatment tanks; therefore, each individual urchin was considered a biological replicate within each treatment (Table A2).

Mortality – Over the course of the experiment there were four mortalities; two in the low salinity treatment (day 10 and day 20); one in the control (day 24) and one accidental death in the medium salinity treatment due to handling error.

Wet weight change, percentage AFDM and physical condition – Salinity had a significant impact on the change in mean ($n=10$) net wet weight (Welch's ANOVA: $F_{(2)}=4.90$, $P=0.021$) (Table A3). There were significant differences between the low and control treatments (Games-Howell: $P=0.019$) (Table A3).

Salinity had a significant impact on mean AFDM as a percentage of total dry mass (Kruskal-Wallis: $X^2_{(2)}=6.86$, $P=0.03$) (Table A3). The low salinity treatment had the lowest mean ($\pm$ s.e.m.) percentage of AFDM to dry mass (low: $16.14 \pm 0.83\%$; medium: $19.5 \pm 1.13\%$; control: $18.11 \pm 0.65\%$); however, this was only significant between low and medium salinity treatments (Dunn: $P=0.0046$) (Table A3).

At the end of the experimental period, urchins in the low salinity treatment had lost a high number of primary spines which appeared to have detached at the tubercles, in addition to damaged tube feet which were dark in colour (Figure 2.4) and appeared limp when underwater. On visual inspection the medium salinity urchins were indistinguishable from the control treatment.

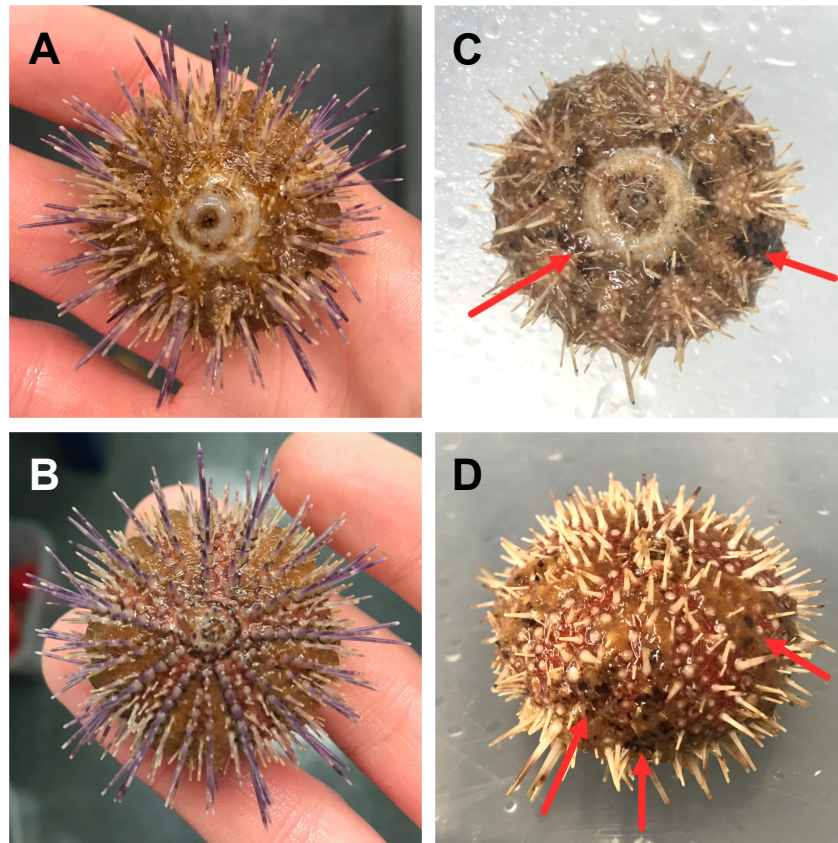


Figure 2.4| A–B Oral and aboral view of *E. esculentus* after 25 days in 31‰ (control treatment), C–D Oral and aboral view of *E. esculentus* after 25 days in 21‰ salinity (low treatment). Note the high primary spine loss and tube feet damage in the low salinity treatment (C–D). Arrows highlight areas of tube feet damage.

Oxygen consumption – Rates of oxygen consumption varied significantly between salinity treatments (two-way ANOVA: $F_{(2)}=7.39$, $P=0.003$) and timepoints (two-way ANOVA: $F_{(4)}=4.74$, $P=0.002$), including their interaction (2-way ANOVA: $F_{(8)}= 8.16$, $P<0.0001$) (Figure 2.5; Table A3). On day 1 oxygen consumption did not differ significantly between salinity treatments (ANOVA: $F_{(2)}=1.29$, $P=0.292$). However, by day 5 oxygen consumption in the low salinity treatment was higher than both medium and control treatments (Tukey: $P<0.001$) (Figure 2.5; Table A3) and remained higher than the control for the duration of the experiment. The medium salinity treatment was not significantly different from the control except on day 17 (Tukey: $P=0.042$) (Figure 2.5; Table A3).

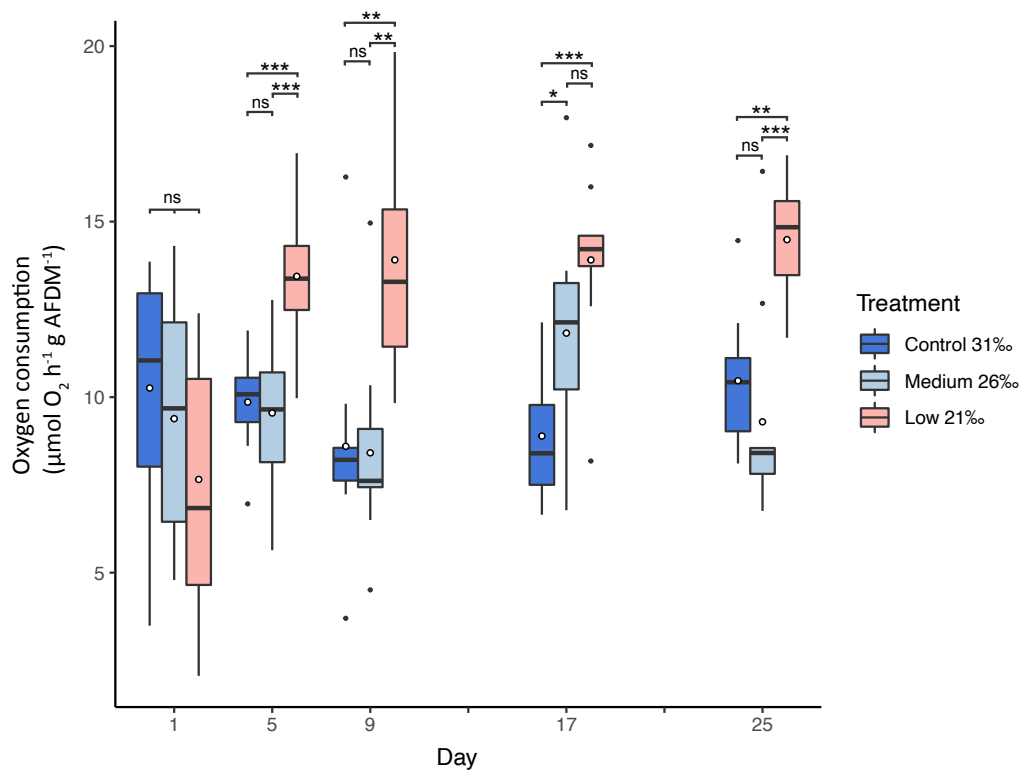


Figure 2.5| Oxygen consumption of *E. esculentus* at timepoints spanning 25 days of exposure to different salinity conditions. Results are shown for one-way ANOVA and post-hoc Tukey test between salinity treatments at each timepoint (ns= not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; $n=10$ biological replicates at each timepoint and treatment). Boxplots show medians (black line), upper and lower quartiles, and maximum and minimum (whiskers). Means are represented by open points and outliers by black points within corresponding box-whisker plots

Righting ability – Mean AC values varied significantly between salinity (two-way ANOVA: $F_{(2)}=69.23$, $P < 0.0001$) and timepoints (two-way ANOVA: $F_{(4)}=5.8$, $P < 0.001$), including their interaction (two-way ANOVA: $F_{(8)}=2.16$, $P=0.036$) (Figure 2.6; Table A3). The time taken for urchins to fully right (expressed as AC values) was significantly longer in the low compared to the control salinity treatment at every sampling point throughout the experimental period (days 1, 9 and 17 Tukey: all $P \leq 0.05$, days 5 and 25 Games-Howell: $P \leq 0.05$) (Figure 2.6; Table A3). AC values also differed significantly between the medium and low salinity treatment on days 9 and 17 (Tukey: $P \leq 0.05$) and day 25 (Games-Howell: $P \leq 0.05$). There was no significant difference in AC values between the medium and control treatments at any sampling point.

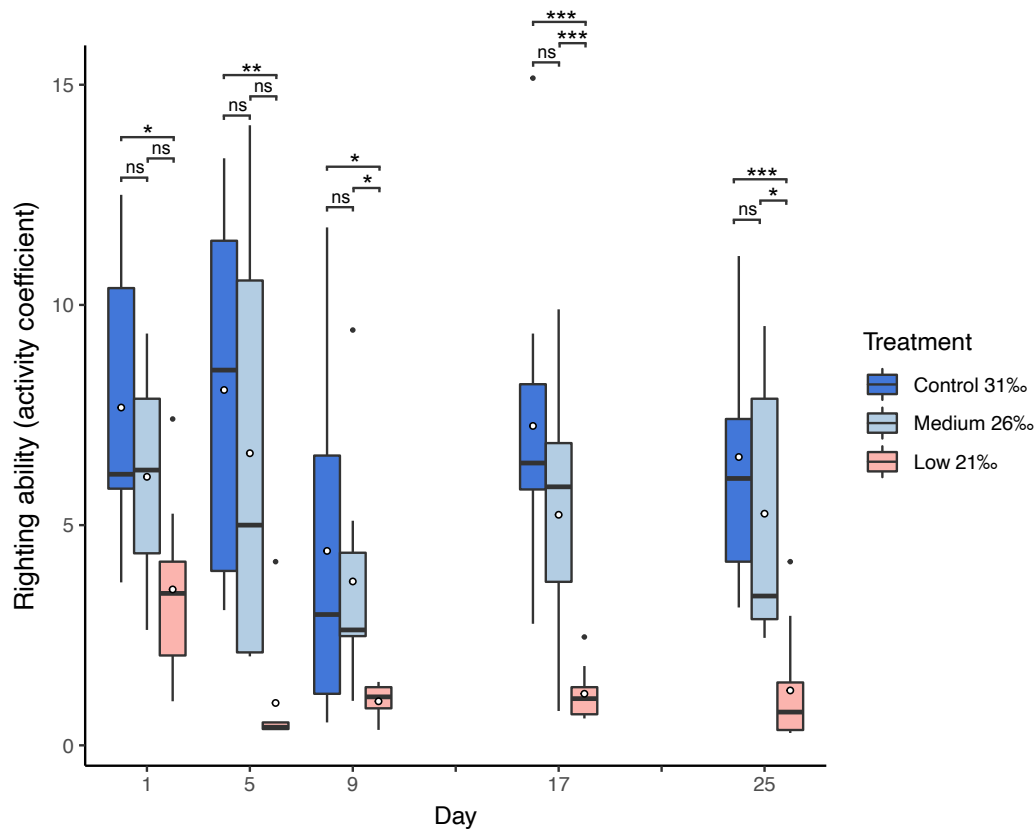


Figure 2.6| Activity coefficient (AC= 1000/righting time in seconds) values of *E. esculentus* at timepoints spanning 25 days of exposure to different salinity conditions (note: a smaller AC value indicates longer righting times). Results are shown for one-way ANOVA and post-hoc Tukey test (days 5,9 and 17) and Games-Howell test (days 1 and 25) between salinity treatments at each timepoint (ns= not significant, * $P<0.05$, ** $P<0.01$, *** $P<0.001$; $n=7-9$, 7-8, 7-10 biological replicates at each timepoint and treatment in control, medium and low salinity respectively). Boxplots show medians (black line), upper and lower quartiles, and maximum and minimum (whiskers). Means are represented by open points and outliers by black points within corresponding box-whisker plots.

Feeding rate – Mean feeding rate varied significantly between salinity treatments (two-way ANOVA: $F_{(2)}=23.06$, $P<0.00001$) and timepoints (two-way ANOVA: $F_{(5)}=6.32$, $P<0.0001$), including their interaction (two-way ANOVA: $F_{(10)}=2.96$, $P=0.002$) (Figure 2.7; Table A3). At each feeding sample point the rate of feeding was significantly different between the control and low salinity treatment (Games-Howell: $P<0.05$) (Figure 2.7; Table A3), except for days 5 and 9. The rate of feeding was also significantly different between the medium and low treatments for all feeding sample days (Games-Howell: all $P<0.05$) (Figure 2.7; Table A3), except on day 1 and 13. There were no significant differences in feeding rate between the control and medium treatment at any point in the experiment.

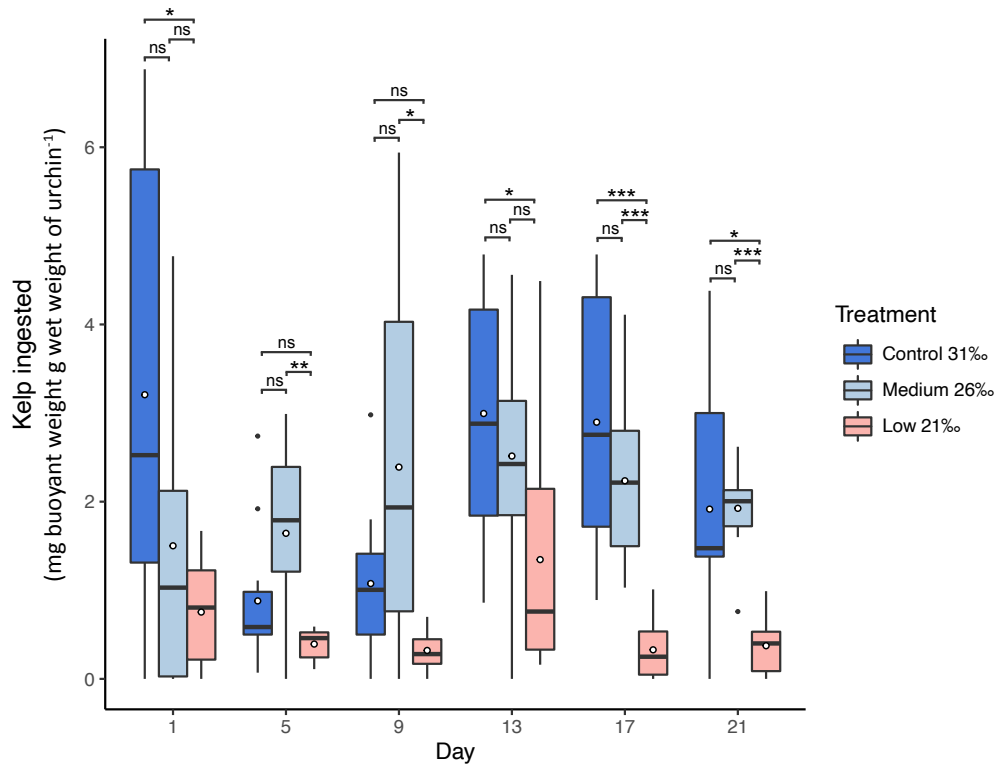


Figure 2.7| Feeding rate of *E. esculentus* at timepoints spanning 25 days of exposure to different salinity conditions. Results are shown for one-way ANOVA and post-hoc Tukey test (day 13) and Games-Howell test (days 1, 5, 9, 17 and 25) between salinity treatments at each timepoint (ns= not significant, * $P<0.05$, ** $P<0.01$, *** $P<0.001$; $n=10$ biological replicates at each timepoint and treatment). Boxplots show medians (black line), upper and lower quartiles, and maximum and minimum (whiskers). Means are represented by open points and outliers by black points within corresponding box-whisker plots.

Coelomic fluid osmolality – Mean coelomic fluid osmolality differed significantly between each treatment at every sample point, including their interaction (two-way ANOVA: $F_{(8)}=194$, $P<0.0001$; Tukey: all $P<0.05$) (Figure 2.8; Table A3). Compared to the tank water, coelomic fluid was always slightly hyperosmotic at every sample point within each treatment, although this was only significant on days 5 and 17 in the control (all one-sample t-tests: day 5: $t_{(4)}=4.43$, $P=0.011$; day 17: $t_{(4)}=7.74$, $P=0.001$), days 1, 5, 17 and 25 in the medium (all one-sample t-tests: day 1: $t_{(4)}=6.88$, $P=0.002$; day 5: $t_{(4)}=3.02$, $P=0.039$; day 17: $t_{(4)}=5.25$, $P=0.006$; day 25: $t_{(4)}=6.55$, $P=0.003$) and days 17 and 25 in the low salinity treatments (all one-sample t-tests: day 17: $t_{(4)}=3.38$ $P=0.028$; day 25: $t_{(4)}=3.94$, $P=0.015$) (Figure 2.8; Table A3)

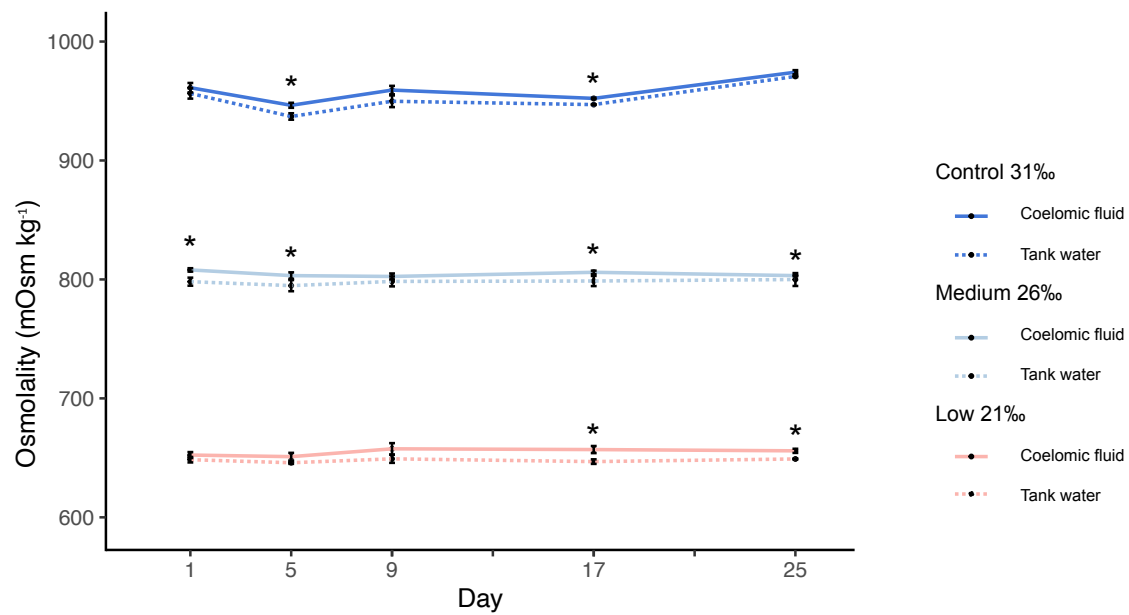


Figure 2.8| Coelomic fluid (CF) osmolality (mean $\pm$ s.e.m.; represented by error bars) of *E. esculentus* ($n=5$ biological replicates for each salinity and timepoint) and tank water ($n=3$ technical replicates for each salinity and timepoint) osmolality at timepoints spanning 25 days of exposure to different salinity conditions. Asterisk indicates significant differences (one-sample t-test: $P<0.05$; $n=5$) between corresponding CF and tank water.

Coelomocyte analysis – Mean total coelomocyte concentrations after 25 days in experimental salinities showed no significant differences between treatments (Table 2.1). The percentage of viable cells and red spherulocytes as a proportion of total coelomocytes also showed no significant differences between treatments (Table 2.1).

Table 2.1| Coelomocyte concentrations from *E. esculentus* after 25 days immersed in different salinities.

Treatment	n	Mean test diameter (mean $\pm$ s.e.m mm)	Mean wet weight (mean $\pm$ s.e.m g)	Cell viability (mean $\pm$ s.e.m %)	Red spherule cells (mean $\pm$ s.e.m % of total coelomocyte concentration)	Total coelomocyte concentration (mean $\pm$ s.e.m $\times 10^6$ cells ml ⁻¹)
Control (31‰)	7	38 $\pm$ 2.6	20 $\pm$ 3.7	87 $\pm$ 1.4	2 $\pm$ 0.3	7.33 $\pm$ 0.8
Medium (26‰)	7	37 $\pm$ 2.5	21 $\pm$ 4.3	91 $\pm$ 1.8	2 $\pm$ 0.6	7.83 $\pm$ 1.7
Low (21‰)	7	41 $\pm$ 2.5	27 $\pm$ 4.5	82 $\pm$ 4.5	4 $\pm$ 1.4	5.71 $\pm$ 0.9

There were no significant differences between salinity treatments in terms of cell viability ($F_2=2.39$, $P=0.14$; Welch's ANOVA), total coelomocyte concentration ($F_2=1.36$, $P=0.28$; ANOVA) and proportion of red spherulocytes ($F_2=1.06$, $P=0.38$; Welch's ANOVA)

2.4.3 Experiment 3. Hypoosmotic shock on acclimated urchins

Following acclimation, mean oxygen consumption after acute immersion in 18‰ salinity showed similar values between acclimated treatments and there were no significant differences (Figure 2.9; Table A4). However, oxygen consumption within the medium salinity treatment increased significantly after acute immersion when compared to its prior acclimated value, while the low treatment conversely decreased significantly (two-sample t-test: Medium, $t_{(15)}=-2.74$, $P=0.015$; Low, $t_{(16)}=2.86$, $P=0.011$) (Figure 2.9; Table A4), indicating a very different response. The control treatment showed no significant change after acute immersion.

After five hours in 18‰ salinity, two urchins from the medium salinity treatment righted fully within 30 minutes (at 17 and 29 minutes). None of the urchins in the low or control salinity treatments righted within one hour or showed any signs of righting (i.e., tube feet remained limp).

Mean coelomic fluid osmolality varied significantly between treatments after six hours in 18‰ (Welch's ANOVA: $F_{(2)}=15.82$, $P=0.0036$), with the low and medium salinity treatments

being significantly lower than the control (Control: 631 ± 10 mOsm kg^{-1} ; Medium: 595 ± 6 mOsm kg^{-1} ; Low 579 ± 1 mOsm kg^{-1}) (Games-Howell: Low v Control, $P=0.008$; Medium v Control, $P=0.034$) (Table A4 and A5). The coelomic fluid osmolality in all treatments was significantly higher than the tank water osmolality (one-sample t-test: Control, $t_{(4)}=7.97$, $P=0.001$; Medium, $t_{(4)}=8.04$, $P=0.001$; Low, $t_{(4)}=25.96$, $P<0.0001$) (Table A4).

After 72 hours in the recovery tanks, all animals had survived. Righting ability was assessed ($n=8$ per treatment) and all animals righted fully within 13 minutes.

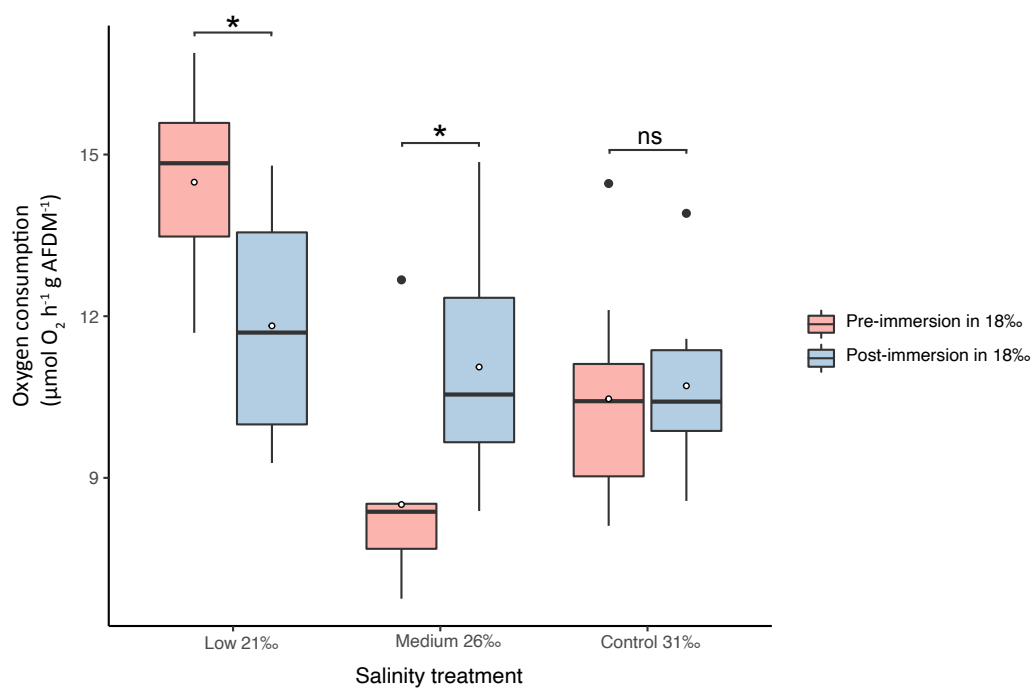


Figure 2.9| Oxygen consumption (mean $\pm$ s.e.m.) of *E. esculentus* after 25 days in different salinity treatments (red boxes: $n=10$ biological replicates) followed by an acute immersion in 18‰ salinity (hypoosmotic shock experiment) (blue boxes: $n=8$ biological replicates). Asterisk indicates significant differences (two-sample t-test: $P<0.05$) between mean oxygen consumption pre- and post- acute immersion for each salinity treatment. Boxplots show medians (black line), upper and lower quartiles, and maximum and minimum (whiskers). Means are represented by open points and outliers by black points within corresponding box-whisker plot

2.5 Discussion

Salinity acclimation is distinct from short-term salinity tolerance, as the former involves a process of cellular reorganisation that aids in long-term survival within the altered environment, while the latter constitutes a temporary ‘reflex’ response to osmotic stress. (Khlebovich, 2017; Rivera-Ingraham and Lignot, 2017). This study revealed that *E. esculentus* has a 24-hour lower tolerance limit of 21‰ after direct transfer from ambient conditions (with a predicted LC_{50} threshold to be 18.5‰), characterised by metabolic depression and a decline in activity. In contrast, exposure to a salinity of 21‰ over 25 days led to an increase in metabolic rate, while activity and feeding were impaired. This resulted in a decline in physical condition, suggesting that this falls below the plasticity limit of acclimation. At 26‰ salinity, there were no measurable impacts on urchin behaviour or physiology after 25 days of exposure, indicating successful acclimation. The hypoosmotic shock trial demonstrated that acclimation offers beneficial functional resistance (righting ability and metabolic capacity) upon exposure to further osmotic challenges.

2.5.1 Survival and physical condition

Acute low salinity tolerance thresholds in echinoids are species and population specific, often reflecting natural distributions (Russell, 2013; Stickle and Diehl, 1987). The lower survival limit of 21‰ established for *E. esculentus* likely reflects their subtidal preferred habitat (Comely and Ansell, 1988). Indeed, LC_{50} salinity data from intertidal populations of *P. miliaris* (collected from the same area as *E. esculentus*) were observed to be substantially lower [$<16‰$ see Appendix A for *P. miliaris* data, Figures A1 and A2; also see also Gezelius (1963)], suggesting that salinity is a significant factor in an echinoids depth distribution.

Although there were no significant impacts on survival in the acclimation experiment, there were observable declines in the physical condition of urchins at 21‰. The high spine loss and appearance of dark colouration in the tube feet indicated severe stress. Significantly, the reduction in mass recorded at the end of the acclimation experiment suggests a cessation of growth and tissue breakdown. If the cost of osmoregulation is too high, energy allocation

for processes such as growth and maintenance may be limited, and when severe, tissue may be catabolised to sustain energetic demands (Podbielski et al., 2022b). The difference in the ratio of AFDM to dry mass between the low and higher salinity treatments suggests salinity had an impact on organic mass despite high spine loss (although spine loss was not quantified), indicating depletion of stored reserves and possibly tissue catabolism. Reductions in mass have previously been observed in echinoids including *P. miliaris* and *Strongylocentrotus droebachiensis* held in low salinities over six weeks (Podbielski et al., 2022b). Spine loss has also been observed in urchins held in long-term low salinity experiments (Sabourin and Stickle, 1981). Physical damage as a result of abiotic stress has been implicated in the reduced survival rates and ability of urchins to recover when ambient conditions return to normal (Minuti et al., 2021). Despite survival at 21‰ over the experimental period, the declines in physical condition observed may have detrimental impacts on urchin recovery abilities and ultimately long-term survival.

2.5.2 Physiological functions

Oxygen consumption varied over the timeframe of exposure and with the degree of salinity reduction. Previous echinoderm studies investigating the effects of acute low salinity exposure on oxygen consumption have showed varied responses (Stickle and Diehl, 1987). Reductions in oxygen consumption have been observed in echinoids, including *Strongylocentrotus purpuratus* (Giese and Farmanfarmaian, 1963) and *S. droebachiensis* (Sabourin and Stickle, 1981). Similar reductions have been observed in an asteroid (Shirley and Stickle, 1982b) an ophiuroid (Talbot and Lawrence, 2002) and a holothurian (Sabourin and Stickle, 1981). Conversely, Stickle and Diehl, (1987) reported an inverse relationship between oxygen consumption and salinity between 30‰ and 15‰ in the ophiuroid *Ophioderma brevispinum*. In the current study, the initial metabolic depression observed under acute low salinity (below 26‰) indicated a reduction in energy utilisation. This is likely a consequence of the rapid influx of water into tissue via osmosis, leading to a temporary slowdown in physiological activity. Metabolic rate depression can be a reaction to extreme stress where an organism reduces activity and energy consumption, prioritising energy allocation in order to maintain homeostasis (Sokolova et al., 2012a). However, it is important to note the current study only recorded the initial metabolic response to a hypoosmotic

exposure (hours 2-5 out of 24), potentially missing a change in the metabolic response once a critical threshold was breached. At 26‰, the absence of a metabolic depression, indicated sufficient energy available to facilitate a relatively quick response to the change in salinity. This may reflect the higher energy demand for osmoregulatory cellular processes entrained to counter the acute osmotic stress (Rivera-Ingraham and Lignot, 2017).

Longer-term studies have demonstrated a reduction in oxygen consumption after acclimation to reduced salinities in echinoderms. For example, oxygen consumption in *S. droebachiensis* after 14 days in low salinity (15‰) was lower than at ambient (30‰) in the summer (Sabourin and Stickle, 1981). A similar response was observed in the asteroid *Leptasterias hexactis*, which exhibited significantly lower oxygen consumption after 28 days of exposure to lowered salinity (15‰ and 20‰) than at ambient (30‰) (Shirley and Stickle, 1982b). In the current study, the higher rates of oxygen consumption after chronic exposure to low salinity diverge from the results seen in previous studies. The impact of the postprandial rise in metabolism can be excluded as a cause because feeding in the low salinity urchins had largely ceased. For *E. esculentus*, therefore, the results suggest that increased energy expenditure was needed to account for costs related to the acclimation process (Sokolova et al., 2012a). These costs may be attributed to cellular rebalancing processes, such as modification of membrane-bound transporters and deamination of amino acids to reduce the cellular osmolytes pool (Gilles, 1987; Gilles and Delpire, 1997; Podbielski et al., 2022b).

Under chronic low salinity (21‰), the increase in oxygen consumption did not correlate with an increased righting response. Righting times were significantly longer compared to control and medium salinities, and did not improve throughout the experimental period. The acute response data showed significant reductions in righting ability below 26‰, suggesting both acute and chronic low salinity disrupts neuro-muscular coordination in *E. esculentus*, perhaps due to tissue swelling and the inability to restore cellular volume. Previous studies have reported a diminished righting ability in echinoids exposed to lowered salinity [e.g., *S. droebachiensis* (Moura et al., 2023b; Sabourin and Stickle, 1981) and *Lytechinus variegatus* (Lawrence, 1975)], a finding supported by this study. The righting response has often been described as a measure of ‘functional health’, with studies demonstrating that it is positively correlated with the energy budget as a function of salinity in echinoderms (Stickle and Diehl, 1987). The decline in righting performance under chronic low salinity, despite the increase in oxygen uptake, implies a limitation in the capacity to

utilise oxygen efficiently. This suggests that salinity is reaching a limiting value, perhaps analogous to the oxygen- and capacity-limited thermal tolerance concept, which proposes that an animal is constrained by physiological limitations in oxygen delivery under thermal stress (see Pörtner et al., 2017).

Crucially, high metabolism must be matched by high food intake or a switch to stored energy reserves. In low salinity, feeding was dramatically lower than at ambient and remained consistently low throughout the experimental period, indicating severe stress. Previous studies have demonstrated reduced feeding rates in echinoderms exposed to low salinity [for example: *L. hexactis*, (Shirley and Stickle, 1982a); *Pisaster ochraceus*, (Held and Harley, 2009)]. Held and Harley (2009) hypothesised that the reduction in activity under salinity stress suppresses the physical ability to feed in asteroids. This is supported by the current study which shows a reduction in righting ability, combined with reduced feeding.

2.5.3 Impact of salinity on coelomic fluids

Throughout the acclimation experiment, coelomic fluid osmolality values were slightly hyperosmotic to the seawater at each salinity (including ambient), indicating a small degree of ionic regulation, consistent with findings in other echinoids like *L. variegatus* (Vidolin et al., 2007). Previous studies have reported that echinoid coelomic fluid is generally isosmotic or slightly hyperosmotic compared to seawater (Diehl, 1986; Santos et al., 2013; Vidolin et al., 2007). Robertson (1980) observed near identical osmolality values between the coelomic fluid from *E. esculentus* and seawater measured at ambient salinity, similar to the current study. With acute exposure, coelomic fluid osmolality was similar to corresponding levels observed in the acclimation study, suggesting that full osmotic rebalancing of the extracellular fluid is complete within 24 hours. Interestingly, coelomic fluid taken from urchins after 24 hours in 16‰ and 11‰ had larger osmotic gradients (i.e., the difference in osmolality between the coelomic fluid and the seawater) than those at 21‰ and above, even though the urchins were likely dying or dead. This warrants further investigation, perhaps through measuring the degree of tissue hydration after immersion in low salinity.

The hypoosmotic shock trial on the acclimated urchins showed that coelomic fluid osmotic gradients in all treatments were substantially larger at 6 hours compared to the 24-hour experiment, indicating that extracellular rebalancing was incomplete and that significant

gradients were maintained. Numerous echinoid species have demonstrated positive ionic and osmotic gradients between the coelomic fluid and seawater after immersion in low salinity which persist for a number of hours (reportedly between 6-12 hours) (Diehl, 1986; Freire et al., 2011a; Vidolin et al., 2007). Despite being considered a subtidal urchin, *E. esculentus* maintains a hyperosmotic gradient for several hours after low salinity immersion. This ability may protect body tissues from sudden extreme hypoosmotic shock, akin to intertidal species (Freire et al., 2011a; Stickle and Ahokas, 1974; Vidolin et al., 2007).

Long-term exposure to low salinity appears to have no demonstrable impact on coelomocyte viability, total cell abundance or percentage of red spherulocytes. Abiotic stressors have previously been shown to affect coelomocyte compositions, with significant increases of red spherulocytes after acute heat stress (Branco et al., 2012). Furthermore, a study examining the impact of acute hypoosmotic stress on coelomocytes in the urchin *Echinometra lucunter*, observed a 122.5% increase in total coelomocyte abundance after 24 hours, but no change in red spherulocytes numbers (Honorato et al., 2017). In the current study, only chronic exposure to low salinity was examined, therefore the impact of acute low salinity stress on the immune cells of *E. esculentus* remains unknown.

2.5.4 Acclimation at 26‰ salinity

Acclimation is commonly defined as ‘The stabilisation of physiological functions after a change in conditions that are experimentally altered’ (Prosser, 1991; Schmidt-Nielsen, 1997). By the end of the experimental period, measures of oxygen consumption, feeding and activity in urchins exposed to 26‰ were similar to those at ambient salinity. Furthermore, there were no significant effects on growth or visible impacts on physical condition. This suggests that acclimation had been achieved in the medium treatment by the end of the experiment, confirming part of the original hypothesis. Additionally, it was predicted that acclimating to reduced salinity would confer functional resistance to subsequent acute hypoosmotic immersion. Following acclimation and immersion in 18‰, righting ability was still functional in a small number of individuals in the medium treatment, while absent in both the control and low treatment. Furthermore, the metabolic response to the osmotic shock varied between the low and medium treatments, with a higher metabolic rate observed in the medium treatment. An increase in metabolic rate indicates sufficient energetic resources

are available, offering an explanation for the ability of some urchins to right. The osmolality data from the hypoosmotic shock trial, showed that the osmotic gradient was greatest in the control treatment (Table A4), suggesting that intracellular osmotic pressure was higher than for the medium treatment. The larger gradient would cause an increase in cell volume, resulting in tissue swelling. This appears to have had a direct impact on physiological functioning, as evidenced by the inability of the control treatment to produce a righting response.

Overall, long-term exposure to 26‰ appears to have facilitated a beneficial advantage to *E. esculentus* in the face of further hypoosmotic exposure and offers further evidence that acclimation to 26‰ was achieved. Successful acclimation to mild stressors (e.g., a small rise in temperature or small reduction in pH) has been previously shown to have beneficial impacts in echinoids, such as improving reproductive outcomes (Suckling et al., 2015). However, further investigations are necessary to establish whether costs related to osmotic acclimation in *E. esculentus* are at a trade-off with investment in reproduction or longer-term somatic growth as demonstrated in other echinoids and osmoconformers (Sanders et al., 2018; Santos et al., 2022).

2.5.5 Lower limit of salinity acclimation

Unlike the medium treatment, stabilisation of physiological functions did not occur in the low salinity urchins. Instead, metabolic rates were substantially elevated to meet the cellular osmoregulatory demands. The increased metabolic costs were clearly not met through food intake. Feeding rates were significantly impacted in the lowest salinity, thereby creating an energetic deficit explaining the mass reduction and possible catabolic tissue breakdown. Furthermore, the resulting damage to tube feet will further restrict locomotion and feeding, while also reducing the surface area available for respiration (Hyman, 1955; Leddy and Johnson, 2000; Moura et al., 2023b). Without a shift towards a positive energy balance through feeding, the metabolic deficit will continue to increase cumulatively, eventually resulting in a decline in urchin health and/or mortality. At the cellular level, the osmolality data from the low salinity treatment confirms that extracellular coelomic fluids were consistently isosmotic or slightly hyperosmotic with the external salinity. Previous studies show that full cellular ionic rebalancing can be achieved within 14 days in many marine

invertebrates [e.g., *Geukensia demissa* (Baginski and Pierce, 1977)], and is therefore also likely here. Under lowered salinity, echinoids reduce their intracellular organic osmolyte pool, which primarily consists of the amino acid glycine (Podbielski et al., 2022b). Therefore, the reduction of available essential amino acids may impact the ability of cells to perform other anabolic processes, such as protein synthesis and may have contributed to the decline of physiological functioning as observed here under low salinity. The physiological and behavioural data demonstrate that urchins were not able to acclimate to the lowest salinity (21‰), indicating that the lower limit for acclimation is between 26‰ and 21‰.

2.5.6 Ecological context

The urchins used in this study originated in Loch Linnhe. Here, estuarine circulations are created when high freshwater input, fed by multiple river systems and terrestrial run-off, mix with incoming saltwater (Rabe and Hindson, 2017). This results in significant stratification, with fresher surface layers flowing seaward, and deeper, saltier layers flowing into the loch (Rabe and Hindson, 2017). Data from the Upper Loch show salinity is most variable in the top ~5 m in Spring, but by Autumn when rainfall is high, strong salinity gradients are present throughout the top 20 m (e.g., minimum surface salinity was 28‰ and 31‰ in May, and 15‰ and 23‰ in October 2011 and 2012 respectively) (Rabe and Hindson, 2017). With knowledge of the salinity tolerance data from the current study and the salinity gradients recorded in Loch Linnhe, it is perhaps unsurprising that *E. esculentus* is known to favour depths below 15m (Comely and Ansell, 1988). This suggests that salinity likely plays a significant role in shaping *E. esculentus* distribution patterns. Therefore, shallow lochs fed by high freshwater inputs are unlikely to be favourable to *E. esculentus* at present and in the future, especially if the rate of extreme freshwater events increase in the UK as a result of changing weather patterns (Adaptation Scotland, 2021). A change in *E. esculentus* distribution may have a cascading effect on the numerous macroalgal species upon which they graze, as previously observed (Bekkby et al., 2015; Jones and Kain, 1967).

2.5.7 Future directions and conclusions

The *E. esculentus* urchins used in this study represent smaller and younger adults, as older individuals can have test diameters up to 150-160 mm (Mortensen, 1927). Echinoid body size is certainly relevant in relation to tolerating reduced salinity (Castellano et al., 2017), however, previous authors have recognised that size is not the only factor defining salinity tolerance and buffering capabilities in echinoderms (Freire et al., 2011a; Santos et al., 2013; Vidolin et al., 2007). Indeed, Stickle and Denoux (1976) note that smaller specimens of *S. droebachiensis* were more tolerant of low salinity than larger specimens, while Vidolin et al (2007) demonstrated that the osmotic and ionic gradients established under reduced salinity were independent of surface area to volume ratio in *L. variegatus*. However, considering the very large size of fully grown *E. esculentus*, it would be of interest to establish how size influences its salinity tolerance.

Controlled salinity acclimation experiments differ from the natural acclimatisation process that *E. esculentus* may exhibit in the wild. Salinity fluctuates on a daily timescale due to tidal changes and weather patterns, while longer term reductions in salinity as a response to changing weather patterns will likely be gradual. What acclimation experiments do offer, is a prediction of how *E. esculentus* may respond to salinity stress *in situ*; however, the current study is unable to determine the long-term success of *E. esculentus* under repeated periods of salinity stress. The impact of a period of extreme stress can be severely detrimental to the long-term health of urchin populations, as vital energy resources can be diverted away from reproduction and growth (Santos et al., 2022). Conversely, successful acclimation to mild stressors can prove beneficial for reproductive success and larval outcomes in echinoids (as shown by Suckling et al., 2015). Furthermore, early life stages are considered more sensitive to environmental stressors than in adults urchins (Bressan et al., 1995). Indeed, even small reductions in salinity have proved to be highly damaging to urchin embryos (Coward et al., 2009). Future research on the impact of periodical low salinity stress on reproduction and growth in *E. esculentus* and other echinoderms would now be valuable. Studies investigating low salinity tolerance and acclimation across a full life cycle are needed to allow predictions of future resilience to change.

The mechanisms underpinning why some echinoderm species have greater euryhaline tolerance and acclimation ability than others, is still largely unknown. Progress has been made

with metabolomic approaches in identifying osmolytes used in cellular osmoregulation as part of the acclimation process (Podbielski et al., 2022b). However, there appear to be no transcriptomic analysis for echinoderms in the context of osmotic stress. This is surprising as such studies exist for many other marine invertebrates in identifying genetic responses to osmotic stress (e.g., Meng et al., 2013; Lou et al., 2019; Barrett et al., 2022). A more recent development in environmental research is the integration of omics techniques to offer a multi-omics approach (e.g., Ichihashi et al., 2020; Bakker et al., 2023). This has the potential to identify causal links between physiological fitness parameters and their underlying genetic and biochemical signatures and would offer a holistic understanding on the nature of osmotic acclimation and tolerance in echinoderms.

In summary, the current study has demonstrated that adult *E. esculentus* can acclimate to lowered salinity with beneficial consequences to further hypoosmotic challenges. However, at the lower threshold of the acute tolerance range (21‰), chronic exposure has severe detrimental impacts on physiological functioning and activity, although innate immune cell composition and concentration were unaffected. Therefore, 21‰ salinity is likely beyond the phenotypic plasticity limit of *E. esculentus*, and continued exposure may lead to mortality. For *E. esculentus* inhabiting coastal areas prone to freshening, its resilient properties will likely enable long-term survival at salinity levels of around 26‰; however lower salinities are likely to represent a barrier to their distribution and survival in the face of extreme climate change, which in turn, may also impact their macroalgae food source.

Chapter 3 – The impact of acute low salinity stress on Antarctic echinoderms

A version of this chapter has been published in *Proceedings of the Royal Society B* as:

Barrett, N. J., Harper, E. M., and Peck, L. S. (2024). The impact of acute low salinity stress on Antarctic echinoderms. *Proc. R. Soc. B Biol. Sci.* 291, 20241038.

<https://doi.org/10.1098/rspb.2024.1038>

The aim of this chapter was to evaluate the response to hypoosmotic shock on a range of Antarctic echinoderms utilising similar methodology developed in Chapter 2. These data also provide species lower tolerance limits that could inform the range of experimental salinities in Chapter 4. I was the primary investigator and contributor to all aspects of this research, including conceptualisation, investigation, formal analysis, writing the original draft and reviewing and editing. All above authors provided supervision and contributed to editing and reviewing.

The data from this chapter is publicly available here: https://github.com/njb202/Chapter_3

3.1 Abstract

Climate change is causing increased coastal freshening in Antarctica, leading to reduced salinity. For Antarctica's endemic echinoderms, adapted to the stable polar environment, the impact of rapid reductions in coastal salinity on physiology and behaviour is currently unknown. Six common Antarctic echinoderms (the sea urchin *Sterechinus neumayeri*; the sea star *Odontaster validus*; the brittle star *Ophionotus victoriae*, and three sea cucumbers *Cucumaria georgiana*, *Echinopsolus charcoti* and *Heterocucumis steineni*), were directly transferred from ambient salinity (34.5‰) to a range of salinity dilutions (29‰ to 9‰) for 24-hours. All species showed reduced activity and the establishment of a temporary osmotic gradient between coelomic fluid and external seawater. Most species exhibited a depression in oxygen consumption across tolerated salinities; however, at very low salinities that later

resulted in mortality, oxygen consumption increased to levels comparable to those at ambient. Low salinity tolerance varied substantially between species, with *O. victoriae* being the least tolerant (24-hour LC_{50} [lethal for 50% of animals]=19.9‰) while *E. charcoti* and *C. georgiana* demonstrated the greatest tolerance (24-hour LC_{50} =11.5‰). These findings demonstrate the species-specific response of Antarctica's endemic echinoderms to short-term hypoosmotic salinity events, providing valuable insight into this phylum's ability to respond to an underreported impact of climate change.

3.2 Introduction

Globally, ecosystems are expected to face widespread impacts due to climate change. In the oceans, the effects of warming and ocean acidification on biodiversity have received considerable attention (Clark et al., 2017; Kroeker et al., 2013; Peck et al., 2009b). However, the impact of freshening (the lowering of seawater salinity) leading to osmotic stress in marine organisms has received less focus. Dramatic freshening events are increasing globally as a result of climate change (Röthig et al., 2023), and in polar regions where there is significant freshwater locked up as ice, warming has the potential to release large amounts into nearshore ecosystems. The Antarctic icesheet, which holds ~70% of global freshwater, is projected to lose mass at an increasing rate throughout the 21st century due to climate change (IPCC, 2022b). Warming in West Antarctica and the Western Antarctic Peninsula, has already led to increased freshwater influx into the Southern Ocean, resulting in coastal freshening (Hellmer et al., 2011; Hobbs et al., 2021; Jacobs and Giulivi, 2010). The coastline here has extensive fjordic systems which are currently rich in biodiversity and benthic abundance (Grange and Smith, 2013). However, there is concern that as marine-terminating glaciers retreat due to warming, the formation of distinct salinity stratification in the water column (Bianchi et al., 2020), may threaten these biodiversity hotspots (Grange and Smith, 2013). Antarctic fjords along the Western Antarctic Peninsula are considered to be at an earlier stage of climate warming than Arctic fjords (Grange and Smith, 2013). In Greenland, inner fjord salinities can regularly persist below 25‰ down to approximately 10 meters in the summer (Sejr et al., 2017), potentially forecasting similar conditions for Antarctic fjords as climate warming progresses. More acutely, the frequency of intense hyposalinity events—where

large volumes of freshwater rapidly enter the marine environment—is increasing due to climate change (IPCC, 2022b; Röthig et al., 2023). For marine animals, salinity is a major environmental variable that impacts their development and survival (Kinne, 1964). Reductions in seawater salinity as a result of freshening can exert stress on marine animals by increasing the cost of osmoregulation (Sokolova et al., 2012a) and also cause direct cellular damage (Jäckle et al., 2001).

Echinoderms are a marine phylum and are generally considered stenohaline with limited control over their extracellular body fluid in response to changes in environmental salinity (Hyman, 1955; Russell, 2013; Stickle and Diehl, 1987). This is thought to be due to the high permeability of their body wall epithelia and the absence of a specific excretory organ (Binyon, 1972; Hyman, 1955; Santos-Gouvea and Freire, 2007). However, many echinoderm species live in intertidal habitats where salinity is highly variable, while numerous species are established in brackish and estuarine environments (Russell, 2013; Stickle and Diehl, 1987; Turner, 2007). Experimental studies have shown that many echinoderms across all classes (with only one example from crinoids) can tolerate short term, acute reductions in salinity, with some displaying remarkable tolerance (Russell, 2013; Stickle and Diehl, 1987). For example, (Barker and Russell, 2008) demonstrated that the sea star *Patiriella regularis* was able to survive for 4 day at 0‰. This approach by Barker and Russell (2008) and others, examines the hypoosmotic shock response to lower salinity for periods typically ranging from 12 to 96 hours. This method replicates the rapid salinity changes that animals may experience in environments such as fjord haloclines or during sudden precipitation changes in intertidal zones (Barker and Russell, 2008; Held and Harley, 2009; Vidolin et al., 2007). In polar regions, these events can be extreme, with meltwater from glaciers and ice shelves rapidly introducing substantial amounts of freshwater into intertidal zones which can persist for extended periods (Davenport, 2001). Here, salinity levels can drop suddenly, occasionally exposing slow-moving benthic invertebrates to pure freshwater (Davenport, 2001; Stockton, 1984). Responses to hypoosmotic shock differ from the gradual cellular adjustments that occur as part of the acclimation process when exposed to low salinity over longer periods, such as weeks to months (Chapter 2; Barrett et al., 2024a; Khlebovich, 2017; Rivera-Ingraham and Lignot, 2017). Numerous studies have demonstrated successful acclimation to reduced salinities over longer durations in echinoderms, however Antarctic species are yet to be examined (Chapter 2; Barrett et al., 2024a; Sabourin and Stickle, 1981; Turner, 2007).

Mechanisms that enable short-term tolerance to reduced salinity in echinoderms may be facilitated through the establishment of transient ionic and osmotic gradients between the extracellular coelomic fluid and the external seawater. These can persist for several hours (between 12–24 hours; Diehl, 1986) offering protection for internal tissue which would otherwise rapidly swell on encountering reduced salinity seawater (Chapter 2; Barrett et al., 2024a; Freire et al., 2011a; Santos et al., 2013). Additionally, some echinoderms appear capable of moderate cell volume or tissue water regulation after hypoosmotic shock, minimising swelling that would occur without regulation (Santos et al., 2013).

Many marine animals are endemic to Antarctica, having adapted to the cold, but stable and predictable environmental conditions of the Southern Ocean (Peck, 2018). Changes in glacial cycles have facilitated periods of high species dispersal around Antarctica, followed by prolonged periods of species isolation in refugia during peak glacial periods. This phenomenon has resulted in a high diversity of colonising founder species, which have later undergone intra-regional speciation aided by the isolation (O’Loughlin et al., 2011). This, in addition to a lack of shell-crushing predators (Aronson and Blake, 2001), is thought to have contributed to Antarctica becoming exceptionally rich in echinoderm diversity (O’Loughlin et al., 2011) where they represent around 10% of the benthic fauna (Clarke and Johnston, 2003). However, the prolonged stability in the Antarctic marine environment may have contributed to a reduced ability for marine species to tolerate rapid change. Their capacity to acclimate to warming temperatures is already known to be poor, while those that can take substantially longer compared to temperate or tropical species (Peck et al., 2014). The ability to tolerate reduced salinities across Antarctic species is less well known. Studies on fish, such as *Harpagifer antarcticus* demonstrate tolerance to modest drops in salinity, but lower salinities result in osmotic imbalances and an inability to maintain homeostasis (Vargas-Chacoff et al., 2021a), dramatically amplified when combined with increased temperature (Vargas-Chacoff et al., 2021b). The limpet, *Nacella concinna* demonstrate limited acute tolerance to low salinity (Davenport and Macalister, 1996) and stenohaline characteristics in regards to acclimation to low salinities (Navarro et al., 2020). Very small reductions in salinity (34 to 30‰) impair behaviour functions critical to long term survival in the isopod *Serolis polita* (Janecki et al., 2010). Multiple studies have demonstrated that low salinity can be a significant modifier of fitness when combined with increased temperature or reduced pH (Martínez et al., 2021; Navarro et al., 2020; Park et al., 2020). For Antarctic echinoderms, there is very little

knowledge of their ability to tolerate reduced salinities, with only two studies to date. Pearse, (1967) demonstrated the common Antarctic asteroid, *Odontaster validus* was able to maintain body volume on immersion in 50‰ seawater, suggesting a degree of control over coelomic fluids that would be beneficial on exposure to summer meltwater. Cowart et al. (2009) demonstrated that embryos of the Antarctic echinoid, *Sterechinus neumayeri*, were highly sensitive to small reductions in salinity (a decrease from 34‰ to 30‰ resulted in 99% mortality), suggesting early life stages of Antarctic echinoderms may be extremely vulnerable to coastal freshening. As the majority of Antarctic echinoderms are considered subtidal, and ice restricts the colonisation of shallow sites (Peck, 2018), it may be expected that either they will not experience such dramatic shifts in salinity levels, or will migrate to deeper, higher salinity waters (e.g., Park et al., 2020). However, changes in community structure as a result of species migrations away from low salinity environments may have cascading effects on the entire Antarctic food web (Sporta Caputi et al., 2020).

Considering the rapid climate changes underway in Antarctica, it is crucial to evaluate the vulnerability of echinoderms to the impact of freshening events and reduced salinity. This assessment is especially pertinent as species such as *S. neumayeri* and *O. validus* are considered keystone species in Antarctic food webs (McClintock et al., 2008a; Sporta Caputi et al., 2020), and Antarctic holothurians account for approximately ~10% of global holothurian diversity (O’Loughlin et al., 2011). In this study, six echinoderms common to the shallow coastal shelf, the echinoid *S. neumayeri*, the asteroid, *O. validus*, the ophiuroid, *Ophionotus victoriae*, and three holothurians, *Heterocucumis steineni*, *Cucumaria georgiana* and *Echinopsolus charcoti*, were collected from Ryder Bay, on the Antarctic Peninsula. Subtidal salinity here is relatively stable [33.4 ± 0.4 ‰ at 15m (mean $\pm$ s.d.)], although surface salinity occasionally drops to ~28.5‰ (Clarke et al., 2022). However, two of these species (*S. neumayeri* and *O. validus*) are occasionally found in the low intertidal (although more common from ~6m depth (Brockington, 2001; Pearse, 2013; Waller et al., 2006a)), where salinities can drop to <12‰ (Davenport, 2001; Stockton, 1984; Vargas-Chacoff et al., 2021a; Waller et al., 2006a). In this regard, it is hypothesised that these two species would have greater acute hyposalinity tolerance than the four species found only subtidally. All animals were assessed for short-term tolerance to reduced salinities over a 24-hour period. Metrics including mortality, oxygen consumption, activity, and osmolality of coelomic fluid were measured to evaluate their response.

3.3 Methods

3.3.1 Experimental animals

All animals were collected in the austral summers of 2020/2021 and 2021/2022 by SCUBA divers at depths of 10–20m near the British Antarctic Survey's (BAS) Rothera research station, Adelaide Island (67°34'07''S, 68°07'30''W). Salinity and temperature (mean±s.e.m.) at 15m depth near the collection site were 33.56±0.06‰ (33.27–33.81‰; $n=13$) and -0.51±0.16°C (-1.28–0.59°C; $n=13$) November 2020 to February 2021, and 33.52±0.05‰ (33.01–33.83‰; $n=20$) and -0.51±0.17°C (-1.58–1.24°C; $n=20$) November 2021 to February 2022 (Clarke et al., 2022). After collection, they were transferred to the Rothera aquarium, then transported to the UK via a temperature-controlled containerised aquarium. Once in the UK, they were transferred to the polar aquarium at BAS Cambridge and held in 280 L tanks in a recirculating aquarium incorporating macro- and biological filtration systems, using seawater from the UK coast. The aquarium was maintained at -0.3 ±0.2°C and 34.5‰, with a 12-hour light/dark photoperiod for 6 to 12 months prior to experiments. *S. neumayeri*, *O. validus* and *O. victoriae*, were fed weekly on a frozen krill diet, while the three holothurians were fed weekly on a ground flake diet (Vitalis, Mixed Reef Food). Prior to all experiments, food was restricted for at least 48 hours. All animals were maintained and handled per UK animal welfare regulations.

3.3.2 Experimental set-up

Six salinity treatments were used to assess echinoderm salinity tolerance to an acute stress over a 24-hour period: 34.5‰ (control), 29‰, 24‰, 19‰, 14‰ and 9‰. Animals were subjected to incrementally lower salinity treatments until the lower lethal concentration for 50% (LC₅₀) of the population was reached; 9‰ was not assessed in *S. neumayeri* and *O. victoriae*. Numbers of animals used for each experimental salinity were, 10 each for *S. neumayeri* (total $n=50$; test diameter 20–34 mm), *O. validus* (total $n=60$; arm radius from the tip of arm to the centre of mouth 36–44 mm) and *O. victoriae* ($n=50$; central disc diameter

16–31 mm), and 8 for *H. steineni* (total $n=48$; length 70–200 mm), *E. charcoti* (total $n=48$; length 40–130 mm) and *C. georgiana* (total $n=48$; length 10–46 mm). Numbers used varied based on the tolerances and total number of individuals available for each species. Within each species, animals were randomly distributed between salinity treatments. Each experimental trial was conducted in a single 30 or 50-L tank fitted with a nano protein skimmer (REEF-Skim Nano 100AC, TMC Ltd) and bio-filter (ZB-150, Ziss) with an attached airline. Distilled water was mixed with filtered natural seawater until experimental salinity levels were achieved. Tank water was maintained at $-0.3\pm0.5^{\circ}\text{C}$. Salinity was measured using a conductivity probe (CDC40101, Hach). For each treatment, oxygen consumption, activity, coelomic fluid osmolality (at 6 and 24 hours) and mortality were assessed. After 24 hours in experimental salinities, animals were immediately moved to an ambient (34.5‰) seawater tank for observation over a minimum 5-day recovery period.

3.3.3 Mortality

Following the 24-hour exposure and minimum of five days recovery in ambient salinity (34.5‰), animals were examined for signs of life (observed movement of tube feet/spines/tentacles) and tested for an activity response (ability to right in *S. neumayeri*, *O. validus* and *O. victoriae*, peristaltic movement in the three holothurians) over several hours using video recording. Animals that showed no signs of life were considered dead.

3.3.4 Oxygen consumption

Oxygen consumption was measured using closed chamber techniques as described in Chapter 2 (Barrett et al., 2024a). Briefly, individual animals ($n=5-8$ per treatment) were immersed directly into experimental salinity tanks and placed within open respirometry chambers (ranging from 80 to 500ml depending on the size of the animal). Animals were left for one hour prior to measurements. Once chambers were sealed, oxygen concentration was measured using a Fibox-4 oxygen sensor (PreSens, Germany). After 4-6 hours a second measurement was taken. Three empty chambers were measured to account for background oxygen changes. All experiments were carried out at mid-morning to minimise any circadian rhythm effects. Individual wet mass ($\pm0.01\text{g}$) and volume ($\pm0.01\text{g}$) were recorded for each

animal. To obtain ash-free dry mass (AFDM; $\pm 0.01\text{g}$), all animals were euthanised by freezing at -20°C and dried in a convection oven at 60°C to constant mass ($\pm 0.01\text{g}$). Dried animals were transferred to a muffle furnace and heated to 475°C for 6 hours. AFDM was obtained by subtraction and used to estimate the mass of respiring organic tissue.

3.3.5 Activity

The righting response in echinoids, asteroids and ophiuroids is a widely used metric for assessing their response to environmental stress (Lawrence and Cowell, 1996; Stickle and Diehl, 1987). The time taken for animals ($n=5$ per salinity treatment) to fully right themselves after 6 hours in experimental salinities was recorded and converted to an Activity Coefficient ($\text{AC} = 1000/\text{righting time in seconds}$) (after Lawrence and Cowell, 1996). Maximum time allocated was 60 minutes for *S. neumayeri* and *O. validus*, which equals a minimum AC value of 0.28 ($1000/3600$ seconds), while for *O. victoriae* (which can right in a few seconds) 15 minutes were allocated, equalling a minimum AC value of 1.11 ($1000/900$ seconds).

Two of the three holothurian species (*H. steineni* and *C. georgiana*) in the current study exhibit external pentaradial symmetry, displaying five equally spaced ambulacra with tube feet (Hyman, 1955). This made righting difficult to assess, necessitating a novel activity assessment. In all three species, after a minor disturbance (e.g., emersion and immersion), movement commenced via peristaltic locomotory waves, as recorded in other holothurians (Heffernan and Wainwright, 1974). Each peristaltic wave begins at the animal's posterior and moves to the anterior. A pre-experimental trial was used to assess whether the number of peristaltic waves within one hour was a repeatable behaviour following emersion and immersion in *H. steineni* and *C. georgiana*. Behavioural repeatability measures variation among individuals vs. variation among measurements within individuals (as per Wolak et al., 2012). Six animals of each species were isolated in floating trays, and each animal was alternately emersed and immersed. Number of peristaltic waves observed within 60 minutes was recorded. Each trial was repeated in succession (three in *C. georgiana* and four in *H. steineni*). A ~20 minutes gap was given between commencing the next trial, comparable to previous studies on echinoderm righting repeatability (Clements et al., 2020; Gutowsky et al., 2016). The intraclass correlation coefficient (ICC) or R (repeatability) values were calculated as,

$$ICC \text{ or } R = \frac{S_A^2}{S_A^2 + S_W^2}$$

where S_A^2 is the variance among groups and S_W^2 is the variance within groups. Data were obtained using the R package ICC (Wolak et al., 2012). The threshold for a repeatable behaviour is commonly taken to be $ICC \geq 0.37$ (Bell et al., 2009; Clements et al., 2020; Gutowsky et al., 2016). *Heterocucumis steineni* produced an $ICC=0.6$ (95% CI=0.21–0.92), with the individual number of waves ranging from 2 to 6 (mean $\pm$ s.e.m.=3.5 $\pm$ 0.2). *Echinopsolus charcoti* produced an $ICC=0.87$ (95% CI=0.60–0.99), with the individual number of waves ranging from 1 to 5 (mean $\pm$ s.e.m.=3.1 $\pm$ 0.3). Upon establishing that this was a repeatable behaviour in *H. steineni* and *C. georgiana* the following protocol was utilised in all three holothurian species. After 6 hours in experimental salinities (*H. steineni* and *E. charcoti*: $n=8$ per treatment; *C. georgiana*: $n=6$ per treatment), each animal was individually removed from the tank and held in the air for ~5 seconds before re-immersing within the same salinity, and the number of peristaltic waves observed within 60 minutes was recorded. A photograph was taken every 30 seconds for detailed assessment. If a wave began at the end of 60 minutes, it was included as a whole wave to ensure count data were recorded as integers for later analysis.

3.3.6 Coelomic fluid osmolality

Coelomic fluid samples were taken by non-lethal extraction to assess osmolality at 6 and 24 hours after immersion in each experimental salinity, utilising a different set of animals at each timepoint (except in *C. georgiana* at 9‰ after 24 hours where samples were not collected). Numbers of animal replicates at each salinity were $n=3$ at 6 h and $n=7-8$ at 24 h for *S. neumayeri*, $n=3-5$ at 6 h and $n=5-6$ at 24 h for *O. validus*, $n=3$ at 6 and 24 h for *H. steineni*, *E. charcoti* and *C. georgiana* (except 9‰ for *C. georgiana* at 6 h, $n=2$). A syringe with a 21-gauge needle was inserted into the coelomic cavity and ~0.5ml of coelomic fluid extracted (as per Chapter 2; Barrett et al., 2024a). Triplicate tank water samples were collected at each sample point and a mean seawater osmolality obtained for use in one-way t-test comparisons to

coelomic fluid. The osmolality of the coelomic fluid and seawater was measured on a Vapro 5600 vapour pressure osmometer (ELITech Group).

3.3.7 Statistics

One-way analysis of variance (ANOVA) tests (including Welch's ANOVA for data with unequal variances) were performed to compare oxygen consumption, osmolality data and activity responses (except for the holothurians) between salinity treatments. Assumptions of normality and equal variance were tested using the Shapiro-Wilk test and Levene's test respectively. When assumptions of normality were violated, data were log transformed. When violations of normality persisted, non-parametric Kruskal-Wallis tests were implemented. Significant ANOVA/Kruskal-Wallis were further examined using post-hoc tests to identify variations among treatments. Tukey tests were used for normally distributed data, Dunn's test for non-parametric data and Games-Howell tests for data with unequal variance. For assessing the number of peristaltic waves per hour in the holothurians, salinity was treated as a continuous variable and a Poisson model fitted to assess significance, with a chi-squared test to assess goodness of fit. If high overdispersion was observed, a quasi-Poisson model was attempted. To assess significance between salinity treatments, two sample Poisson tests were carried out between all salinity treatment pairings with Bonferroni-adjusted P values. The comparison between coelomic fluid values and the mean osmolality of tank water was conducted using one-sample t -tests. Second order regressions were fitted to all 6- and 24-hour osmolality data as this consistently produced lower AIC values and higher r^2 values when compared to a linear model, in addition to the clear non-linear arrangement of the 6-hour data. However, additional linear models were fitted to the 24-hour data to assess the degree of osmoconforming. LC_{50} values were calculated by fitting a linear model to the mortality data across salinities from the point prior to where mortality first occurred and where mortality reached 100%. Statistical tests were considered significant if $P < 0.05$. All analysis were carried out in R (version 4.2.3).

3.4 Results

3.4.1 Mortality

After 24 hours in experimental salinities, mortalities were recorded at $\leq 19\text{‰}$ in *O. victoriae*, at $\leq 14\text{‰}$ in *S. neumayeri*, *O. validus* and *H. steineni*, and 9‰ for *E. charcoti* and *C. georgiana* (Table B1). For *O. victoriae*, *S. neumayeri* and *H. steineni*, mortalities were observed directly after the 24-hour period with no animals showing any signs of recovering when placed back in ambient salinity (34.5‰). For *O. validus*, some individuals survived for up to 7 days in ambient salinity, including a single animal at 9‰ . For *E. charcoti* and *C. georgiana*, all animals survived for at least 7 days after the 9‰ trial before dying, with one individual of *E. charcoti* surviving for 14 days before dying.

The calculated LC_{50} value is 19.9‰ for *O. victoriae*, 16.5‰ for *S. neumayeri*, 13.4‰ for *H. steineni* and *O. validus* and 11.5‰ for *E. charcoti* and *C. georgiana* (Table B1).

3.4.2 Oxygen consumption

Salinity had a significant impact on oxygen consumption in *O. validus* (One-way ANOVA: $F_{(5,25)}=9.1$, $P<0.0001$), *C. georgiana* (One-way ANOVA: $F_{(5,29)}=9.2$, $P<0.0001$), *H. steineni* (One-way ANOVA: $F_{(5,40)}=6.2$, $P<0.001$) and *E. charcoti* (One-way ANOVA: $F_{(5,40)}=3.7$, $P=0.008$) (Figure 3.1; Table B2). For *O. victoriae* there was no significant impact on oxygen consumption when all five treatments were included, however on removal of the lowest two salinity treatment (14‰ and 19‰), there was a significant decline in oxygen consumption between 34.5‰ and 24‰ (One-way ANOVA: $F_{(2,12)}=7.3$, $P=0.008$; Tukey 34.5‰ v 24‰ , $P=0.007$). Salinity had no significant impact on oxygen consumption in *S. neumayeri*. Post hoc testing demonstrated that oxygen consumption was significantly lower compared to animals in controls at salinities 24‰ , 19‰ and 14‰ in *O. validus*, at 24‰ and 19‰ in *H. steineni*, at 14‰ and 19‰ in *C. georgiana*. At very low salinities, oxygen consumption was significantly higher at 9‰ compared to 14‰ , 19‰ and 24‰ in *O. validus*, at 9‰ compared to 19‰ , and at 14‰ compared to 19‰ and 24‰ in *E. charcoti*, and at 14‰ compared to 19‰ in *H. steineni* (Table B2). Across all species, there were no significant differences in oxygen

consumption between the lowest salinity treatment (14‰ in *S. neumayeri* and *O. victoriae*; 9‰ for all other species) and the control (Table B2).

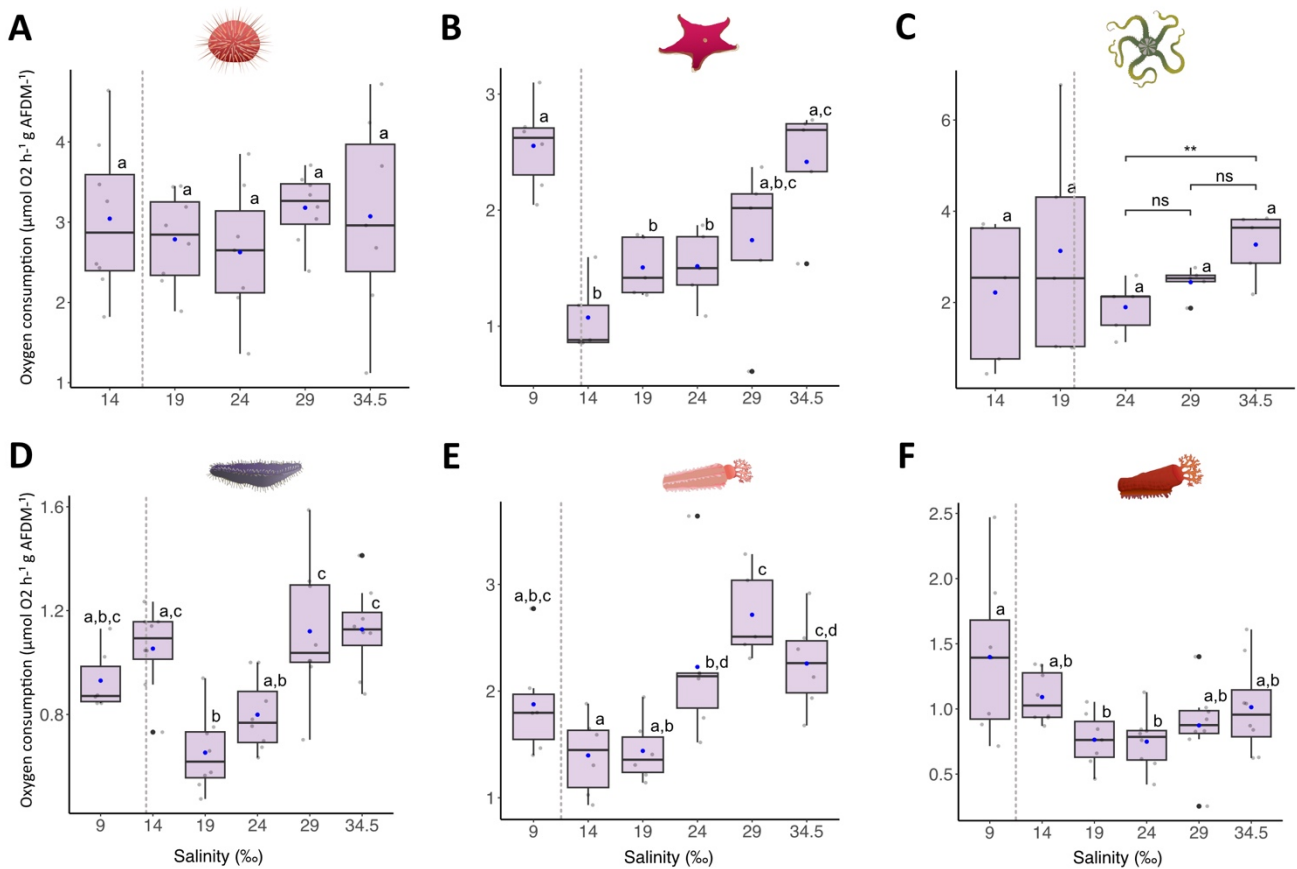


Figure 3.1| Oxygen consumption measured over the first 5-7 hours after transfer from ambient salinity (34.5‰) in 6 Antarctic echinoderms; (A) *Sterechinus neumayeri*, (B) *Odontaster validus*, (C) *Ophionotus victoriae*, (D) *Heterocucumis steineni*, (E) *Cucumaria georgiana*, (F) *Echinopsolus charcoti*. Results are shown for one-way ANOVA and *post hoc* Tukey test, with different letters indicating significant differences [$P < 0.05$; $n = 7-8$ (A), $n = 5$ (B,C), $n = 8$ (D,F), $n = 5-6$ (E) biological replicates for each treatment] between treatments. Bars on C show results between 24‰–34.5‰ only (n.s., not significant; $^{**}P < 0.01$). Box plots show medians (black line), upper and lower quartiles, and maximum and minimum (whiskers). Biological replicates are represented by grey circles, means by blue circles and outliers by filled black circles. AFDM, ash-free dry mass. Grey dashed line represents the calculated LC₅₀ value.

3.4.3 Activity

Salinity had a significant impact on activity values across all species (*S. neumayeri*: Kruskal-Wallis $\chi^2_4 = 14.58$, $P < 0.01$; *O. validus*: Kruskal-Wallis $\chi^2_5 = 26.29$, $P < 0.0001$; *O. victoriae*: Kruskal-Wallis $\chi^2_4 = 21.27$, $P < 0.001$; *C. georgiana*, *E. charcoti* and *H. steineni*: all Poisson $P < 0.0001$) (Figure 3.2, Table B2).

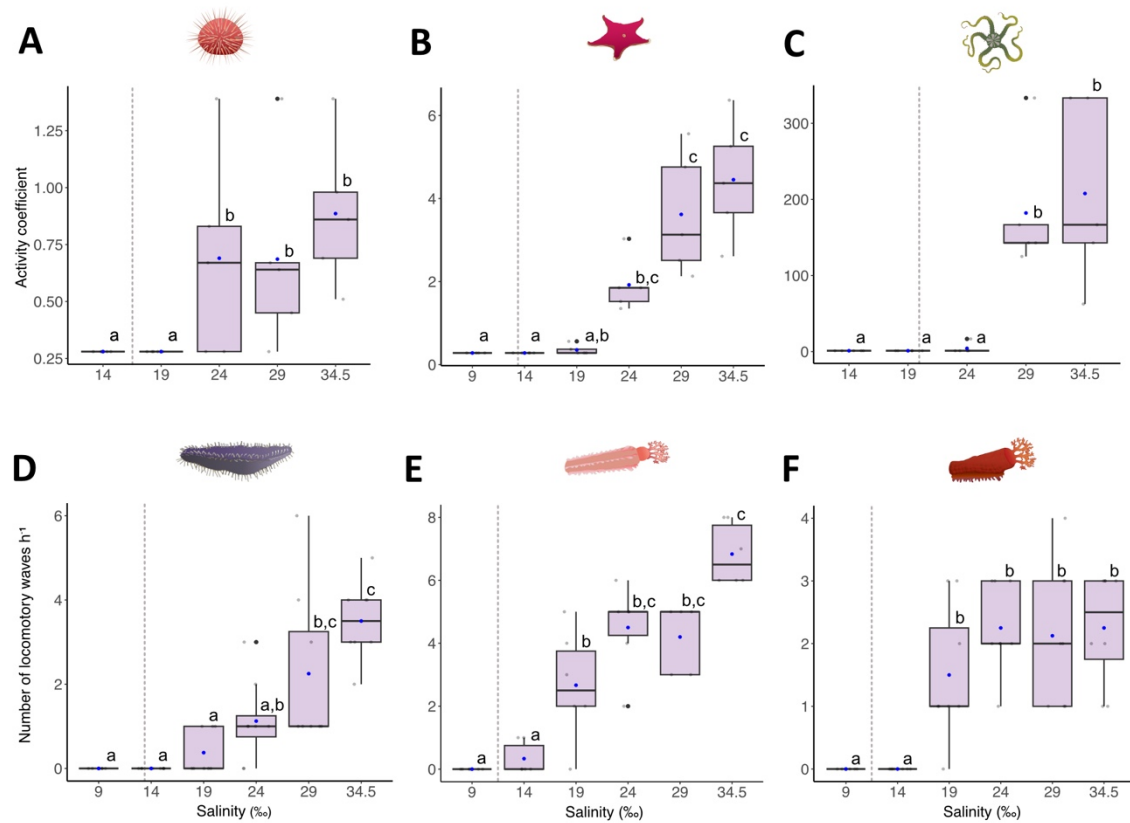


Figure 3.2 | Activity rates 6 hours after transfer from ambient salinity (34.5‰) into low salinity treatments in 6 Antarctic echinoderms; (A) *Sterechinus neumayeri*, (B) *Odontaster validus*, (C) *Ophionotus victoriae*, (D) *Heterocucumis steineni*, (E) *Cucumaria georgiana*, (F) *Echinopsolus charcoti*. (A–C) Righting ability was converted to an activity coefficient, calculated as $AC = 1000 / \text{righting time in seconds}$; a smaller AC value indicates a longer righting time. (D–F) Number of peristaltic locomotory waves recorded over 1 hour. Results are shown for Kruskal–Wallis and *post hoc* Dunn’s test (A–C), and two-sample Poisson test (D–F) with different letters indicating significant differences [$P < 0.05$; $n = 5$ (A–C), $n = 6$ (E), $n = 8$ (D, F) biological replicates for each treatment]. Box plots show medians (black line), upper and lower quartiles, and maximum and minimum (whiskers). Biological replicates are represented by grey circles, means by blue circles and outliers by filled black circles. Grey dashed line represents the calculated LC₅₀ value.

Mean AC values were significantly different from the control salinity (34.5‰) at $\leq 19\%$ in *S. neumayeri* and *O. validus*, and $\leq 24\%$ in *O. victoriae*. For the holothurians, the number of locomotory waves recorded in one hour was significantly different from the control salinity (34.5‰) at $\leq 24\%$ in *H. steineni*, $\leq 19\%$ in *C. georgiana* and $\leq 14\%$ in *E. charcoti* (Figure 3.2, Table B2).

3.4.4 Coelomic fluid osmolality

Coelomic fluid osmolality was significantly affected by salinity in each echinoderm species after 6 hours (all $P < 0.01$) and after 24 hours immersion (all $P < 0.0001$) (see Table B3).

Coelomic fluid osmolality was either isosmotic or hyperosmotic to the tank water under all salinities in each species (Figure 3.3). Mean osmolality gradients (difference between coelomic fluid and external tank sea water) after 6 hours in salinity treatments were significant at salinities $\leq 29\text{‰}$ in *S. neumayeri*, *E. charcoti* and *C. georgiana* (except treatment 24‰ and 9‰ in *C. georgiana* which were not significant), at $\leq 24\text{‰}$ in *O. victoriae*, at $\leq 19\text{‰}$ for *H. steineni* and 14‰ only for *O. validus* (Figure 3.3; Table B3; see Table B4 for mean osmolality gradients). The most osmoconforming species at 6 hours was *O. validus*, which did not have significant gradients between the coelomic fluid and tank water except at 14‰ (Figure 3.3; Table B3, B4). After 24 hours, mean osmolality gradients were significant at $\leq 24\text{‰}$ in *S. neumayeri*, *O. validus*, *O. victoriae* and *E. charcoti* and at $\leq 19\text{‰}$ in *C. georgiana* and at 19‰ only in *H. steineni* (Figure 3.3; Table B3, B4).

After 24 hours at 14‰ (the lowest measured treatment where species survived) *E. charcoti* had the largest mean osmolality gradient compared to other species (244 ± 1 mOsm/kg) which was significantly different from all other species, excluding *O. victoriae* where data were absent (Tukey test: all *E. charcoti* comparisons $P < 0.05$) (Table B3).

Linear regressions of the 24-hour data were used to assess the most osmoconforming species (all had r^2 values ≥ 0.96) (Figure B1). *C. georgiana* was the most osmoconforming species with the closest slope value to 1 ($b_1 = 0.93$) and the lowest intercept ($b_0 = 73$) of all species. The most osmoconforming species to least, were: *C. georgiana* > *O. victoriae* > *O. validus* > *H. steineni* > *S. neumayeri* > *E. charcoti*.

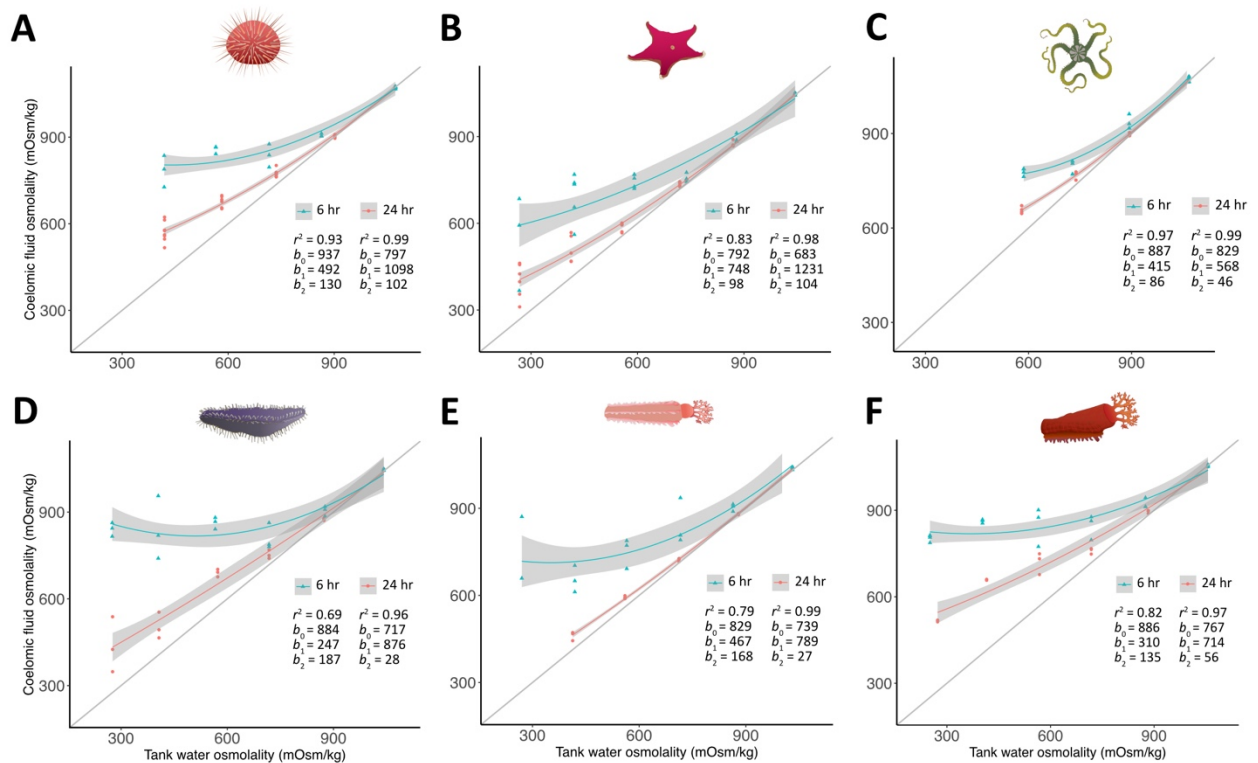


Figure 3.3 | Coelomic fluid osmolality of Antarctic echinoderms at 6 and 24 h after transfer from ambient salinity (34.5‰) versus that of the corresponding tank water; (A) *Sterechinus neumayeri*, (B) *Odontaster validus*, (C) *Ophionotus victoriae*, (D) *Heterocucumis steineni*, (E) *Cucumaria georgiana*, (F) *Echinopsolus charcoti*. Number of animal replicates at each experimental salinity treatment were $n=3$ at 6 h and $n=7-8$ at 24 h for *S. neumayeri*, $n=3-5$ at 6 h and $n=5-6$ at 24 h for *O. validus*, $n=3$ at 6 and 24 h for *H. steineni*, *E. charcoti* and *C. georgiana* (except 9‰ for *C. georgiana* at 6 h, $n=2$). Mean ($\pm$ s.e.m.) tank water osmolality (mOsm kg^{-1}) in each salinity experiment was: 34.5‰= 1051 ± 6 , 29‰= 876 ± 3 , 24‰= 721 ± 2 , 19‰= 576 ± 2 , 14‰= 414 ± 2 and 9‰= 269 ± 3 . A second order polynomial regression line (blue line) and the standard error of the mean (shaded grey area) were fitted to the data. The iso-osmotic line is represented by a grey line. Regression coefficients and r^2 values are included to the right of the isosmotic line.

3.5 Discussion

Short-term low salinity tolerance varied dramatically between the six Antarctic echinoderm species examined. The brittle star, *O. victoriae*, showed the least tolerance to low salinity, with 80% mortality at 19‰ after 24-hours exposure. In contrast, two holothurian species, *E. charcoti* and *C. georgiana* demonstrated 100% survival for at least a week after 24 hours in 9‰ before dying. At lower salinities, all species exhibited reduced activity and the development of a temporary osmotic gradient between coelomic fluid and external seawater. The typical metabolic pattern in all species exhibited a depression in oxygen consumption across tolerated low salinities; however, at extremely low salinities, oxygen consumption

increased to levels similar to those at ambient salinities, and this increase corresponded to critical salinity tolerance limits.

3.5.1 Mortality and LC_{50}

Evaluating the tolerance limit to low salinity has been a prominent feature in echinoderm salinity studies (Russell, 2013; Stickle and Diehl, 1987). This assessment provides a reference point of where the salinity gradient becomes lethal, leading to the organisms demise, while also aiming to determine whether such limits align with the degree of freshening that species may encounter in their natural habitats (Binyon, 1972; Stickle and Diehl, 1987).

In previous studies on asteroids, the record for the lowest tolerated salinity was observed in *Patiriella regularis* which survived at 0‰ for up to 4 days (Barker and Russell, 2008). However, this is considered exceptional and the majority of lower tolerance limits range from 14–27‰ (Russell, 2013). Therefore, *O. validus*, which tolerated $\geq 14‰$, demonstrates a comparatively high tolerance of hypoosmotic stress relative to other species of asteroids.

In a global review of echinoids, Russell (2013) showed lower tolerance limits are in the range of 14–27‰. For example *Echinus esculentus* survived for 25 days at 21‰ and had an estimated 24-hour LC_{50} of 18.5‰ (Chapter 2; Barrett et al., 2024a). The majority of studies have used longer durations to assess LC_{50} , for example Stickle et al. (1990) observed a 28-day LC_{50} of 13‰ and 21‰ for *Strongylocentrotus droebachiensis* and *Strongylocentrotus pallidus* respectively. The results reported here for adult *S. neumayeri* (24-hour LC_{50} =16.5‰) suggest that it has moderate resilience to acute hypoosmotic stress relative to other echinoids, and contrasts with their extremely stenohaline embryonic form where 99% mortality was recorded at 30‰ (Cowart et al., 2009).

The Antarctic holothurians demonstrated the greatest resilience to low salinity out of the four echinoderm classes. However, there was notable interspecies variation. Mortality in *H. steineni* (from $\leq 14‰$) was observed immediately after 24-hour exposure, marked by evident epithelial rupture and pigment loss. Interestingly, *E. charcoti* and *C. georgiana* initially appeared to recover from 9‰, demonstrating full peristaltic movements for at least seven days prior to mortality. Examples of high tolerance to low salinity has been observed in other holothurians, for example *Eupentacta quinquesemita* survived for 30 days at $\sim 12.5‰$

(Sabourin and Stickle, 1981), while other studies report tolerance ranging from 14–26‰ (Russell, 2013; Stickle and Diehl, 1987). In this respect, Antarctic holothurians appear exceptionally tolerant of low salinity relative to other holothurians.

In contrast, the ophiuroid *O. victoriae* was the least euryhaline species examined with a calculated 24-hour $LC_{50} \sim 20‰$. Interestingly, ophiuroids are considered to have the greatest tolerance to low salinity of any echinoderm class (Stickle and Diehl, 1987), with examples of species endemic to brackish water environments [e.g., *Ophiophragmus filograneus* (Turner and Meyer, 1980)]. Indeed, laboratory studies demonstrate that *O. filograneus* is able to survive 6 weeks of continuous exposure to 10‰, with those at 14‰ demonstrating full acclimation (Turner, 2007). Whether *O. victoriae* is capable of acclimating to lower salinity is currently untested, but the current study suggests that it deviates from previous predictions about ophiuroid tolerance (Stickle and Diehl, 1987).

3.5.2 Oxygen consumption

Changes in oxygen consumption in response to external stressors are indicative of the need to either utilise more energy to respond to moderate stress (in the case of an increase) or conserve energy in response to extreme stress (in the case of a decrease) (Sokolova et al., 2012a). In the current study, four out of the six species demonstrated significant reductions in oxygen consumption between 34.5‰ and 19‰ while for *O. victoriae* reductions were between 34.5‰ and 24‰. This suggests that when salinity decreases from ambient levels to the lower end of the animal's tolerance range, it is characterised by metabolic depression—a reduction in energy expenditure and activity in response to extreme stress (Sokolova et al., 2012a). This is likely caused by the large osmotic gradient drawing water into tissue and causing swelling. By reducing physiological activity and diverting energy expenditure away from digestion, growth, reproduction etc, energy resources can be solely allocated to homeostatic maintenance, thus maximising survival chances until favourable osmotic conditions return (Sokolova et al., 2012a). Reductions in oxygen consumption in response to acute hypoosmotic immersion have been observed in other echinoderms, for example in echinoids, including *S. purpuratus* (Giese and Farmanfarmanian, 1963), *S. droebachiensis* (Sabourin and Stickle, 1981) and *E. esculentus* (Chapter 2; Barrett et al., 2024a) and a holothurian, *E. quinquesemita* (Sabourin and Stickle, 1981).

Common to most species was the noticeable sharp increase in metabolism to levels similar to controls when animals were exposed to salinities past the lower end of their tolerance range. This was most evident in *E. charcoti*, *O. validus* and *H. steineni* where there were significant differences in oxygen consumption between the lowest salinity treatments and those preceding them. Furthermore, across all species mean oxygen consumption at the lowest salinity was similar to that at ambient salinity. This indicates that a crucial threshold had been crossed, prompting an uptake in metabolic activity to maximise survival. Mechanisms driving the rise in oxygen consumption at these very low salinities are likely distinct from those at ambient salinity. In ambient conditions, these will comprise the whole organism routine energy demands (e.g., maintenance, growth, activity, reproduction, storage etc) which are expected to remain suppressed under high osmotic stress (Sokolova et al., 2012a). Instead, other processes appear to be upregulated resulting in the net increase in oxygen consumption. These could include processes regulating cell volume adjustment (e.g., the reduction of intracellular free amino acids (Goolish and Burton, 1989), the implementation of cellular stress kinase pathways leading to apoptosis (Jäckle et al., 2001) and even stress responses to mechanical cell lysis due to extreme swelling. Indeed, the LC₅₀ value is closely associated with the salinity treatment where oxygen consumption spikes, suggesting that salinity reduction has passed a critical tipping point.

In the case of *S. neumayeri*, the lack of significant change in metabolic rate was surprising. The absence of a metabolic depression (as observed in all other species examined), may indicate that *S. neumayeri* is well adjusted to low salinity immersion and can rapidly reallocate resources to maintain homeostasis. Indeed, *S. neumayeri* is considered to have one of the lowest recorded metabolic rates of any echinoid (Brockington, 2001) which may provide a buffering effect for the initial shock response.

3.5.3 Activity

Activity rates have been widely used in echinoderm studies to assess functional well-being (Stickle and Diehl, 1987). Righting time is the most commonly used metric to assess responses to environmental stress, especially in asteroids, echinoids and ophiuroids (Stickle and Diehl, 1987), and occasionally holothurians (Clements et al., 2020). However, as righting ability was difficult to measure in two of the three holothurian species (*H. steineni* and *C. georgiana*), a

peristaltic wave counting metric was employed to assess activity rates under reduced salinity. This novel metric, which is highly repeatable, may be of use in future studies in assessing activity rates in holothurian species without a central orientation, while also applicable to those that do.

Common to all species was a significant decline in activity in relation to reduced salinity. Previous studies have observed similar findings in relation to low salinity, for example in the echinoids *S. droebachiensis* (Moura et al., 2023b) and *E. esculentus* (Chapter 2; Barrett et al., 2024a), in the ophiuroid *Ophiothrix angulate* (Stancyk and Shaffer, 1977) and the asteroids *Leptasterias hexactis* (Shirley and Stickle, 1982a) and *Pisaster ochraceus* (Held and Harley, 2009). In the holothurian *Holothuria scabra*, reductions in salinity from 35‰ to 20‰ induced burrowing behaviour while at 15‰ there was a reduction in the ability to burrow (Mercier et al., 1999). The effects of rapid influxes of osmotically drawn water into tissue is likely to cause cellular swelling and appears to disrupt neuro-muscular coordination in echinoderms (Moura et al., 2023b). Furthermore, the reduction in metabolic activity implies the animals are in a state of energy preservation, prioritising homeostatic mechanisms over activity levels. Interestingly, even when metabolic rate is elevated at salinity levels near the animal's critical tolerance threshold, there is no apparent increase in activity. Increased activity at these very low salinities may have been predicted as a mechanism for the animal to seek higher salinities (e.g., Park et al., 2020). Instead, the lack of activity, despite a high metabolic rate suggests there is a direct physiological and behavioural cost at very low salinity. Similar increases in oxygen consumption and reductions in activity at very low salinities have been observed in copepods, attributed to the energetic costs associated with reducing the intracellular osmolyte pool to maintain osmotic balance (McAllen and Taylor, 2001). The current study suggest similar processes may be present in echinoderms at very low salinity.

3.5.4 Coelomic fluid osmolality

At ambient salinity, all species had coelomic fluid osmolality that was either isosmotic or slightly hyperosmotic to seawater, consistent with findings from other echinoderms (Diehl, 1986; Freire et al., 2011a; Santos et al., 2013). After 6 hours in reduced salinity, coelomic fluid was hyperosmotic in all species with wide intraspecies variation. Osmotic gradients that

persist for several hours after exposure to reduced salinities has been widely noted in previous echinoderm studies (Chapter 2; Barrett et al., 2024a; Santos et al., 2013; Vidolin et al., 2007). It has been suggested that echinoderms may have control over the permeability of epithelial membranes enabling the establishment of ionic and osmotic gradients (Santos et al., 2013). This is hypothesised to protect internal tissue from varying salinity, aligning with cyclical tidal fluctuations (Santos et al., 2013; Vidolin et al., 2007). However, the formation of osmotic gradients under low salinity is not necessarily associated with an intertidal lifestyle. For example, the intertidal echinoid *Echinometra lucunter*, which is able to tolerate unstable salinity conditions, demonstrated a lack of an osmotic gradient over 35‰ to 25‰ (Freire et al., 2011a). All echinoderm species in the current study are largely subtidal, suggesting that the ability to maintain high osmotic gradients could be the result of prior adaptation or may even serve a different purpose.

After transfer to lower salinities, it is commonly considered to take between 12-24 hours for coelomic fluids to equilibrate with the external medium (Diehl, 1986). This was observed at 29‰ only in all species after 24 hours in the current study. Below this salinity level, large gradients were still present across the majority of species after 24 hours. It appears 24 hours was insufficient for full osmotic rebalancing at lower salinities, similar to results observed in *E. esculentus* at low salinities (Chapter 2; Barrett et al., 2024a). Notably, the least osmoconforming species, *E. charcoti*, maintained coelomic fluid osmolality equivalent to a salinity of ~17‰ (516 ± 2 mOsm/kg) after 24 hours in 9‰. Further research is necessary to establish whether the mechanism that enables the maintenance of this remarkable gradient is mediated through control over the permeability of epithelial membranes, ion specific transport (Santos et al., 2013), or an unknown mechanism. Furthermore, the ability to maintain a large osmotic gradient may not be the sole reason for high tolerance to low salinity, as both the least and most osmoconforming echinoderms (*C. georgiana* and *E. charcoti* respectively) demonstrated the same LC_{50} of 11.5‰ after 24 hours. Santos et al. (2013) showed that urchins with the least capacity to maintain a high osmotic gradient (therefore the most osmoconforming) exhibited the greatest capacity to regulate tissue volume (i.e., the subtidal echinoid, *Arbacia lixula*). The ability to resist tissue swelling through cell volume regulation under hypoosmotic shock may be an important mechanism untested in this study. Indeed, *O. validus* has previously been observed to have control over body volume on exposure to reduced salinity (Pearse, 1967). This may explain its high

tolerance to acute low salinity while also being a strong osmoconformer, similar to other echinoderms (e.g., *A. lixula*; Santos et al., 2013).

Interestingly, at 6 hours exposure to reduced salinities, coelomic fluid osmolality was at similar levels at $\leq 24\text{‰}$ in all species. For example, the mean coelomic fluid osmolality of *E. charcoti* after 6 hours in 24‰ was 845 ± 25 mOsm/kg, while after 6 hours in 9‰ , it was 805 ± 6 mOsm/kg (Games-Howell 24‰ v 9‰ : $q=2.3$, $P=0.65$). This suggests that there is a temporal limitation on osmotic adjustments, regardless of the size of the osmotic imbalance, perhaps due to constraints on diffusion. However, oxygen consumption (which was also measured over the first 6-7 hours) increased to comparable levels with the control at the lowest salinities, departing from the observed metabolic depression seen at salinities in between. This suggests that although internal tissues are buffered from the full osmotic gradient, there must be a physiological cost elsewhere, perhaps from exposed external epithelial tissue such as tube feet. With a lack of a buffer, epithelial cells are exposed to the full osmotic gradient (e.g., the coelomic fluid of an echinoderm acclimated to 34.5‰ is ~ 1050 mOsm kg^{-1} , while 9‰ seawater has an osmolality of ~ 270 mOsm kg^{-1} , therefore the osmotic gradient is ~ 780 mOsm kg^{-1}). Therefore, various processes, from volume regulation to cell damage responses, as previously described, may account for the increased metabolic rate at very low salinities. Further work is needed to assess the impact of very low salinities on external echinoderm epithelia and the extent to which they can volume regulate.

3.5.5 Ecological perspective and conclusions

To my knowledge, this study is the first to assess the impact of acute low salinity on four of the major classes of Antarctic echinoderms. They were collected from the subtidal (10-20m) in Ryder Bay, Adelaide Island, on the Antarctic Peninsula. Here, subtidal salinity at 15m has remained relatively stable over the past ~ 20 years at $33.4 \pm 0.4\text{‰}$ (mean $\pm$ s.d.) ranging from 32.2‰ to 34.1‰ (Clarke et al., 2022). However, closer to the shoreline, the intertidal zone has more dramatic variations in salinity, where runoff from melting ice can expose benthic organisms to salinities down to 12‰ (Waller et al., 2006a), and lower if species become trapped in a hyposaline lens or meltwater stream (e.g., Davenport, 2001; Stockton, 1984). Of the six echinoderms, *S. neumayeri* and *O. validus* are occasionally found in the intertidal (Pearse, 2013; Waller et al., 2006a).

The results observed in this study demonstrate that Antarctic echinoderms are not uniformly osmoconformers, and that wide species variations exist in their salinity tolerances. Interestingly, the pattern of tolerance did not correlate with habitat as originally hypothesised. Factors such as phylogenetic or intrinsic biological traits over habitat are likely influencing species tolerance. *Heterocucumis steineni* and *O. validus* exhibit a relatively broad tolerance to changes in salinity, whereas *E. charcoti* and *C. georgiana* display exceptional short-term tolerance to low salinity for echinoderms. These species are expected to demonstrate significant resilience to rapid freshening within the Antarctic environment, both presently and in the future. Indeed, they are unlikely to experience levels of very low salinity if populations remain in the deeper subtidal. It may be predicted that they will simply migrate to deeper, higher salinity waters if freshening persists, potentially impacting ecosystem dynamics negatively (Sporta Caputi et al., 2020). Although predominately subtidal, *S. neumayeri* is known to occasionally inhabit the low intertidal, where salinity is already known to drop to as low as 12‰. This is well below the calculated LC_{50} for this species, perhaps accounting for its preference for deeper water. Furthermore, *S. neumayeri* embryos are highly sensitive to reductions in salinity (Coward et al., 2009), likely restricting larval recruitment to deeper, higher salinity environments. The most stenohaline echinoderm examined, *O. victoriae* would certainly be vulnerable to the impact of acute freshening. Its vulnerability to climate change is compounded by its inability to acclimate to warming at +2°C, which is currently only <0.5°C above summer maximum temperatures at Rothera (Peck et al., 2009a). When multiple environment stressors are applied in tandem (e.g., warming and low salinity), the effect on organisms can be additive or even synergistic (Barrett et al., 2022; Gunderson et al., 2016). Multiple stressor studies on Antarctic echinoderms have so far not included freshening which, for a phylum clearly vulnerable to low salinity, should be a focus for future research. Furthermore, attention should be paid to Antarctic crinoids and their ability to tolerate hyposalinity. Worldwide, crinoids are the least studied of all echinoderm classes in relation to salinity tolerance, and have not been found living in low salinity environments (Russell, 2013) suggesting they may be vulnerable to future Antarctic freshening.

The aim of the current experiment was to examine the hypoosmotic shock response to low salinity through acute direct transfer. However, alongside the current approach, incorporating a more gradual, step-wise dilution may better represent the ecologically realistic scenario of acute low salinity in the Antarctic. This method, which could be described

as a diurnal acute response, combined with longer-term acclimation experiments, would help decouple the different mechanisms underlying the capacity for low salinity tolerance in Antarctic echinoderms.

Climate change poses concern regarding the increased frequency of freshening events in nearshore coastal ecosystems (Röthig et al., 2023). Fjordic regions, such as the Western Antarctic Peninsula, are particularly vulnerable to future freshening events due to the presence of marine terminating glaciers (Moon et al., 2015). These marine areas are considered to be highly biodiverse (Grange and Smith, 2013), and the Antarctic region as a whole is considered a biodiversity hotspot for echinoderms (O'Loughlin et al., 2011). However there is fear that future warming will increase freshwater input into Antarctic fjords diminishing species richness and diversity, as has been observed in Arctic fjords (Grange and Smith, 2013). Ultimately, for Antarctic echinoderms to prosper in a reduced salinity environment over the long-term, they will need to demonstrate the ability to acclimatise. Capacity for acclimatisation in Antarctic marine species is considered paramount under a climate change context (Peck, 2018; Somero, 2010). Many are slow growing and have long generations times, meaning that survival will depend on their flexibility to adjust through acclimatisation and ensure populations survive long enough for genetic adaptation to take place (Peck, 2018). Priority now should be focused on the capability for Antarctic echinoderms to acclimatise to low salinity which reflects future climate change projections.

In summary, these findings highlight the species-specific response of Antarctic echinoderms to short-term low salinity, providing valuable insights into their potential resilience to climate change induced freshening.

Chapter 4 – Low salinity acclimation in two keystone Antarctic echinoderms: *Odontaster validus* and *Sterechinus neumayeri*

A version of this chapter has been accepted for publication in the journal *Science of The Total Environment*.

This Chapter utilised the acclimation methodology developed in Chapter 2 to evaluate the capacity of two Antarctic species to acclimate to low salinity. Lower salinity limits observed in these two species from Chapter 3 informed the salinity levels examined. Both Chapters 2 and 3 provided comparative data that could be incorporated in the analysis. I was the primary investigator and contributor to all aspects of this research, including conceptualisation, investigation, formal analysis, writing the original draft and reviewing and editing. All above authors provided supervision and contributed to editing and reviewing.

The data from this chapter is available here:

https://github.com/njb202/Chapter_4

4.1 Abstract

Coastal freshening in the Southern Ocean is expected to increase under projected climate scenarios. As a major environmental stressor, prolonged reduced salinity could pose a significant challenge to Antarctica's endemic echinoderms. Acclimatising to low salinity may be crucial for their continued survival as climate change accelerates, yet little is currently known about their capacity to do so. The sea star *Odontaster validus* and sea urchin *Sterechinus neumayeri*, two of the most ecologically important and abundant echinoderms of the shallow Antarctic seas, were exposed to reduced salinities (29‰ and 24‰) for at least 71 days after a stepwise dilution from 34.5‰. Feeding, faecal production (*S. neumayeri* only) and activity coefficient were significantly impacted at 24‰ and did not recover to control levels in either species. Oxygen consumption remained similar to control levels (34.5‰)

across both treatments and species until day 85, when a significant increase was observed in *S. neumayeri* at 24‰. Coelomic fluid osmolality was near isosmotic with external salinities in both species, while coelomocyte composition and concentration were unaffected by reduced salinities (*S. neumayeri* only). Both species demonstrated the capacity to tolerate lower salinities that may be expected with climate change, with successful acclimation demonstrated at 29‰. Although survival rates were high at 24‰, significant reductions in mass and the failure of metrics to return to control levels suggest that long-term survival at 24‰ is unlikely, potentially impacting Antarctic food-web dynamics and ecological interactions.

4.2 Introduction

Accelerated warming in Antarctica is driving an increase in the rate of coastal freshening (the lowering of seawater salinity) due to melting of sea ice, glaciers and ice-shelves, reduced sea ice production and increased precipitation (Haumann et al., 2016; Hellmer et al., 2011; Jacobs et al., 2002). In particular, the Western Antarctic Peninsula (WAP) has already experienced the highest rate of warming of any region globally (González-Herrero et al., 2022; Vaughan et al., 2003), which has contributed to reductions in coastal salinity (Haumann et al., 2016; Hellmer et al., 2011). Varying salinity gradients can be a major environmental stressor for marine invertebrates (Freire et al., 2011b; Kinne, 1964), due to the metabolic costs associated with adjusting cellular level compositions in order to balance osmotic gradients (Rivera-Ingraham and Lignot, 2017; Sokolova et al., 2012a) among other factors. For this reason salinity plays a crucial role in determining the physiology, distribution and survival of marine invertebrates (Kinne, 1964). The fjordic coastline of the WAP is currently rich in biodiversity and benthic abundance (Grange and Smith, 2013). However, predictions from Arctic fjords (which are considered to be at a later stage of climate warming than Antarctic fjords), suggest that coastal freshening could lead to distinct salinity stratification in the water column (Bianchi et al., 2020), potentially threatening these biodiversity hotspots (Grange and Smith, 2013). For example, in Greenland, inner fjord salinities often remain below 25‰ down to at least 10 meters (Sejr et al., 2017; Sejr et al., 2022) indicating that Antarctic fjords may experience similar conditions as climate warming advances.

Reductions in seawater salinity due to coastal freshening is a concern for Antarctica's endemic marine organisms which have evolved in a thermally stable and predictable environment over millions of years (Peck, 2018). Their capacity to acclimatise to environmental change, particularly warming, is generally considered poor and the time required for acclimation is considerably longer compared to similar temperate species (Peck et al., 2014). Evidence of low salinity acclimatisation abilities in Antarctic marine organisms, is sparse. Studies that have investigated long-term tolerance to low salinity demonstrate poor acclimation capacity. For example the Antarctic limpet *Nacella concinna* reached 50% mortality at 20‰ after 60 days (Navarro et al., 2020), while the intertidal Antarctic nototheniid teleost, *Harpagifer antarcticus* demonstrated reduced capacity to maintain homeostasis when exposed to low salinities for one week (Vargas-Chacoff et al., 2021a).

Of particular concern are Antarctica's endemic echinoderm species. As a strictly marine phylum, echinoderms are often considered to have poor euryhaline abilities, lacking distinct excretory organs and possessing highly permeable body walls (Binyon, 1972; Hyman, 1955; Santos-Gouvea and Freire, 2007). However, numerous species live in low salinity environments, such as brackish waters (Russell, 2013; Turner and Meyer, 1980) and the intertidal (Castellano et al., 2018; Freire et al., 2011a). Aquarium studies have demonstrated wide variation in short-term low salinity tolerance levels across different taxa (Russell, 2013; Stickle and Diehl, 1987), while several studies have demonstrated that echinoderms have the capacity to acclimate to lower salinity over longer periods (Chapter 2; Barrett et al., 2024a; Russell, 2013; Shirley and Stickle, 1982a). However, the physiological and behavioural response of echinoderms to low salinity, can differ dependant on the duration and rate of salinity reduction (e.g., Chapter 2; Barrett et al., 2024a). This correlates with mechanistic differences in the acute response to low salinity, compared to that of the gradual process of cellular re-organisation necessary for acclimation in osmoconformers (Khlebovich, 2017; Rivera-Ingraham and Lignot, 2017). In echinoderms under acute hypoosmotic stress, the establishment of transient ionic and osmotic gradients between the coelomic fluid and external seawater, appear to provide a 'buffer' to internal tissue, allowing time for cellular volume control to take place (Chapter 2; Barrett et al., 2024a; Freire et al., 2011a; Santos et al., 2013). Over longer periods of exposure to low salinity in osmoconformers, cellular solute composition and concentration can be modified through changes to membrane bound transporters favouring the release of specific 'osmolytes' from the cytoplasm (Barrett et al.,

2022; Gilles, 1987; Meng et al., 2013; Podbielski et al., 2022b). In addition, structural changes to the cell cytoskeleton may support changes in osmotic pressure (Barrett et al., 2022; Pedersen et al., 2001). However, the genetic mechanisms underlying acclimatisation to lower salinity in echinoderms is yet to be established.

There have been no long-term studies on their capacity of Antarctic echinoderms to acclimatise to low salinity. However, short-term tolerance to hypoosmotic exposure has been evaluated in a number of species with varying results (Chapter 3; Barrett et al., 2024b; Cowart et al., 2009; Pearse, 1967). For example, multiple holothurian species demonstrate high tolerance to low salinity (24-hr LD₅₀ = 11.5‰) in contrast to the Antarctic brittle star *Ophionotus victoriae*, where mortality was >50% at 19‰ after 24 hours (Chapter 3; Barrett et al., 2024b).

The sea star, *Odontaster validus* and sea urchin *Sterechinus neumayeri* are both common circumpolar inhabitants of the nearshore Antarctic benthic ecosystem (Dell, 1972; Pearse, 2013). *Odontaster validus* is a subtidal asteroid normally found at depths of between 15 and 200 meters, however it can be found from the shoreline down to depths of over 2000 meters (Dearborn, 1977; Pearse, 2013). It is considered an opportunistic omnivore with diverse feeding strategies from predatory to detrital feeding (see McClintock, 1994), and has been considered a keystone species (Kidawa et al., 2010; McClintock et al., 1988; McClintock et al., 2008; although see Pearse, 2013). *Sterechinus neumayeri* is a shallow-water regular echinoid which inhabits both the intertidal and subtidal zones down to 450 meters (Brey, 1991). It is most abundant in depths of less than 35 meters (Barnes and Brockington, 2003; Dell, 1972; Waller et al., 2006a) where it is the dominant echinoid in the near-shore benthic ecosystem (Brockington, 2001). Considered an omnivorous grazer and predator, its diet ranges from diatoms and macroalgae to scallops, bryozoans and even seal faecal material (McClintock, 1994; Pearse and Giese, 1966). Due to its high abundance and diverse feeding strategies, it significantly impacts community structure (Brey, 1991) and plays a key role in regulating the food web in Antarctic benthic ecosystems (Sporta Caputi et al., 2020). Consequently, it is considered a keystone species (Détrée et al., 2023; Ortiz et al., 2017; Sporta Caputi et al., 2020). Tolerance to acute low salinity has been demonstrated in both species, with *O. validus* the more tolerant of the two (Chapter 3; Barrett et al., 2024b; Pearse, 1967). The calculated 24-hour LD₅₀ (salinity which is lethal to 50% of exposed animals) was 13.4‰ for *O. validus* and 16.5‰ for *S. neumayeri* (Chapter 3; Barrett et al., 2024b). However, early

life stages of *S. neumayeri* appear to be highly sensitive to subtle decreases in salinity, with 99% mortality observed in embryos exposed to a drop in salinity from 34 to 30‰ (Coward et al., 2009).

This study aims to assess the acclimation capacities of *O. validus* and *S. neumayeri*, to lower salinity levels (29‰ and 24‰) over a period of at least two and a half months, as may be predicted under future freshening scenarios in Antarctica. Assessments of whole animal physiological metrics [mortality, oxygen consumption, feeding, faecal production (*S. neumayeri* only) and changes in mass] and behavioural assessments (ability to right), will be combined with cellular and body fluid measurements (coelomic fluid osmolality and coelomocytes composition and concentration) to assess evidence of low salinity acclimation. This is the first study to assess the low salinity acclimation capacity of any Antarctic echinoderm. It will also provide samples for downstream molecular analysis, enabling a mechanistic accompaniment to the current study.

4.3 Methods

4.3.1 Experimental animals

Both species were collected in the austral summer of 2021/2022 by SCUBA divers at depths between 10-20m near the British Antarctic Survey's (BAS) Rothera research station, Adelaide Island (67°34'07''S, 68°07'30''W). After collection, they were transferred to the Rothera aquarium, and subsequently transported to the UK in a temperature-controlled, containerised aquarium. In the UK, they were kept in 280-liter tanks within a recirculating system that included macro- and biological filtration. Seawater was obtained from the UK coast. The system was maintained at $-0.3 \pm 0.2^\circ\text{C}$ and 34.5‰, in a 12:12 hour light: dark regime, for approximately 6—12 months prior to the experiment. Both species were fed once a week on a frozen krill diet.

4.3.2 Experimental set-up

Three salinity treatments were selected to evaluate acclimation capacity of the two species over a 71-day (*O. validus*) and 85-day (*S. neumayeri*) period: 34.5‰ (control), 29‰ (medium), 24‰ (low). The two reduced salinity treatments were selected based on the results of their 24-hour short-term tolerance (Chapter 3; Barrett et al., 2024b), data from the Rothera Time Series [surface salinity ranged from $28.4 \pm$ to 35.2 ± 0.03 ‰ from December 2012 to January 2021 (Clarke et al., 2022)], approaches from previous salinity studies on Antarctic marine animals (e.g., Navarro et al., 2019; Navarro et al., 2020; Park et al., 2020) and future freshening predictions from Arctic fjords (e.g., Grange and Smith, 2013; Sejr et al., 2017). For *O. validus*, 108 animals with an arm radius (from the tip of arm to the centre of mouth) of 25–63 mm were randomly divided between the three salinity treatments and distributed between three replicate 60-litre tanks per treatment ($n=12$ per tank; $n=36$ per treatment, 9 tanks in total). For *S. neumayeri*, 120 animals with a test diameter of 15–37 mm were split between four replicate 50-litre tanks per treatment ($n=10$ per tank; $n=40$ per treatment; 12 tanks in total). Tank replication ensured that unintended variation or bias introduced by using different tanks could be assessed. A protein skimmer (REEF- Skim Nano 100AC, TMC Ltd) and bio-filter (ZB-150, Ziss) with an attached airline were fitted to each tank.

Animals were habituated for 8 (*O. validus*) or 12 days (*S. neumayeri*) before tanks were diluted using distilled water in a stepwise approach, reducing salinity by 2‰ per day until experimental salinities were reached (as per Chapter 2; Barrett et al., 2024a; Shirley and Stickle, 1982b). A conductivity probe (CDC40101, Hach) was used for measuring salinity. Within each experiment floating trays were added to tanks to isolate individual animals for repeated measurements. For *O. validus*, 3 animals per tank were isolated in trays, equalling 9 per treatment; for *S. neumayeri* 2 animals per tank were isolated in trays, equalling 8 per treatment. In the *S. neumayeri* experiment, data could not be collected during the week of day 43. Therefore, the data from day 50 represents two weeks' worth of observations, which have been averaged.

Water chemistry was monitored twice weekly using JBL aquarium test kits (JBL, Germany). Ammonium (NH_4^+), nitrates (NO_3^-) and nitrites (NO_2^-) were kept below recommended levels ($\text{NH}_4^+ < 0.05 \text{ mgL}^{-1}$, $\text{NO}_3^- \sim 1 \text{ mgL}^{-1}$, $\text{NO}_2^- < 0.025 \text{ mgL}^{-1}$), for each treatment by refreshing seawater (pre-diluted and chilled) twice weekly between 20–30 litres per tank.

Water pH was monitored weekly using a pH probe prior to water changes (Hach, Germany) (*S. neumayeri* control: pH 7.97 ± 0.05 , medium: 7.93 ± 0.05 , low: 7.92 ± 0.05 , mean $\pm$ s.e.m, $n=12$; *O. validus* control: pH 7.98 ± 0.03 , medium: 7.92 ± 0.03 , low: 7.93 ± 0.03 , mean $\pm$ s.e.m, $n=10$). Tank temperatures were maintained at $\sim -0.4^\circ\text{C}$ (*O. validus*: $0.45 \pm 0.03^\circ\text{C}$ mean $\pm$ s.e.m $n=72$; *S. neumayeri* $-0.42 \pm 0.02^\circ\text{C}$ mean $\pm$ s.e.m, $n=158$) and checked daily.

4.3.3 Mortality

Mortality was assessed daily. Animals unable to right within 24 hours and lack of movement of tube feet (*S. neumayeri*) or arms (*O. validus*) after stimulus were declared dead.

Buoyant weight change and Percentage ash free dry mass (AFDM)

To assess for changes in mass in *O. validus*, buoyant weight was measured in each isolated sea star before and after the experimental period following seven days without feeding. Buoyant weight is considered insensitive to changes in water content of the animal (Jokiel et al., 1978), which can occur in sea stars due to the lack of an exoskeleton. For *S. neumayeri*, percentage AFDM was used as an end point assessment of the difference in organic mass to inorganic mass per isolated urchin over the experimental period (following Chapter 2; Barrett et al., 2024a). This was expressed as:

$$\% AFDM = \frac{AFDM}{(AFDM + ash\ mass)} \times 100$$

4.3.4 Oxygen consumption

Oxygen consumption was measured in the isolated urchins seven days before salinity dilution began, and then on day 1, 8, 15, 29, 57, 71 (*O. validus* only) and 85 (*S. neumayeri* only) after the experimental salinity dilution was reached (hereafter referred to as sample points). Closed bottle respirometry techniques were used to measure oxygen consumption following Chapter 2 (Barrett et al., 2024a). Each animal was placed in a 300ml glass respirometry chamber with a 4mm mesh covering, allowing oxygenated water to circulate but preventing

animals from escaping. Animals were left for one hour to habituate within the chamber and ensure the metabolic effects of handling had subsided, before the lids were sealed and a first measurement of the water oxygen saturation levels were measured. A Fibox-4 fibre optic oxygen sensor system (PreSens, Germany) was used to record the chamber water oxygen saturation. After approximately four hours for *O. validus* or six hours for *S. neumayeri*, a second oxygen saturation measurement was taken. Three additional empty chambers were sealed and assessed over the same time period to measure any background microbial metabolic activity. These values were then used to correct the calculated rates for each animal. Animal volume was measured by way of water displacement following Morley et al. (2016) and each chamber volumes was adjusted for animal volume to produce a respired water volume for each calculated value of oxygen consumption. At the end of the experimental period animals were selected for destructive sampling to obtain AFDM. Animals were sacrificed by being placed in a -80°C freezer, then dried in a convection oven at 60°C for at least 48 hours until a consistent mass was obtained. Dried animals were weighed, then placed in a muffle furnace for six hours at 475°C to obtain an ash mass value. AFDM was calculated by subtracting the ash mass from dry mass. For *O. validus*, AFDM was measured in all isolated animals ($n=9$ per treatment). For *S. neumayeri*, AFDM was determined from a wet mass vs. AFDM regression using a combination of isolated and tank animals ($n=10$ per treatment). Due to the change in ratio of organic mass to inorganic mass observed at the end of the experiment, linear regressions between pre-experiment wet mass and post-experiment mass were used to determine modelled AFDM at each timepoint.

For additional confirmation of the impact of salinity on oxygen consumption in *S. neumayeri*, a repeat experiment was implemented. A single end point sample of oxygen consumption was measured after 78 days of exposure to experimental salinities which was consistent with the previous trial (see Figure C1).

4.3.5 Righting ability (Activity coefficient)

Sea urchins and sea stars will attempt to right themselves if inverted with the time taken to right being a widely used metric to assess echinoderm activity rates (Russell, 2013; Stickle and Diehl, 1987). Time taken to right in seconds was converted to an activity coefficient ($AC=1000/\text{righting time in seconds}$) (after Lawrence and Cowell, 1996) and assessed weekly in all

treatments for both species including seven days before salinity dilution began. For *O. validus* repeat measurements were made on the same isolated sea stars ($n=9$ per treatment), while for *S. neumayeri* between 8-12 urchins were randomly sampled per treatment. The maximum time allowed for righting was 30 minutes for *O. validus* and 60 minutes for *S. neumayeri*, which equals a minimum AC value of 0.55 for *O. validus* (1000/1800 s) and 0.28 for *S. neumayeri* (1000/3600 s).

4.3.6 Feeding

Feeding trials began within the habituation period while all treatments were at ambient salinity (34.5‰) to provide a baseline feeding rate (two weeks for *S. neumayeri* and one week for *O. validus*), and then commenced weekly. Feeding was not measured in the final week in either species. The artificial diet fed to the animals (Vitalis Marine Grazer) constituted 28% protein obtained from fish, algae, molluscs and crustacean derivatives and had been previously used in *S. neumayeri* feeding experiments (De Leij, 2021). The feed was also tested on *O. validus* in a pre-experiment feeding trial to ensure suitability. The isolated animals were fed pre-dried and weighed pellets once a week throughout the experimental period and allowed to feed for 48 hours before any remaining food was removed. The remaining food was rinsed twice in distilled water to remove saltwater, dried for 24 hours in a convection oven and weighed. The feeding rate was calculated by subtracting the remaining dry mass of food from the initial dry mass and dividing by the ADFM of the animal (mg food ingested mg animal ADFM⁻¹ week⁻¹). The free roaming tank animals were fed weekly and allowed to feed *ad libitum*. The amount of food provided was at least $\geq 50\text{mg animal}^{-1}\text{ week}^{-1}$ for *S. neumayeri* and $\geq 100\text{mg animal}^{-1}\text{ week}^{-1}$ in *O. validus* to ensure consumption was not limiting. In *O. validus*, an additional metric of feeding behaviour was recorded after 24 hours by turning over each sea star and visually assessing whether food was being ingested.

4.3.7 Faecal production

Faecal collection for *S. neumayeri* began on day 1 after acclimation salinities were reached and represented faecal production from the prior week of feeding. Faeces were collected weekly from all isolated urchins ($n=8$ per treatment) by pipette and transferred to a

microcentrifuge vial. Faeces were then rinsed twice with distilled water to remove saltwater and dried in a convection oven for 24 hours and weighed. Dried faeces were then ashed in a muffle furnace for 6 hours at 475°C and weighed. Ash free dried mass (AFDM) was calculated by subtracting the ash mass from the dried mass. Faecal production was not assessed in *O. validus* due to the lack of coherence in the faeces produced by this species.

4.3.8 Coelomic fluid osmolality and coelomocyte analysis in *S. neumayeri*

Coelomic fluid was extracted from randomly selected free-roaming tank animals ($n=4$ for *O. validus*; $n=5$ for *S. neumayeri*) in each treatment on days 1, 8, 15, 29, 57 and 85 for *S. neumayeri* and days 1, 8, 22, 36, and 64 for *O. validus* after the experimental salinity dilution was reached. Sub-lethal sampling was conducted by inserting a syringe with a 21-gauge needle into the coelomic cavity (after Reinardy and Bodnar, 2015). Between 0.5-1ml of coelomic fluid was extracted per urchin. Triplicate tank water samples were taken at the same sampling point to provide a mean tank water osmolality value. Coelomic fluid and seawater osmolality was measured on a Vapro 5600 vapour pressure osmometer (ELITech Group).

Coelomocytes were collected at the last sample point (day 85) from *S. neumayeri* ($n=5$ per treatment). Coelomic fluid was collected as previously described, with samples kept on ice until measurements were taken to prevent cell clumping. Cell concentration, differential cell counts (red spherulocytes and clear cells) and cell viability were assessed on a Neubauer haemocytometer using a light microscope. The percentage viable cells was assessed by mixing coelomic fluid with 0.5% Trypan Blue (ratio 1:1) and the number of dead blue cells and clear live cells counted (as per Barrett et al., 2024a; Liu et al., 2022). Coelomocyte analysis was not performed on *O. validus*.

4.3.9 Hypoosmotic shock

To assess the resilience of the acclimated animals to hypoosmotic shock, a subset of animals ($n=6$ per treatment for *O. validus*; $n=5$ per treatment for *S. neumayeri*) were directly transferred to a single 19‰ salinity tank per treatment for a duration of seven hours. Oxygen consumption was assessed over the first 3 hours in *O. validus* and the first 6 hours in *S. neumayeri* as previously described. Activity coefficient was assessed after 6 hours in 19‰ as

previously described. For *S. neumayeri*, the righting challenge was additionally extended to 90 minutes which equals a minimum AC value of 0.19 (1000/5400 s).

4.3.10 Statistics

One-way analysis of variance (ANOVA) was implemented when comparing the response means between salinity treatments at specific timepoints, followed by Holm's adjusted correction to account for multiple comparisons. Assumptions of normality and homogeneity of variance were formally tested using Shapiro–Wilk test and Levene's test, respectively. When the residuals of the data were not normally distributed, data were either log, square or cube root transformed and reassessed. With continued violations of normality in the residuals, non-parametric Kruskal-Wallis tests were implemented. For data where the variance of residuals across groups was unequal, Welch's ANOVA tests were used. For significant ANOVA, Welch's ANOVA, or Kruskal-Wallis tests, post-hoc tests were performed to determine significant differences between group pairings, using Tukey's test, Games-Howell test, and Dunn's test, respectively. Two-way ANOVA tests were performed when comparing the response variable over different time points including their interaction. When repeat measurements were performed on the same animal, the model was adapted to include the individual animal as a random factor. When the assumptions of normality were violated in two-way models and data transformation was unsuccessful, an aligned rank transformation ANOVA (ART ANOVA) was implemented as a non-parametric alternative. One-sample *t*-tests were performed when comparing coelomic fluid osmolality values with mean tank water osmolality. Two sample *t*-tests were performed when comparing oxygen consumption values before and after hypoosmotic shock. Chi-squared tests were performed on mortality data. Percentage data (e.g., percentage AFDM and coelomocyte metrics) were arcsine transformed prior to statistical analysis. Statistical tests were considered significant if $P < 0.05$. All analyses were carried out in R (version 4.2.2).

4.4 Results

4.4.1 Tank effects

There were no significant variations between tanks regarding oxygen consumption, activity coefficient and feeding rate in either the *O. validus* or *S. neumayeri* experiment, therefore each individual animal was treated as a biological replicate (Table C1 and C2).

4.4.2 Mortality

O. validus – Over the experimental period, there were three recorded mortalities in the low salinity treatment. There were no mortalities in the medium and control salinities. Overall, salinity had a marginal but significant impact on mortality (Chi-squared: $\chi^2(2)=6.2$, $P=0.046$) (Figure 4.1A; Table C1).

S. neumayeri – Mortalities were recorded in all three treatments over the experimental period: four in both the control and medium salinities, and nine in the low salinities. However, salinity had no significant impact on mortality (Chi-squared: $\chi^2(2)=3.4$, $P=0.18$) (Figure 4.1B; Table C2).

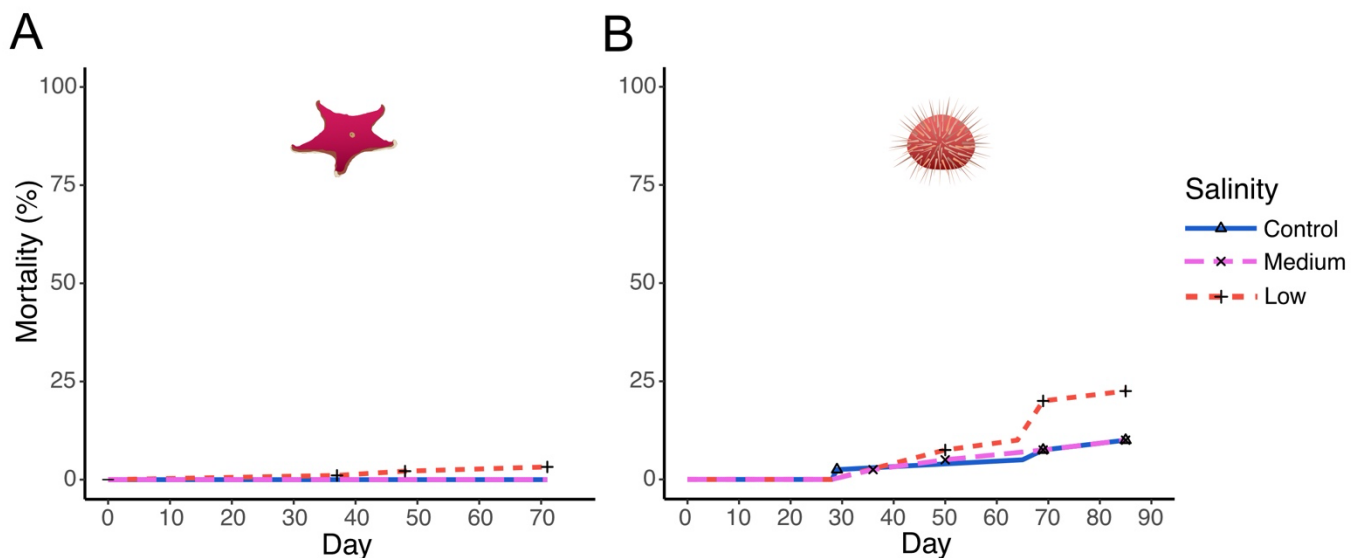


Figure 4.1| Cumulative percentage mortality of A) *Odontaster validus* and B) *Sterechnus neumayeri* over the course of the experimental period. Symbols (triangle, cross and plus sign) correspond to recorded mortalities.

4.4.3 Buoyant weight change

O. validus – Salinity had a significant impact on the change in mean ($\pm$ s.e.m.; $n=8-9$) buoyant weight (low: -1.09 ± 0.03 g; medium: -0.004 ± 0.01 g; control: $-0.006-0.02$ g) (One-way ANOVA: $F_{(2,23)}=6.9$, $P=0.004$). There were significant differences between the low and control (Tukey: $P<0.01$) and low and medium salinities (Tukey: $P=0.009$) (Table C1).

4.4.4 Percentage AFDM

S. neumayeri – Salinity had a significant impact on the ratio of organic mass to inorganic mass (AFDM to dry mass) following the acclimation period (expressed as the mean percentage AFDM: low $17.51\pm0.86\%$; medium $27.1\pm1.28\%$; control $26.84\pm1.55\%$) (One-way ANOVA: $F_{(2,54)}=17.4$, $P<0.0001$). Animals in low salinity had a significantly lower percentage of AFDM compared to animals in control salinity and medium salinity (Tukey: both $P<0.0001$), while there were no significant differences between animals in medium and control salinity (Table C2).

4.4.5 Oxygen consumption

O. validus – The impact of salinity on rates of oxygen consumption had no overall significant effects (Two-way ANOVA with repeated measures: $F_{(2,24)}=1.1$, $P=0.34$); however, the interaction between time points and salinity was significant (Two-way ANOVA with repeated measures: $F_{(12,144)}=1.96$, $P=0.03$), in addition to the effects of time (Two-way ANOVA with repeated measures: $F_{(6,144)}=10.9$, $P<0.0001$). Analysis of oxygen consumption at each timepoint demonstrated no significant impact between salinity treatments (Figure 4.2A; Table C1).

S. neumayeri – The impact of salinity on rates of oxygen consumption had no overall significant effects (Two-way ART ANOVA with repeated measures: $F_{(2,23)}=0.2$, $P=0.8$); however, the interaction between time points and salinity was significant (Two-way ANOVA with repeated measures: $F_{(12,126)}=4.6$, $P<0.0001$), in addition to the effects of time (Two-way ANOVA with repeated measures: $F_{(6,126)}=14.5$, $P<0.0001$). Analysis of rates of oxygen consumption at each

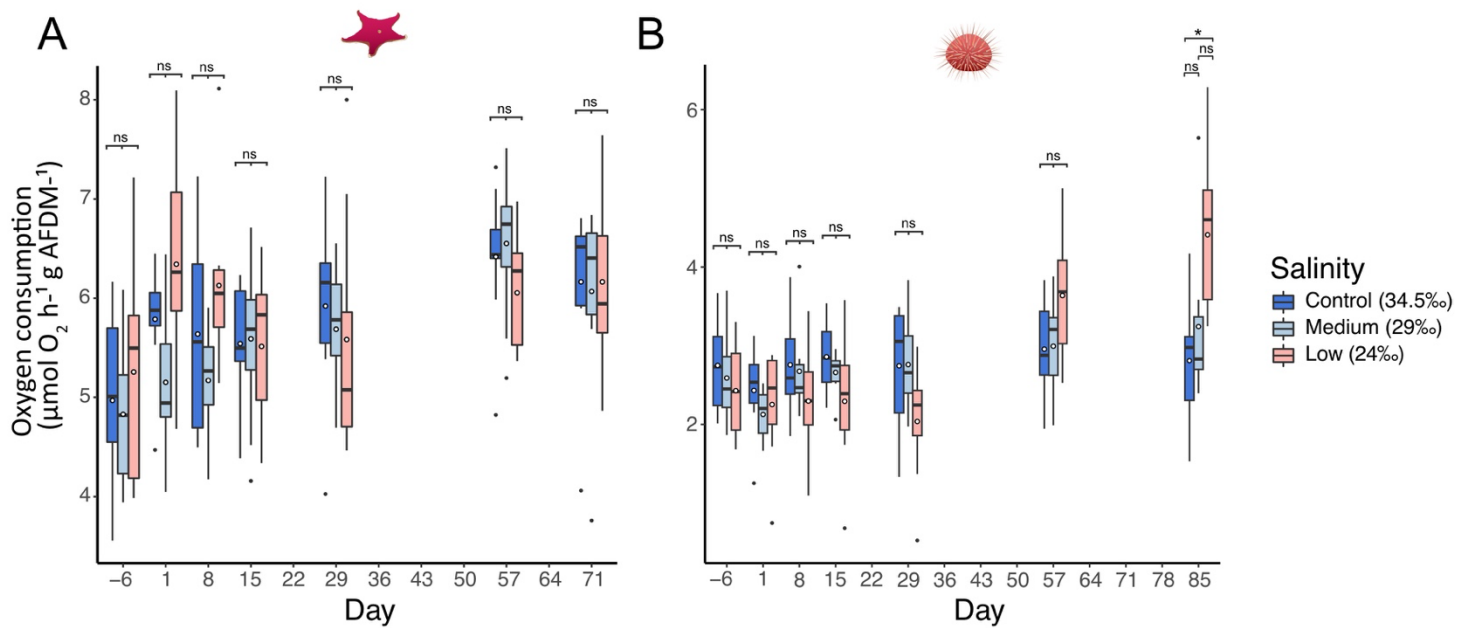


Figure 4.2| Rate of oxygen consumption of A) *Odontaster validus* and B) *Sterechninus neumayeri* at time points during exposure to different salinity conditions. Results are shown for one-way ANOVA/Welch's ANOVA and post hoc Tukey test (*S. neumayeri*: day 85) between salinity treatments at each time point (n.s., not significant; * $P < 0.05$; $n = 9$ for *O. validus* and $n = 8$ for *S. neumayeri* biological replicates at each time point and treatment). Box plots show medians (black line), upper and lower quartiles, and maximum and minimum (whiskers). Means are represented by open circles and outliers by filled circles.

timepoint demonstrated salinity had a significant impact on day 85 only (One-way ANOVA: $F_{(2,21)} = 6.4$, $P = 0.046$), where there were significant differences between the control and low salinity treatments (Tukey: $P = 0.01$) (Figure 2B; Table C2). In the repeat experiment (day 78), salinity had a significant impact on the rates of oxygen consumption (One-way ANOVA: $F_{(2,20)} = 9.6$, $P = 0.001$), with significant differences between the control and low salinity treatments (Tukey: $P = 0.007$) and between the medium and low salinity treatments (Tukey: $P = 0.001$) (Figure C1; Table C2).

4.4.6 Righting ability (Activity coefficient)

O. validus – Mean AC rates varied significantly between salinity treatments and time points, including their interaction (Two-way ANOVA with repeated measures: Salinity $F_{(2,24)} = 30.8$, $P < 0.0001$; Time points $F_{(11,264)} = 14.6$, $P < 0.0001$; Salinity:Time points $F_{(22,264)} = 3.5$, $P < 0.0001$). At each time point, except on day -6, salinity had a significant impact on mean AC (One way

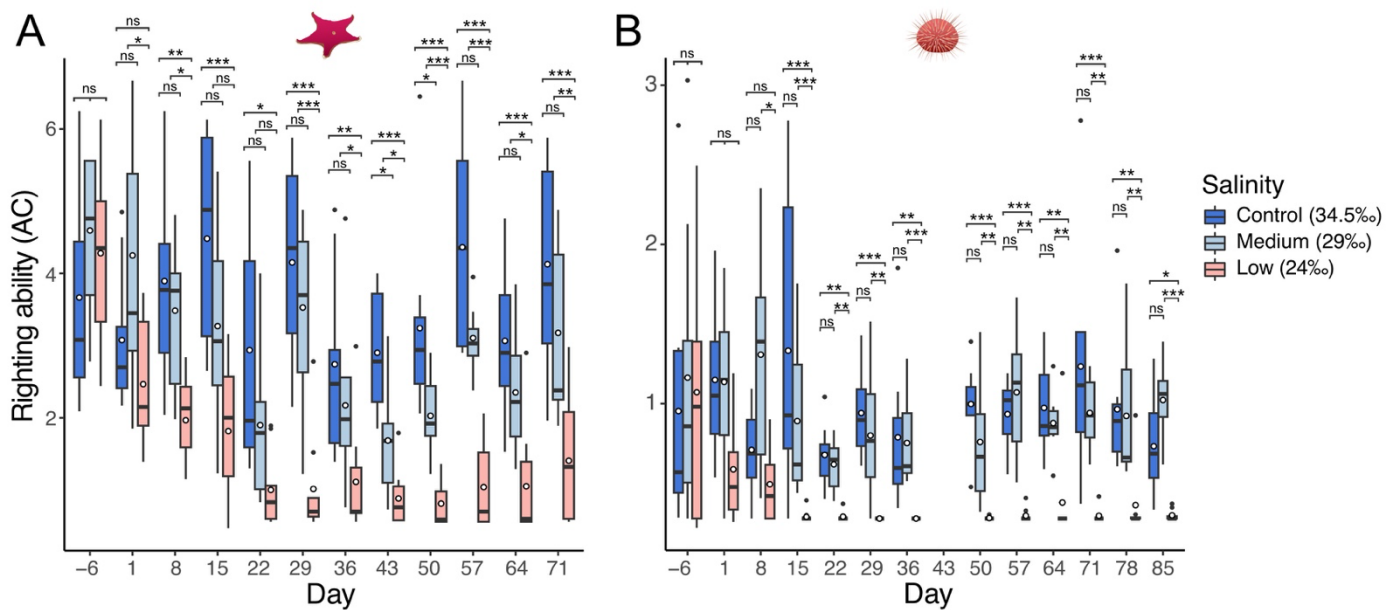


Figure 4.3| Righting ability (activity coefficient) of A) *Odontaster validus* and B) *Sterechnus neumayeri* at time points during exposure to different salinity conditions. Results are shown for one-way ANOVA/Welch's ANOVA/Kruskal-Wallis and relevant post hoc test between salinity treatments at each time point (n.s., not significant; * $P<0.05$, ** $P<0.01$, *** $P<0.001$; $n=9$ for *O. validus* and $n=8-12$ for *S. neumayeri* biological replicates at each time point and treatment). Note: a smaller AC value indicates longer righting times. Box plots show medians (black line), upper and lower quartiles, and maximum and minimum (whiskers). Means are represented by open circles and outliers by filled circles.

ANOVA and Welch's ANOVA: all $P<0.05$). Righting times were significantly longer in low salinity compared to the control at all time points from day 8 (Tukey and Games-Howell: all $P<0.05$) and compared to medium salinity from day 1 (Tukey and Games-Howell: all $P<0.05$), with the exception of day 15 and 22 where they were not significant. Righting times were significantly longer in medium salinity compared to the control on day 43 (Games-Howell: $P=0.02$) and day 50 (Tukey: $P=0.02$) only (Figure 4.3A; Table C1).

S. neumayeri – Mean AC rates varied significantly between salinity treatments and time points, including their interaction (Two-way ART ANOVA: Salinity $F_{(2,296)}=107.4$, $P<0.0001$; Time points $F_{(12,296)}=2.8$, $P<0.01$; Salinity:Time points $F_{(24,296)}=1.1$, $P=0.04$). At each time point, except on day -6 and 1, salinity had a significant impact on mean AC (One way ANOVA and Welch's ANOVA and Kruskal-Wallis: all $P<0.05$). Righting times were significantly longer in low salinity compared to the control at all time points from day 15 (Dunn and Games-Howell: all $P<0.02$), and compared to medium salinity from day 8 (Dunn and Games-Howell: all $P<0.02$).

There were no significant differences in AC between medium salinity and control at any point in the experiment (Figure 4.3B; Table C2).

4.4.7 Feeding

O. validus – Mean feeding rates varied significantly between salinity treatments, time points and their interactions (Two-way ART ANOVA with repeated measures: Salinity $F_{2(2,256)}=81.1$, $P<0.0001$; Time points $F_{(10,256)}=4.2$, $P<0.01$; Salinity:Time points $F_{(20,256)}=1.76$, $P=0.025$). At each time point, except on day -6 and 50, salinity had a significant impact on feeding (One way ANOVA and Welch's ANOVA: all $P<0.05$). Feeding rates were significantly lower in low salinity compared to the control at all time points from day 1 (Tukey and Games-Howell: all $P<0.04$), and the medium salinity at all time points from day 1 (Tukey and Games-Howell: all $P<0.04$), except on day 50 where they were not significant. There were no significant differences in feeding between medium salinity and control at any point in the experiment (Figure 4.4A; Table C1). The number of individual sea stars feeding after 24 hours dropped from 100% before dilution to 0% after dilution in the low salinity treatment, before stabilising at 44% from day 44 to 64. Feeding in the medium group followed a similar pattern to the control group, stabilising at 100% in both groups from day 44 to 64 (Figure 4.5).

S. neumayeri – Mean feeding rates varied significantly between salinity treatments, time points and their interactions (Two-way ART ANOVA with repeated measures: Salinity $F_{(2,23)}=63.6$, $P<0.0001$; Time points $F_{(12,252)}=18.2$, $P<0.0001$; Salinity:Time points $F_{(24,252)}=7.9$, $P<0.0001$). At each time point, except on day -13 and -6, salinity had a significant impact on feeding (One way ANOVA, Welch's ANOVA and Kruskal-Wallis: all $P<0.01$). Feeding rates were significantly lower in low salinity compared to the control (Tukey, Dunn and Games-Howell: all $P<0.002$) and medium salinity (Tukey, Dunn and Games-Howell: all $P<0.05$) at all time points from day 1. There were no significant differences in feeding between medium salinity and control at any point in the experiment (Figure 4B; Table C2).

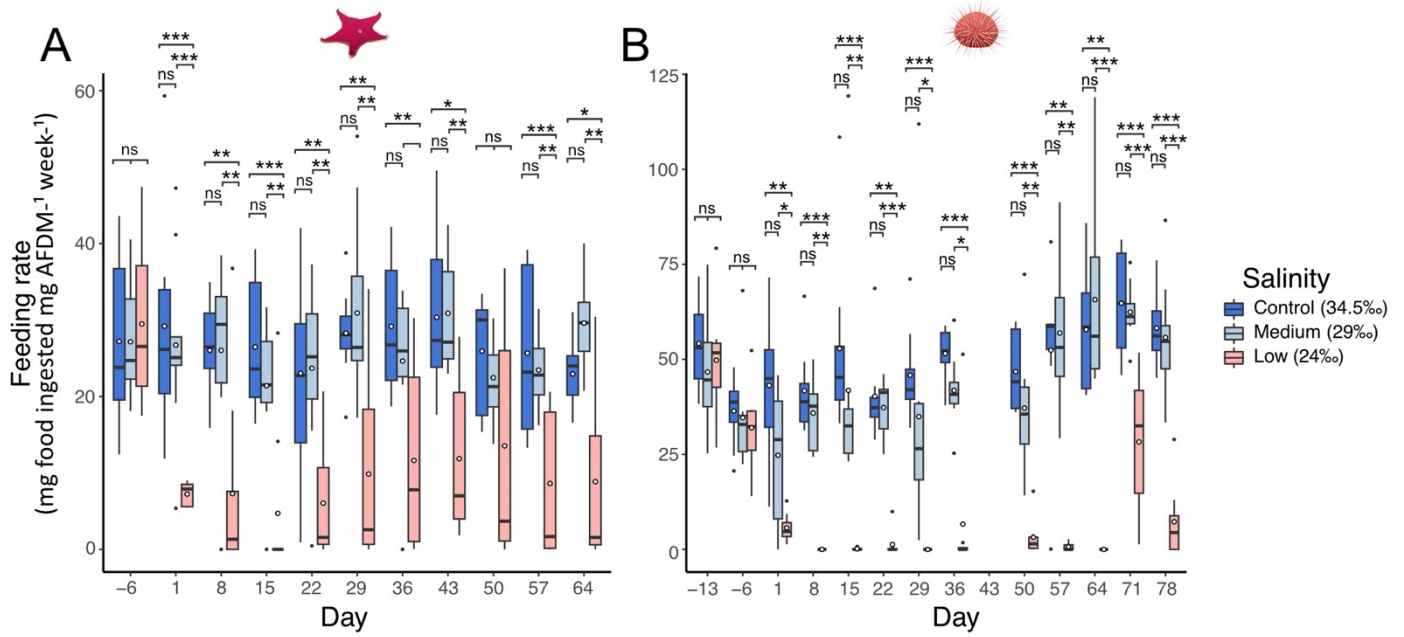


Figure 4.4 | Feeding rate of A) *Odontaster validus* and B) *Sterechnus neumayeri* at time points during exposure to different salinity conditions. Results are shown for one-way ANOVA/Welch's ANOVA/Kruskal-Wallis and relevant post hoc test between salinity treatments at each time point (n.s., not significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; $n = 9$ for *O. validus* and $n = 8$ for *S. neumayeri* biological replicates at each time point and treatment). Feeding was calculated as mg food ingested/mg animal AFDM/week. Box plots show medians (black line), upper and lower quartiles, and maximum and minimum (whiskers). Means are represented by open circles and outliers by filled circles.

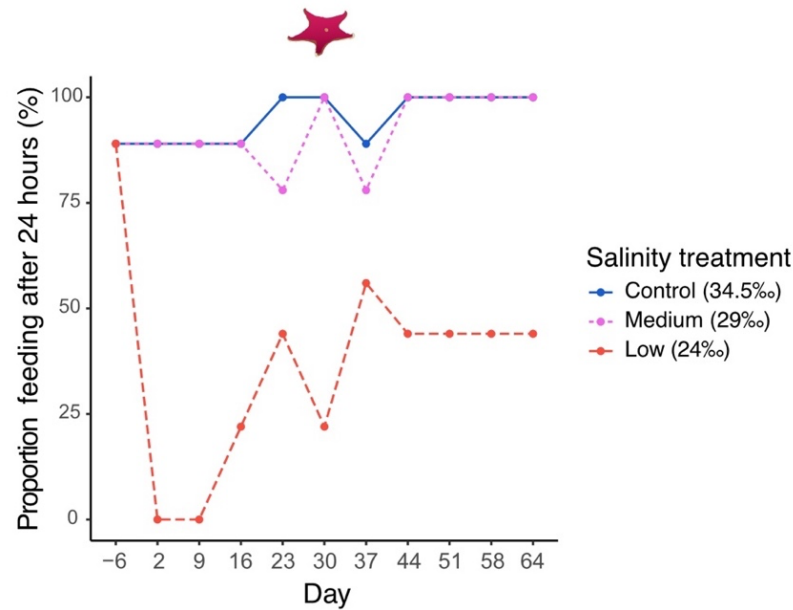


Figure 4.5. Percentage of *Odontaster validus* feeding 24 hours after food given over the experimental period.

4.4.8 Faecal production

S. neumayeri – Mean faecal production varied significantly between salinity treatments, time points and their interactions (Two-way ART ANOVA with repeated measures: Salinity $F_{(2,21)}=111.5$, $P<0.0001$; Time points $F_{(11,231)}=8.8$, $P<0.0001$; Salinity:Time points $F_{(22,231)}=6.2$, $P<0.0001$). At each time point, except on day -6 and 1, salinity had a significant impact on faecal production (One way ANOVA, Welch's ANOVA and Kruskal-Wallis: all $P<0.05$). Faecal production was significantly lower in low salinity compared to the control at all time points from day 8 (Tukey, Dunn and Games-Howell: all $P<0.006$), and the medium salinity at all time points from day 15 (Tukey and Games-Howell: all $P\leq 0.05$). Faecal production was significantly lower on day 22 in the medium group compared to the control (Games-Howell: $P=0.011$), but there were no significant differences at any other timepoint. (Figure 4.6; Table C2).

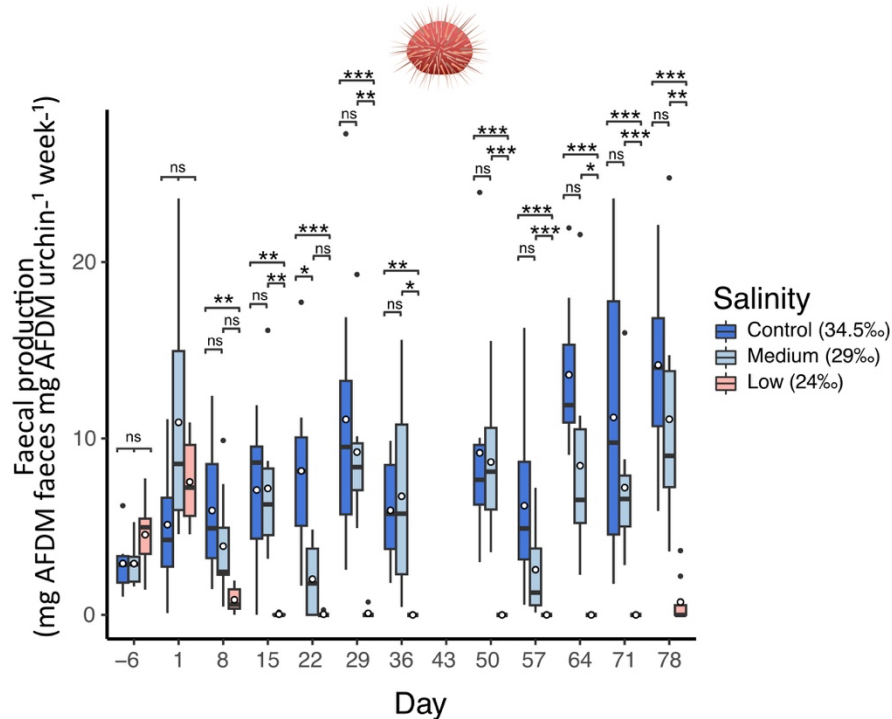


Figure 4.6| Rate of faecal production of *Stereochinus neumayeri* at time points during exposure to different salinities. Results are shown for one-way ANOVA/Welch's ANOVA/Kruskal-Wallis and relevant post hoc test between salinity treatments at each time point (n.s., not significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; $n = 8$ biological replicates at each time point and treatment). Box plots show medians (black line), upper and lower quartiles, and maximum and minimum (whiskers). Means are represented by open circles and outliers by filled circles.

4.4.9 Coelomic fluid osmolality

O. validus – Mean coelomic fluid osmolality differed significantly between salinity treatments at each sample time point (Two-way ANOVA: Salinity $F_{(2,30)} = 68903.22$, $P < 0.0001$; Tukey: all $P < 0.0001$). At each sample time point within each salinity treatment, coelomic fluid was always isosmotic or slightly hyperosmotic compared to the tank water. Hyperosmotic coelomic fluid was only significantly different (all one-sample t-tests) from tank water on day 65 in the control ($t_2 = 8.7$, $P = 0.01$) and days 8 ($t_2 = 5.3$, $P = 0.03$), 35 ($t_2 = 12.1$, $P = 0.007$), and 65 ($t_2 = 22$, $P = 0.002$) in medium salinity (Figure 4.7A; Table C1).

S. neumayeri – Mean coelomic fluid osmolality differed significantly between salinity treatments at each sample time point (Two-way ANOVA: Salinity $F_{(2,50)} = 44450.6$, $P < 0.0001$; Tukey: all $P < 0.0001$). At each sample time point within each salinity treatment, coelomic fluid was always isosmotic or slightly hyperosmotic compared to the tank water. Hyperosmotic

coelomic fluid was only significantly different (all one-sample t-tests) from the tank water on day 29 ($t_2=4.6$, $P=0.02$) and 85 ($t_2=3.2$, $P=0.03$) in the control, days 1 ($t_2=3.4$, $P=0.043$), 8 ($t_2=5.4$, $P=0.006$) and 15 ($t_2=4.8$, $P=0.009$) in medium salinity, and days 1 ($t_2=2.8$, $P=0.047$) and 85 ($t_2=3.8$, $P=0.02$) in the low salinity (Figure 4.7B; Table C2).

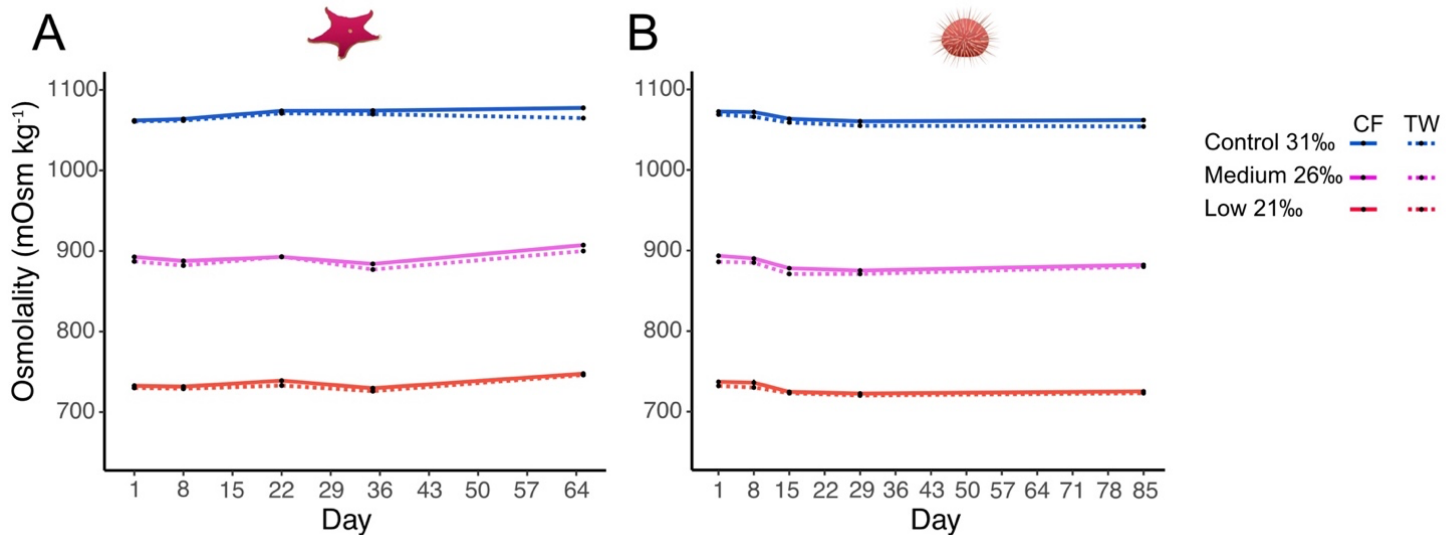


Figure 4.7| Coelomic fluid osmolality of A) *Odontaster validus* and B) *Stereochinus neumayeri* versus tank water osmolality. Data are mean $\pm$ s.e.m. coelomic fluid (CF; $n=4$ for *O. validus* and $n=5$ for *S. neumayeri* biological replicates for each salinity and time point) and tank water (TW; $n=3$ technical replicates for each salinity and time point) osmolality at time points during exposure to different salinity conditions.

4.4.10 Coelomocytes

S. neumayeri – Salinity had no significant impact on total coelomocyte number, percentage viable cells or percentage red spherulocytes (Table 4.1, Table C2).

Table 4.1| Coelomocyte analysis after 85 days of exposure of in *Sterechinus neumayeri* to different salinities.

Treatment	n	Mean test diameter (mm)	Mean wet weight (g)	Cell viability (%)	Red spherule cells (% of total coelomocyte concentration)	Total coelomocyte concentration (cells ml ⁻¹)	s.e.m.
Control (34.5‰)	5	23±1	5.7±0.7	96.58±1.2	10.42±4.3	1.45E+07	1.45E+07
Medium (29‰)	5	24±1.5	6.0±0.6	95.22±0.7	13.05±3.2	3.69E+06	8.25E+05
Low (24‰)	5	21±1.2	4.3±0.7	95.98±0.8	12.88±3.4	1.50E+06	5.31E+05

Data are means±s.e.m. There were no significant differences (one-way ANOVA) between salinity treatments in terms of cell viability ($F_{(2,12)}=0.31$, $P=0.74$), proportion of red spherulocytes ($F_{(2,12)}=0.31$, $P=0.74$) and total coelomocyte concentration ($F_{(2,12)}=3.88$, $P=0.05$).

4.4.11 Hypoosmotic shock

O. validus – Following the acclimation period, mean oxygen consumption after direct immersion in 19‰ varied significantly between treatments (One-way ANOVA: $F_{(2,15)}=7.6$, $P=0.005$), with a significantly higher rate in the low salinity compared to both control and medium (Tukey: both $P<0.04$); however, there was no significant difference in oxygen consumption between control and medium salinity. Both the control and medium salinities had significantly lower rates of oxygen consumption after immersion in 19‰ compared to their prior mean value on day 71 (Two sample t-test: Control $t_5=4.8$, $P<0.001$; Medium $t_{13}=4.4$, $P<0.001$), while there was no significant difference in the low salinity treatment (Figure 4.8A; Table C1).

There was no significant difference in mean AC between treatments following direct immersion in 19‰ (Figure 4.8C; Table C1). Righting was completed in 3 of 6 control, 5 of 6 in medium salinity and 4 of 6 in low salinity. Both the control and medium salinities had significantly lower mean AC values after immersion in 19‰ compared to their prior mean

value on day 71 (Two sample t-test: Control $t_8=6.8$, $P<0.001$; Medium $t_{13}=3.8$, $P<0.01$) while there was no significant difference in the low salinity treatment (Figure 4.8C; Table C1).

S. neumayeri – There were no significant differences in mean oxygen consumption between salinity treatments after direct immersion in 19‰ following the acclimation period. Oxygen consumption was significantly lower in the low treatment after immersion in 19‰ compared to the prior mean value on day 85 (Two sample t-test: $t_9=3.5$, $P<0.01$), however there were no significant differences in the medium and control treatments (Figure 4.8B; Table C2).

Following acclimation, mean AC values measured over 60 minutes were not significantly different between treatments after immersion in 19‰ (Figure 4.8D; Table C2). One animal from the low salinity group completed righting after 60 minutes, while none did in the control or medium treatments. However, mean AC values measured over 90 minutes were significantly different between treatments (Kruskal-Wallis: $\chi^2(2)=6.9$, $P=0.03$), with significantly faster righting times (higher AC values) in low salinity compared to the control and medium (Dunn: both $P<0.02$) (Figure C2; Table C2). Three urchins in the low treatment righted themselves within 90 minutes, while none in the control or medium treatments did so. Both the control and medium salinities had significantly lower mean AC values after immersion in 19‰ compared to their prior mean value on day 85 (Two sample t-test: Control $t_7=4$, $P<0.01$; Medium $t_7=8.3$, $P<0.0001$) while there was no significant difference in the low salinity treatment (Figure 4.8D; Table C2).

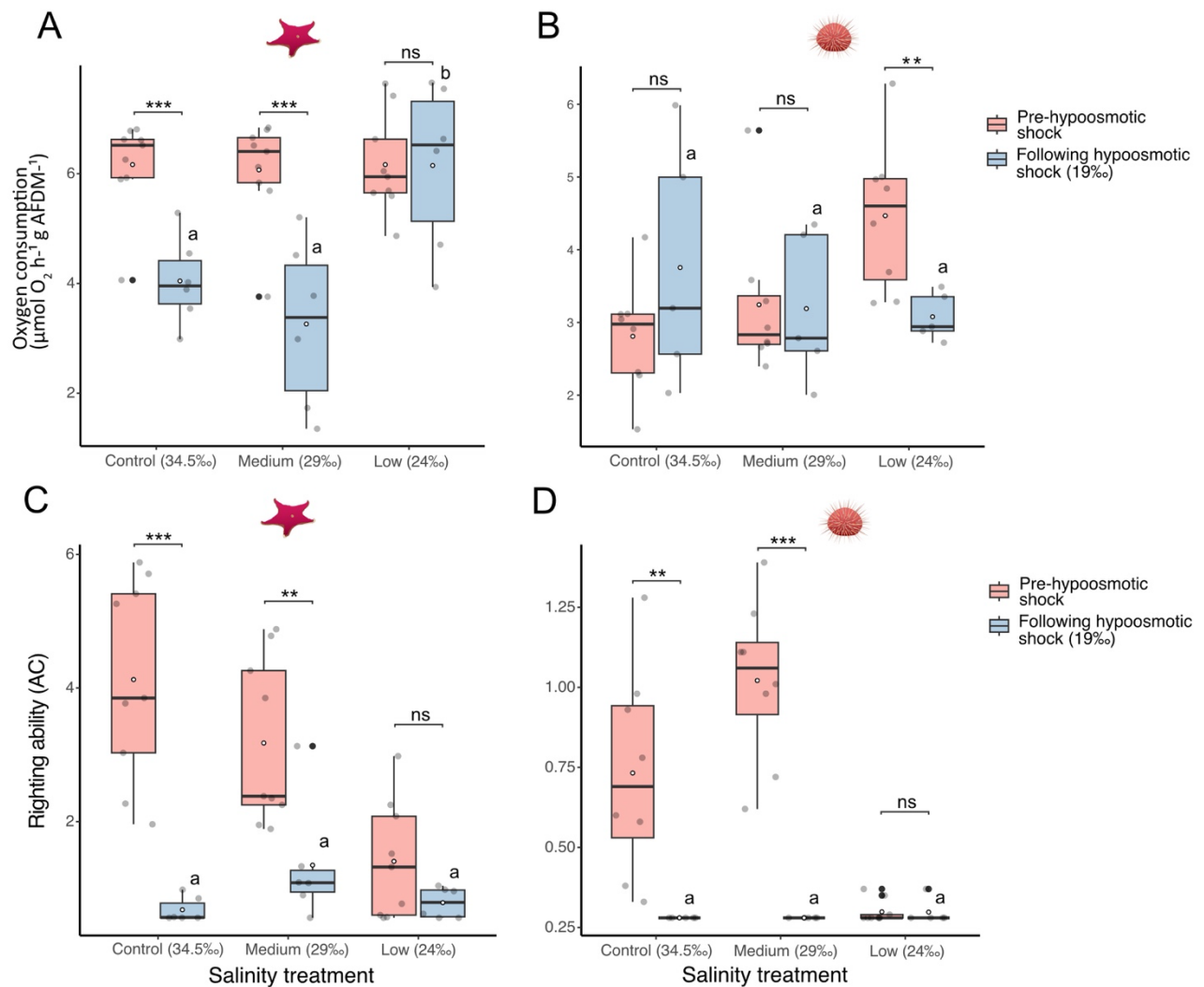


Figure 4.8| Hypoosmotic shock—animals were exposed to different salinity treatments for 71 days (*O. validus*) and 85 days (*S. neumayeri*) followed by an acute immersion in 19‰ salinity. Oxygen consumption of A) *Odontaster validus* and B) *Sterechinus neumayeri* before (red boxes: $n=9$ for *O. validus* and $n=8$ for *S. neumayeri* biological replicates) and after hypoosmotic shock (blue boxes: $n=6$ for *O. validus* and $n=5$ for *S. neumayeri* biological replicates) following acclimation to reduced salinity. Righting ability (activity coefficient (AC)) of C) *O. validus* and D) *S. neumayeri* before (red boxes: $n=9$ for *O. validus* and $n=8$ for *S. neumayeri* biological replicates) and after hypoosmotic shock (blue boxes: $n=6$ for *O. validus* and $n=5$ for *S. neumayeri* biological replicates) following acclimation to reduced salinity. Results are shown for two-sample t -test between pre-hypoosmotic shock and following hypoosmotic shock for each salinity treatments (n.s., not significant; * $P<0.05$, ** $P<0.01$, *** $P<0.001$; and results for one-way ANOVA/Kruskal-Wallis and post hoc Tukey test between salinity treatments following hypoosmotic shock (different letters indicating significant differences $P<0.05$). Box plots show medians (black line), upper and lower quartiles, and maximum and minimum (whiskers). Biological replicates are represented by grey circles, means by open circles and outliers by filled black circles.

4.5 Discussion

The capacity to acclimate in response to rapid environmental change is considered of paramount importance for slow-growing organisms with long generation times, such as Antarctic marine species, to counter the threat posed by climate change. (Peck et al., 2014; Somero, 2010). An ability to acclimate to low salinity, has clearly been demonstrated in both species of Antarctic echinoderm in this study, with animals in the medium salinity treatment (29‰) showing no significant behavioural or physiological differences compared to control animals by the end of the experimental period. However, at the lower salinity (24‰), although survival was high, both species displayed responses which suggested that the stabilisation of physiological functions had not occurred within the experiment.

4.5.1 Mortality, buoyant weight change and percentage AFDM

Overall mortality rates were relatively low indicating a high capacity to tolerate the lowest salinity for an extended period in both species. Barrett et al. (2024b; Chapter 3) demonstrated acute 24-hour LD₅₀ limits to be 13.4‰ for *O. validus* and 16.5‰ for *S. neumayeri*, substantially below the low salinity level tested in this study. However, in the current study mortality rates were higher in both low salinity groups by the end of the experimental period compared to control animals (significantly higher in *O. validus*), suggesting extended exposure at 24‰ may be unsustainable and below acclimation limits for both species. This is exemplified by significant reductions in mass observed in both species at low salinity, indicating impaired growth and likely tissue catabolism. These findings mirror observations from previous studies on chronic exposure to low salinity in both echinoids [*Echinus esculentus* (Chapter 2; Barrett et al., 2024a); *Psammechinus miliaris*, and *Strongylocentrotus droebachiensis*, (Podbielski et al., 2022)] and asteroids [*Asterias rubens* (Podbielski et al., (2022); *Leptasterias hexactis* (Shirley and Stickle, 1982a)].

4.5.2 Oxygen consumption, activity, feeding and faeces production

In both species, oxygen consumption remained at similar levels to control animals for the first 71 days under reduced salinities. Under acute hypoosmotic shock, Barrett et al. (2024b; Chapter 3) also demonstrated no change in oxygen consumption at 29‰ in both species and at 24‰ in *S. neumayeri*. However, in *O. validus* they observed a significant reduction in oxygen consumption at salinities ≤ 24 ‰, which was also a common pattern in other Antarctic echinoderm species (Chapter 3; Barrett et al., 2024b). A reduction in metabolic rate in response to stress is considered a mechanism to conserve energy and allocate resources to core homeostatic functions (Sokolova et al., 2012b). The return to metabolic rates similar to controls at 24‰ in *O. validus* over a longer period in the current study suggests either a stabilising adjustment due to acclimation or additive costs related to osmoregulation that balance out the overall metabolic rate. Exposure to chronic low salinity in temperate echinoderms have caused varying response patterns in regard to oxygen consumption. Reductions were observed in the echinoid *L. hexactis* when exposed to 28 days at 15‰ and 20‰ (Shirley and Stickle, 1982b) and *S. droebachiensis* after 14 days at 15‰ (Sabourin and Stickle, 1981). However, Barrett et al. (2024a; Chapter 2) demonstrated increased oxygen consumption in the echinoid *E. esculentus* after 5 days at low salinity (21‰), which persisted over the 25-day experiment. For *S. neumayeri*, there was a significant rise in oxygen consumption for animals held at low salinity by day 85 (similarly observed in the repeat experiment on day 78), matching the response pattern seen in *E. esculentus* by Barrett et al. (2024a; Chapter 2), but only after a substantially extended period of stasis. An increase in oxygen consumption implies an increase in energy requirements for metabolic functions. As echinoderms are osmoconformers with extracellular fluids that are generally isosmotic with the external medium, acclimation involves active cellular volume regulation to adjust the osmotic and ionic balance within cells (Rivera-Ingraham and Lignot, 2017). These changes may include modifications to membrane-bound transporters, structural changes and amino acid metabolism, all incurring metabolic costs (Gilles and Delpire, 1997; Podbielski et al., 2022b; Rivera-Ingraham and Lignot, 2017). Resting metabolic rates in Antarctic marine invertebrates are generally substantially lower than for temperate and tropical species (Peck, 2005), which consequently means a slower accumulation of detrimental metabolic waste, while stored reserves can stretch further (Peck et al., 2010). The substantial delay in the rise in oxygen

consumption for *S. neumayeri* at low salinity, compared to the relatively rapid increase seen in the temperate urchin *E. esculentus*, suggests that Antarctic urchins were able to offset the increased metabolic demands of osmoregulation. Instead, their low metabolic rate as a consequence of the cold environment, enabled reliance on stored reserves for a protracted period before reserves ran critically low (demonstrated by the loss in organic matter), and homeostatic metabolic demands increased. Barrett et al. (2024b; Chapter 3) demonstrated that metabolic rates in multiple Antarctic echinoderm species increase at critical lower salinity limits, suggesting that *S. neumayeri* in the current study were near failure. Indeed, mortality reached ~25% at the time of increased oxygen consumption.

Although metabolic rates across the first 71 days appears to be unaffected by reduced salinity, activity and feeding rates were severely impacted under the lowest salinity in both species. In both species a similar pattern was observed; significant reductions in AC after the first 8-15 days in the low salinity treatment compared to control, with levels remaining low over the first 50 days. This was followed by slight increases in AC towards the later weeks, especially in *O. validus*, however, rates did not return to control levels. Reductions in AC have been demonstrated in both species under acute hypoosmotic stress (Chapter 3; Barrett et al., 2024b). For *O. validus* this was at $\leq 24\text{‰}$ (Chapter 3; Barrett et al., 2024b) differing from the current study on day 1 (where AC rates were undistinguishable across treatments), but consistent from day 8, suggesting that the stepwise dilution process provides time for cellular volume regulation to occur. AC rates for *S. neumayeri* under acute low salinity (Chapter 3; Barrett et al., 2024b) were consistent with the current study over the first two weeks of treatment. Reduced righting rates are a frequent observation in echinoderm salinity studies upon exposure to reduced salinity [e.g., in echinoids: *S. droebachiensis* (Moura et al., 2023a; Sabourin and Stickle, 1981), *E. esculentus* (Chapter 2; Barrett et al., 2024a) and asteroids *Lytechinus variegatus* (Lawrence, 1975) and *Luidia clathrata* (Forcucci and Lawrence, 1986)]. Barrett et al. (2024a; Chapter 2) and Sabourin and Stickle (1981) demonstrated that echinoderms could not return to control AC levels after chronic low salinity exposure, a finding similar to both Antarctic echinoderms in the current study. The inability to recover righting performance under chronic low salinity implies a reduction in functional capacity and suggests that energy allocation to activity is constrained (Stickle and Diehl, 1987). This is likely due to an increase in homeostatic maintenance as a result of elevated osmoregulatory costs. Even a significant uptake in oxygen consumption at the end of the experimental period in *S.*

neumayeri did not correlate with increased AC rates, similar to the findings for *E. esculentus* (Chapter 2; Barrett et al., 2024a). This implies a tipping point has been reached where there is a physiological limitation to resisting the continued impact of salinity stress and suggests that continued exposure, at least in *S. neumayeri*, will result in failure.

Feeding was impacted directly after step-wise dilution to low salinity and failed to recover to comparable control rates in both species. Similar results were observed in the echinod *E. esculentus* following exposure to low salinity over 25 days (Chapter 2; Barrett et al., 2024a), while a reduction in feeding in response to low salinity has also been demonstrated in the asteriods *L. hexactis* (Shirley and Stickle, 1982a), *Pisaster ochraceus* (Held and Harley, 2009) and *Luidia clathrata* (Forcucci and Lawrence, 1986). Since feeding in urchins and sea stars involves neuromuscular coordination (Hyman, 1955), the parallel reduction in activity suggests an increasing inability to feed due to decreased movement, as previously observed in echinoderms under hypoosmotic stress. (Chapter 2; Barrett et al., 2024a; Forcucci and Lawrence, 1986; Held and Harley, 2009).

The cessation of feeding in *S. neumayeri* under low salinity (24‰) was reflected in the cessation of faeces produced. Under medium salinity (29‰) there was a reduction in faecal production after the first three weeks, with subsequent recovery to control levels. However, mean faecal production remained consistently lower than the control in the medium animals from day 57, although this was not significant. Changes in faecal production as result of environmental change can reflect the speed of movement of food through the gut as reported in *S. neumayeri* (De Leij et al., 2022). As feeding in the medium salinity treatment was very similar to rates in the control, the lower mass of faeces produced, at least on day 22, suggests either a slower movement of food through the gut or a greater absorbance of organic material as a response to reduced salinity. The subsequent return to levels similar to controls , indicates successful acclimation after the initial adjustment.

In both species at low salinity, there was an increase in feeding after an initial drastic reduction. In *O. validus*, this was observed from day 22; however, it appears that just under half of the animals exhibited this response (Figure 4.5), suggesting a degree of acclimation in some individuals. In *S. neumayeri* there was an increase in the last two weeks of the experiment, coinciding with an increase in oxygen consumption compared to control levels. The specific dynamic action of feeding (SDA) which represents the energy expended on ingestion, digestion and absorption of food is often characterised by a rapid rise in metabolic

rate following feeding (Secor, 2009). Although measures to minimise the effect of SDA on oxygen consumption were taken by leaving a five day gap between the removal of food and respirometry measurements being made, Antarctic marine ectotherms are known to have post-prandial rises in metabolism that persist for considerably longer than those in warmer species (Peck, 1998). This, in addition to the likely slower movement of food through the gut (as seen in the medium treatment) may go some way to accounting for the observed higher rates of oxygen consumption in *S. neumayeri*. However, the drastic reduction in organic body composition by over one-third in the low salinity urchins suggests that high catabolism – needed to counter the lack of calorie intake while maintaining metabolic rates similar to controls – may ultimately prove fatal over longer periods if the high metabolic rates persist.

4.5.3 Coelomic fluid osmolality and coelomocytes

In both species coelomic fluid values were consistently isosmotic or slightly hyperosmotic to the treatment salinity, comparable to findings by Barrett et al. (2024b; Chapter 3) for both species and the general pattern found in other echinoderms (Chapter 2; Barrett et al., 2024a; Diehl, 1986; Vidolin et al., 2007). The maintenance of near-isosmotic osmolality with the treatment salinity throughout the experiment confirms that osmoconforming strategies are present in both species. Exposure to long periods of low salinity had no observable effects on the innate immune cells in *S. neumayeri* suggesting that urchin immune system functioning is unlikely to be affected by environmental freshening. However, comparisons of total coelomocyte concentration were borderline significant between treatments, which may warrant further investigation with a higher sample size. Changes in coelomocyte composition have been reported in response to acute hypoosmotic stress in echinoids (Honorato et al., 2017), however, similar findings to the current study were observed in the temperate urchin *E. esculentus* over 25 days at low salinity (Chapter 2; Barrett et al., 2024a).

4.5.4 Hypoosmotic shock

Exposure to hypoosmotic shock after the experimental acclimation period had differing responses between species. In *O. validus*, the reduction in oxygen consumption in

the medium and control group mirrored the results observed by Barrett et al. (2024b; Chapter 3) for direct immersion from the acclimated salinity to 19‰ over 24-hours. This likely reflects the suppression of all metabolic activity to devote all available resources to homeostatic maintenance in a bid to prolong survival, while cell volume re-balancing takes place (Sokolova et al., 2012b). In the low salinity animals, the maintenance of oxygen consumption rates similar to their pre-exposure values could mean one of two things. Either, there is sufficient energy available to maintain homeostatic functioning without the need to further suppress metabolic activity; or, suppression of non-essential functions (e.g., growth, reproduction, storage etc.) is accompanied by an increase in homeostatic functions related to cellular volume re-balancing [e.g., metabolism of intracellular free amino acids (Goolish and Burton, 1989)], resulting in no net change in oxygen consumption. For *S. neumayeri*, the reduction in oxygen consumption in the low group could be indicative of the suppression of metabolic activity to prolong survival. Metabolic depression has previously not been recorded in *S. neumayeri* following acute low salinity immersion (Chapter 3; Barrett et al., 2024b). However, since oxygen consumption of the low salinity group was elevated prior to the hypoosmotic shock, the values were only suppressed back to the control group levels, or 'normal' levels.

AC levels in the *S. neumayeri* low group were substantially higher than either medium or control animals after hypoosmotic shock within 90 minutes (see Figure C2). The ability to mount a righting response in 19‰ indicates sufficient energy reserves available. As the osmotic change is substantially less from 24‰ to 19‰ than from either 29‰ or 34.5‰, there is likely to be less swelling and consequently less immediate stress. This was also observed in *O. validus* with 4 of 6 animals from the low group able to complete righting, providing a higher mean AC compared to the control (Low=0.79±0.09; Control=0.68±0.08, mean±s.e.m, $n=6$). Although this comparison was not significant, the fact that righting was still possible indicates sufficient energy was available. The significantly higher AC in the medium group compared to the control implies that, despite the apparent metabolic depression, energy could still be allocated to righting. This matches results observed in the echinoid *E. esculentus* where there was an improved ability to right in animals acclimated to reduced salinity (Chapter 2; Barrett et al., 2024a). Perhaps the smaller osmotic change on immersion in 19‰ and the resulting reduced osmotic stress, allow sufficient energy to be allocated to righting in the low salinity group, which is considered a critical function (i.e., offering immediate protection from predation) with a high failure threshold (De Leij et al., 2022). The exception occurs when the

osmotic gradient is large, likely causing significant swelling and temporarily impeding the ability to right.

4.5.5 Ecological context and conclusions

Both echinoderm species in this study were collected from Ryder Bay, Adelaide Island, on the WAP at a depth of 10-20m. Although subtidal salinity (15 m) in the bay has remained relatively stable over the past 20 years [e.g., $33.4 \pm 0.4\text{‰}$ (mean $\pm$ s.d.) between 2001 and 2021], surface salinity (0 m) occasionally drops to $\sim 28.5\text{‰}$ (Clarke et al., 2022). Closer to the shoreline, and especially in enclosed regions, meltwater runoff can reduce salinity to $<12\text{‰}$ (Stockton, 1984; Vargas-Chacoff et al., 2021a; Waller et al., 2006a). The extensive fjordic systems of the WAP, have been described as biodiversity hotspots for Antarctica benthic fauna due to high productivity, low impact of meltwater intrusion and low sedimentation disturbance (Grange and Smith, 2013). Increased warming and glacial melting, in addition to increased precipitation in the region (González-Herrero et al., 2024; Hellmer et al., 2011) can substantially increase the extent and thickness of low salinity layers in fjords (Bianchi et al., 2020). This may lead to long-term reductions in salinity in the fjords of the WAP, similar to the low salinities observed in the Arctic, such as those found in inner Greenland fjords where salinity levels can drop below 25‰ at depths of up to 10 meters (Sejr et al., 2017; Sejr et al., 2022). Based on the current study, reductions in salinity to 29‰ appear to be well tolerated by both echinoderms species. Successful acclimation to 29‰ was evident across all metrics measured by the end of the experimental period, with no differentiation between the medium and control groups. In most cases, this occurred directly after the stepwise dilution, implying that osmotic adjustments were rapid and not detrimental to the energy budget of either species. Similar results were observed in the temperate echinoid *E. esculentus* in a mid-level salinity within their tolerance range (26‰ previously acclimatised to 31‰), using a comparable experimental set up to that used here, but over a shorter timespan (Chapter 2; Barrett et al., 2024a). In the current study, the lower salinity of 24‰ was clearly tolerated by both species, however, acclimation was absent in both. *Odontaster validus* appeared the more tolerant of the two at 24‰ , based on the return to feeding in some animals, improvement in righting and stable oxygen consumption, which is consistent with it having a lower 24-hr low salinity threshold compared to *S. neumayeri* (Chapter 3; Barrett et al., 2024b).

However, it was apparent that the increased rate of feeding in *O. validus* from day 22, was only exhibited by just under half the sea stars at 24‰ (see Figure 4.5). It is unknown whether a return to previous control levels of feeding and righting ability would have been possible if the experiment had continued for longer, but it seems likely that only a subset of the population would have achieved this. From an evolutionary perspective, if a significant proportion of a population has the capacity to acclimatise, selection for these traits under increased freshening may enable longer term survival, especially for species with very large population sizes such as *O. validus* and *S. neumayeri*. However, caution should be applied to evaluating surviving individuals that have not displayed full physiological acclimation, especially in polar marine ectotherms, where innate low metabolic rates can mask other critical changes (Peck et al., 2010).

For both species, persistent coastal freshening may induce migration to deeper, higher salinity waters, relinquishing the need for acclimation capacity. Considering both are keystone species, changes in community structure as a result of migration may disrupt ecosystem interactions and food web dynamics (Sporta Caputi et al., 2024). However, neuromuscular coordination was substantially reduced under low salinity in the current study, indicating that migration abilities may still depend on the capacity to acclimate.

Future research on longer-term survival, growth and reproductive capabilities of Antarctic echinoderms under low salinity would now be beneficial. However, this would be logistically challenging, requiring multi-year, multi-generation experimental systems to incorporate full lifecycles. A fuller picture of acclimation could be provided by examining genetic and biochemical level changes utilising different ‘omics approaches such as RNA-seq and metabolomic analysis, which have yet to be examined in Antarctic echinoderms. Attention should also focus on how low salinity interacts with other climate change stressors—such as temperature, pH, and oxygen saturation—which may have additive or synergistic effects on marine organisms (Barrett et al., 2022; Gunderson et al., 2016). Multiple stressor studies on Antarctic echinoderms have yet to include low salinity, which has proven to significantly modify fitness in other Antarctic species when combined with other stressors (Navarro et al., 2020; Park et al., 2020).

What may be an advantage to Antarctic echinoderms in tolerating long durations of low salinity compared to their temperate species, is the ability to maintain low metabolic rates, with *S. neumayeri* having one of the lowest on record for a regular echinoid

(Brockington, 2001). Indeed, the temperate echinoid *E. esculentus*, which has a resting metabolic rate at least three times greater than *S. neumayeri* displayed elevated oxygen consumption after only five days at low salinity compared to controls (Chapter 2; Barrett et al., 2024a). The low temperatures experienced in the Antarctic marine environment, enable these echinoderms to maintain low metabolic rates under reduced salinity for a substantial period (i.e., > 2.5 months in the current experiment). Low metabolic rates require lower calorific input from feeding, and instead can be sustained through metabolising stored reserves, as demonstrated by the loss of mass. These adaptations are well known to be advantageous in the Antarctic winter environment when food is scarce. For example *S. neumayeri* can survive for up to six months without feeding, in part due to their very low metabolic rate (Brockington, 2001). Under long-term reduced salinity, this ability could allow sufficient time for a return to better salinity conditions or greater time for acclimation adjustments.

In summary, two of the most abundant macroinvertebrates in Antarctic benthic ecosystems *O. validus* and *S. neumayeri*, demonstrate long-term tolerance to the reduced salinities that may occur due to climate change-induced freshening. Both species successfully acclimated to 29‰, but at 24‰, while survival remained relatively high, neither species had fully acclimated after more than 2.5 months. This suggests that 24‰ is near the physiological limits of both species, and prolonged exposure could lead to population decline which may impact ecosystem dynamics. Nevertheless, the inherently low metabolic rates of Antarctic echinoderms may allow them to endure extended seasonal low salinity, which may be advantageous under a warming climate.

Chapter 5 – Low salinity acclimation and intracellular osmolyte adaptations in two Antarctic echinoderms: a metabolomic approach

This chapter utilised samples from Chapter 4 to investigate biochemical mechanisms underlying acclimation in two Antarctic echinoderms. External collaborators contributions included: Prof Roy Goodacre and Dr Adam Burke at the Centre for Metabolomics Research (CMR) at The University of Liverpool. Prof Roy Goodacre and Dr Adam Burke developed and drafted part of the methodology (section 5.2.3.3 to 5.2.3.7). Dr Adam Burke carried out the sample extraction process, GC-MS analysis and data processing. I was the primary investigator and contributor to all aspects of this research. Additional funding for this work was gained through the NERC NEOF Pilot Competition; grant number NEOF1503.

The data from this chapter is available upon request:

https://github.com/njb202/Chapter_5

5.1 Introduction

Micromolecules (low-molecular-mass molecules found in biological systems e.g., inorganic ions, small organic molecules, protons etc.) have been demonstrated to play numerous and overlapping roles in biological processes such as protein stability, osmotic regulation, freeze tolerance/avoidance and desiccation resistance (Olsson et al., 2016; Somero et al., 2017). For example, in osmoconforming invertebrates, small organic molecules, termed ‘osmolytes’ are utilised to control cell volume regulation (Somero and Yancey, 2011; Somero et al., 2017). In osmoconformers, extracellular fluids are maintained isosmotic to external seawater [see Chapters 2 and 4; Barrett et al. (2024a)], with intracellular fluids following the same pattern (Yancey, 2005). At average strength seawater (~35‰), osmoconformers accumulate organic osmolytes within the cell cytoplasm in a colligative manner (i.e., based on their quantity over their intrinsic qualities) to balance intracellular osmolality with that of the extracellular fluids

and external seawater (Somero and Yancey, 2011). Four classes of organic osmolytes are commonly identified: free amino acids and their derivatives (e.g., glycine, alanine, proline, taurine etc.), polyols (e.g., glycerol, trehalose etc.), methylammonium and methylsulfonium compounds (e.g., glycine-betaine, dimethylsulphoniopropionate (DMSP) etc.) and urea (Podbielski et al., 2022a; Somero et al., 2017; Yancey, 2005). Upon exposure to lowered salinity, organic osmolytes can be reduced through efflux, catabolism or synthesis of proteins and other macromolecules (Gilles, 1987), effectively lowering intracellular solute concentration to regulate cellular volume and prevent swelling.

Parallel to their colligative role in cellular osmoregulation, the specific properties of accumulated osmolytes can play a substantial role in promoting protein stability within the intracellular environment (Arakawa and Timasheff, 1985; Somero et al., 2017; Taneja and Ahmad, 1994) and act as thermoprotectants under heat or cold stress (Somero et al., 2017). If intracellular ionic balance were maintained solely using inorganic ions (such as K^+ and Cl^-) at full strength seawater (~35‰), protein function and stability would be severely compromised (Somero and Yancey, 2011; Somero et al., 2017), while also leading to DNA double strand breaks (Dmitrieva et al., 2006). Instead, organic osmolytes with non-perturbing properties are accumulated and referred to as compatible osmolytes (Yancey, 2005). For example, osmolytes with strong hydrophobic properties are considered disruptive to protein stability, as hydrophobic interactions increase the degree of protein denaturing (Taneja and Ahmad, 1994). Furthermore, the hydrophobic effect is enhanced with increasing temperatures and concentration (Sun et al., 2022). Therefore, the selection of organic osmolytes with low hydrophobic properties (e.g., glycine) is likely beneficial for both euryhaline and eurythermal marine organisms (Somero and Yancey, 2011).

In addition to their intrinsic properties that function to stabilise proteins under heat or cold stress, osmolytes can also function in a colligative manner to lower the freezing point in organisms to prevent freezing. As an example of supercooling—lowering the temperature of a liquid below its thermodynamic freezing point—, some organisms such as insects and plants, but also some Arctic teleost fish (Raymond, 1992), accumulate high quantities of polyols (in particular glycerol) and sometimes amino acids, in extracellular and intracellular fluids when exposed to sub-zero conditions (Ansart and Vernon, 2003; Lee, 2010; Raymond, 1992; Somero et al., 2017). This has the effect of maintaining body fluids in a liquid state below their freezing point and thus avoid freezing (Ansart and Vernon, 2003; Somero et al.,

2017). Antarctic notothenioid fish also use the colligative action of solutes for freezing point depression, in combination with antifreeze glycoproteins (DeVries and Wohlschlag, 1969; Peck, 2015). Although not considered as common in marine invertebrates as in insects (Ansart and Vernon, 2003), the use of organic solutes as cryoprotectants has been suggested by some researchers [e.g., taurine, strombine and alanine in the blue mussel *Mytilus edulis* (Loomis et al., 1988; Williams, 1970)].

For marine animals living in permanently cold environments, such as Antarctic marine ecosystems, synthesising and retaining functional proteins is considered to be substantially harder than for temperate and tropical species (Fraser et al., 2022). This may be due to decreases in protein stability at low temperatures and difficulties in protein folding associated with the increased viscosity at temperatures below 0°C (Peck, 2016; Peck, 2018). In Antarctic marine species, adaptations at the macromolecular level are believed to play a key role in attempts to minimise the challenge of protein synthesis. For example, Antarctic marine species have substantially higher ratios of RNA-to-protein compared to temperate or tropical species, suggesting there is an overcompensation of RNA production to account for inefficiencies in peptide synthesis (Peck, 2018). The role of micromolecules in stabilising proteins and facilitating protein folding in Antarctic marine animals has been largely unexplored. However, evidence from other cold-adapted species suggests that the composition of the osmolyte pool plays a crucial thermoprotective role in maintaining protein structure under extreme conditions (Somero et al., 2017; Stefels, 2000). For example, the organic osmolyte DMSP and its breakdown products, are compounds found mainly in species of marine algae and some plants, but also corals, and play important roles in osmoregulation, ROS detoxification and coral-to-microbe signalling (Somero et al., 2017; Stefels, 2000). In the polar macroalga *Acrosiphonia arcta*, strong upregulation of DMSP and increased enzyme stability have been observed after exposure to low temperature, suggesting it acts as a protein cryoprotectant (Stefels, 2000).

The sea star *Odontaster validus* and the sea urchin *Sterechinus neumayeri* are both widespread inhabitants of the nearshore Antarctic benthic ecosystem, with a circumpolar distribution (Dell, 1972; Pearse, 2013). The seasonal melting of sea-ice, precipitation and glacial and ice-sheet meltwater, all contribute to the lowering of Southern Ocean seawater salinity, which is predicted to further reduce under climate change (IPCC, 2022a; Haumann et al., 2016). Both species have shown moderate tolerance to acute low salinity (Chapter 3;

Barrett et al., 2024b), the ability to acclimate to a reduced salinity of 29‰ and can tolerate a lower salinity of 24‰ for extended periods (Chapter 4). However, the mechanisms underlying this resilience have only been explored in studies on temperate echinoderms (e.g., Podbielski et al., 2022b; Schmittmann, 2017). In temperate echinoderms and other marine osmoconforming invertebrates, the primary mechanism is believed to be cellular volume regulation, achieved by reducing the concentration of organic osmolytes within the intracellular pool (Podbielski et al., 2022a; Somero et al., 2017). In the polar environment of the Antarctic, osmolytes may also function colligatively as freezing point depressants (e.g., DeVries and Wohlschlag, 1969; Peck, 2015). Both species demonstrate supercooling abilities, capable of tolerance down to a median air temperature of $\sim -7^{\circ}\text{C}$ (Waller et al., 2006b). However, the mechanisms enabling this are unknown. In Antarctic echinoderms, osmotic stress, freeze avoidance, and challenges in protein synthesis and stability may shape the specificity of a species micromolecular osmolyte profile. This profile likely results from overlapping functions and is subject to strong selective pressure from multiple environmental stressors (Somero et al., 2017). Our current understanding and knowledge of adaptations at the micromolecular level in Antarctic marine animals is very limited, yet these species are likely to face some of the strongest global pressures to develop specific micromolecular profiles that fulfil multiple and overlapping roles.

The aim of this chapter is to investigate the organic osmolyte profile of tissue from *O. validus* and *S. neumayeri* acclimated to different salinity regimes, using gas chromatography-mass spectrometry (GC-MS). The aims were fourfold: 1) assess if organic osmolyte concentration decreases during acclimation to low salinity, 2) assess if osmolyte profiles in Antarctic echinoderm species differ from non-polar species, 3) identify which osmolytes are utilised for cell volume regulation, and 4) assess whether osmolytes have overlapping functions related to osmoregulation, freeze tolerance and protein stability.

5.2 Methods

5.2.1 Experimental animals

Odontaster validus and *S. neumayeri* were collected in the austral summer of 2021/2022 by SCUBA divers at depths between 10-20 m near the British Antarctic Survey's (BAS) Rothera research station, Adelaide Island (67°34'07"S, 68°07'30"W). For more details see Chapter 4.

5.2.2 Salinity acclimation experiment

Both species were exposed to three salinity treatments: 34.5‰ (control), 29‰ (medium salinity) and 24‰ (low salinity). Salinity dilutions were achieved via stepwise reductions at 2‰ per day using deionised water until experimental salinities were reached. Experiments were maintained over a 71-day (*O. validus*) and 85-day (*S. neumayeri*) period to evaluate acclimation capacity of the two species. For each treatment, 8 animals of each species were isolated in floating trays and split between four replicate tanks (12 tanks in total). For a detailed methodology see chapter 4 (note that the *O. validus* animals used in this experiment were maintained in the same *S. neumayeri* tanks).

5.2.3 Tank effects

An analysis to identify tank effects on each species determined that there was no significant effect (see Appendix C, Table C1 and C2), and therefore, each individual was treated as a biological replicate.

5.2.4 Metabolomics

5.2.4.1 Tissue extraction

Eight individual replicates per species and treatment were selected for metabolomic sampling, with the exception of the *O. validus* low salinity treatment, where there was a single mortality resulting in only seven samples. In *O. validus* the pyloric caecum was selected for tissue analysis because of its accessibility and large size. For *S. neumayeri* gut tissue was chosen as this provided the largest somatic organ. Following the acclimation experiment, tissue was dissected from live animals, washed in phosphate-saline buffer to remove coelomic fluid and immediately flash frozen in liquid nitrogen and stored at -80°C. Samples

were freeze dried for at least 24 hours until at constant mass (± 0.01 mg). A ~ 30 mg sample was aliquoted into a separate 2 ml homogenisation tube. Lyophilised samples were stored at -80°C then shipped frozen to the Centre for Metabolomics Research (CMR) at The University of Liverpool for further processing, GC-MS analysis, and statistical evaluation. Dr Adam Burke carried out the sample extraction process, GC-MS analysis, and data processing.

5.2.4.2 Sample Processing

The methodology below (section 5.2.3.3 to 5.2.3.7) was used to prepare and analyse *O. validus* and *S. neumayeri* samples. It was originally written and developed by Prof Roy Goodacre and Dr Adam Burke at the CMR. Sample processing, method optimisation and data acquisition were carried out by Dr Adam Burke.

5.2.4.3 Extraction

A solvent mixture of 80% methanol and water was prepared and chilled on dry ice for one hour. Samples were taken from storage at -80°C and transferred to dry ice for immediate processing. The extraction solvent was added to each sample vial at a volume of 50 μL to normalise the sample concentrations. Samples were homogenised using a Fisherbrand Bead Mill 24 with two 10-second bursts at 6 m/s, with an 8-second dwell time between bursts. Following homogenization, samples were immediately placed on dry ice and centrifuged at $30130g$ for 15 minutes at 4°C . A fixed volume of 800 μL of supernatant (tissue extracts) was collected from each sample and transferred into fresh 2 mL microcentrifuge tubes. Samples were spiked with 50 μL of an internal standard mix (1.67 mg/mL of each succinic acid- d_4 , L-lysine- d_4 , glycine- d_5 and L-alanine- d_7 in water) before vortex mixing for 30 s. An additional 65 μL of each sample was aliquoted into a sample pool. The sample pool was then sub-aliquoted into fresh 2 mL tubes for use as quality control (QC) injections. Process blanks were generated by labelling empty vials and following the same extraction procedure as samples. Samples, QCs and process blanks were briefly spun in a centrifuge to remove liquid from vial lids. The vials were then placed into a Savant SpeedVac SPD130DLX, connected to a Savant vapor trap RVT5105, (ThermoFisher Scientific) for eight hours to lyophilise, and subsequently stored at -80°C until further processing.

5.2.4.4 Derivatisation

The lyophilised samples were split into randomised batches prepared on consecutive days. A two-step derivatisation procedure was followed immediately before analysis by GC-MS to enhance metabolite volatility and stability through methoximation and trimethylsilylation. Samples were removed from -80°C storage and placed into the vacuum centrifuge for one hour to remove residual water. Dried extracts were redissolved in 50 µL of a 20 mg/mL *O*-methoxylamine hydrochloride in pyridine solution, vortex mixed for 10 s, and placed into a digital heat block (Eppendorf) for 40 min, 65°C. 50 µL *N*-acetyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) was then added to each sample, vortex mixed for 10 s, and placed into a digital heat block (Eppendorf) for 40 min, 65°C. The volumes of the derivatisation agents were reduced for samples with insufficient supernatant volume during the aliquot step. QC extracts and process blanks were derivatised at the same time.

5.2.4.5 Retention Index Ladder

A retention index marker was prepared using a Kovats alkane ladder. Solution containing 0.3 mg/mL of each decane, dodecane, pentadecane, nonadecane and docosane in anhydrous pyridine was prepared and 50 µL added to each sample vial. Samples were vortex mixed for 10 s and centrifuged at 15 800 *g* for 15 min. Then, 100 µL supernatant was transferred to high recovery GC-MS vials for analysis.

5.2.4.6 Instrument methods

GC-MS instrument calibration and tuning was completed prior to sample analysis (see Table D1 for equipment details). A system suitability test mixture, containing 10 standard components, was used to manually evaluate instrument performance. The run order for each extraction batch was randomized. Column conditioning began with the injection of seven QCs, followed by a process blank and the samples, with additional QC injections used to bracket the samples. The run sequence is shown in Appendix D (Table D2). A 1 µL aliquot of the derivatised sample was injected using an autosampler through a split/splitless inlet set at

280°C, with a 30:1 split ratio for tissue samples. To avoid contamination through sequential needle solvent washes and avoid bubble entrainment, autosampler parameters were utilised. A constant helium carrier gas flow of 1 mL/min was maintained with electronic pressure control through the GC column. An oven temperature gradient of 70°C, 4 min hold, 20°C/min to 300°C, 4 min hold, was programmed, with a 300°C transfer line temperature. The mass spectrometer was operated at 70eV ionisation energy, 200°C source temperature, 40-600 m/z acquisition range at 10 Hz. At the end of each batch, retention index peaks were manually inspected to ensure that peak shape, height, and retention time were consistent and free of any systematic drift.

5.2.4.7 Data Processing

A list of software versions is shown in Appendix D (Table D3). The raw data in vendor format was converted to mzML open format using MSConvert, with a peak picking filter applied to extract centroid data. The files were then imported into MS-DIAL for peak deconvolution and alignment. Data collection focused on the acquired mass range with a retention time filter of 4-20 minutes to exclude the solvent front. Peak detection was carried out in accurate MS mode, with masses associated with alkanes and trimethylsilyl moieties added to an exclusion list. A retention index dictionary was created by manually integrating peaks from the injected Kovats ladder using MassHunter Qualitative Analysis. The corresponding retention times for each compound were recorded in an index file, which was then uploaded into MS-DIAL. For peak annotation, a retention index tolerance of 20 and m/z tolerance of 0.75Da was utilised, with an EI similarity score cut-off of 70%. The public KovatsRI-VS3 reference library was used, concatenated with purchased NIST, Fiehn and internally generated libraries (Table D3).

After deconvolution, alignment, and peak annotation, each peak spot was manually reviewed for quality. This involved checking the integrated peak shape, extraction ion chromatograms, raw and deconvolved peak spectra, and alignment results. Peak annotations were verified for accuracy by examining the closest matches and manually comparing retention index similarity and EI similarity scores. All annotations were assigned with two orthogonal properties (spectral and retention index matches) to achieve MSI Level 2 confidence, unless otherwise specified in the processed data matrices. Labelled internal standards were used for quality

checks by evaluating deconvolved peak area and retention index consistency across samples. The internal standard L-lysine-*d*4 was selected as the alignment target and utilised for intensity normalisation. Pooled quality control based locally weighted scatterplot smoothing (LOWESS) normalisation was applied to correct for drift and batch effects, in addition to blank subtraction based on a five-fold change threshold sample average/blank average filter.

Raw and processed data were exported from MS-DIAL into a tabular matrix that included peak annotations, retention time, assigned retention index, and peak area. Peaks were exported without filtering to ensure that potentially significant features were not inadvertently excluded. The processed data were then reformatted into TidyData format and saved as an Excel xlsx file before being loaded into QCMxP software for post-normalization quality checks.

5.2.5 Statistics

The full data matrix data was log transformed and checked for normal distribution (see Appendix D, Table D4). One-way analysis of variance (ANOVA) and a Fisher's LSD post-hoc analysis were carried out for each metabolite using an FDR corrected *P* value threshold of ≤ 0.05 . These analyses were performed in MetaboAnalyst 6.0. Heatmaps were generated in R (version 4.2.3) using the pheatmap package (1.0.12) on significantly differentially expressed metabolites (DEMs) after the removal of likely synthetic compounds and unknown metabolites (Table D4).

Metabolites identified as possible organic osmolytes were subjected to one-way ANOVA tests and Tukey post-hoc analysis to compare mean total organic osmolytes (sum of peak values for each osmolyte) between salinity treatments in each species (Table D5). Assumptions of normality and equal variance were tested using the Shapiro-Wilk test and Levene's test respectively. This was carried out in R (version 4.2.3).

5.3 Results

5.3.1 Metabolites

In total, 209 annotated metabolites were identified across both species tissue spectra, with an additional 106 unknown metabolites. Unknown metabolites, synthetic compounds and those that were unlikely to be naturally occurring (artefacts and contaminants) were removed. Derivatives of the same molecules (e.g., Serine_2TMS_minor and Serine_3TMS_major) were combined into one metabolite entry, resulting in a total of 121 metabolites.

5.3.2 Univariate data

Statistical analysis of univariate data showed numerous significantly differentially expressed metabolites (DEM) between different salinity treatments in each species. Multi-group data subjected to one-way ANOVAs with Fisher's LSD post-hoc analysis (FDR corrected P value threshold ≤ 0.05) revealed 8 significantly DEMs in *O. validus* tissue and 20 DEMs in *S. neumayeri* tissue. In *O. validus*, 4 were downregulated and 4 upregulated in response to low salinity, while in *S. neumayeri* 12 were downregulated and 8 upregulated in response to low salinity (Figure 5.1).

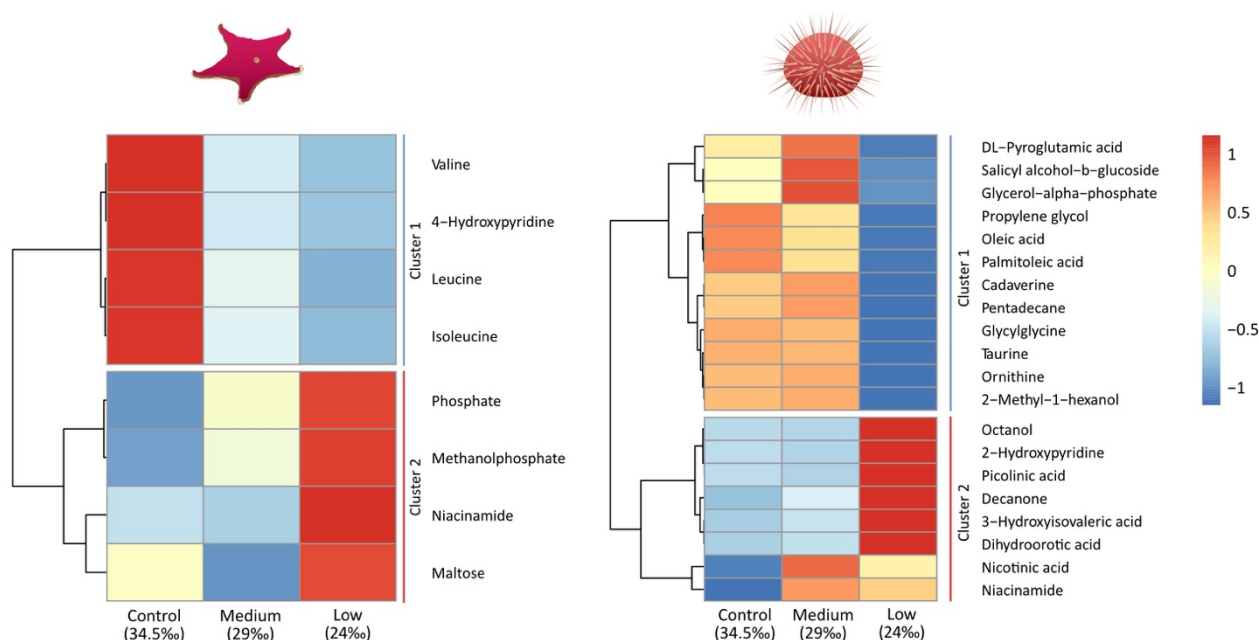


Figure 5.1| Heat map showing significantly differentially expressed metabolites in *Odontaster validus* pyloric caecum (left) and *Stereichinus neumayeri* gut tissue (right) among three salinity treatments after acclimation. Columns show the mean normalised peak expression for animals ($n=8$, except *O. validus* at 24‰ $n=7$) in each salinity treatment, and the rows show each metabolite. Colour scale indicates relative expression, red represents high expression and blue low expression. There were two main clusters in each heatmap; cluster 1 shows metabolites downregulated in response to low salinity and cluster 2, metabolites upregulated in response to low salinity.

5.3.3 Organic osmolytes

The 121-metabolite dataset was filtered to remove compounds that were unlikely to be osmolytes (e.g., alcohols, fatty acids and hydrocarbons etc) resulting in 56 organic compounds that could function as osmolytes, and are from here on referred to as osmolytes (Table 5.1). Within the 56 identified osmolytes, 3 were significantly downregulated in *O. validus*: valine, leucine and isoleucine, while 2 were significantly upregulated, maltose and niacinamide. In *S. neumayeri* 6 were significantly downregulated: glycylglycine, cadaverine, taurine, glycerol-alpha-phosphate, propylene glycol and ornithine, while niacinamide was upregulated. The mean sum total of peak values within each salinity treatment were considered to be the total intracellular organic osmolytes pool (total pool). Osmolyte concentrations that significantly decreased with decreasing salinity were assigned as major osmolytes (>5% of the total pool), intermediate osmolytes ($\geq 1\%$ to $\leq 5\%$ of the total pool) and minor osmolytes (<1% of the total pool) following similar approaches in Somero and Yancey (2011) and Podbielski et al. (2022b).

Utilising the normalised mean peak data, osmolytes were ordered by decreasing value (i.e., their relative concentration) at control salinity to demonstrate their contribution to the intracellular osmolyte pool (Table 5.1). The top 20 constituted 98.34% and 98.31% of the total osmolytes pool for *O. validus* and *S. neumayeri* respectively (Figure 5.2 and 5.3). Within the top 20 osmolytes in *O. validus* tissue, there were three major osmolytes; valine (8.2% of total pool), leucine (7.3% of total pool) and isoleucine (5.4% of total pool). Collectively these represented 20.9% of the total pool at control salinity dropping to 5.4% at low salinity (Table 5.1). Within the top 20 osmolytes in *S. neumayeri* tissue, there were three intermediate osmolytes, these were glycylglycine (3.1% of total pool), cadaverine (2.3% of total pool) and taurine (1.1% of total pool). Collectively these represented 6.4% of the total pool at control salinity, dropping to 3.9% at low salinity (Table 5.1). Minor osmolytes identified in the total pool in *O. validus* were niacinamide and maltose contributing <0.04% to the total pool. In *S. neumayeri*, minor osmolytes were glycerol-alpha-phosphate, propylene glycol and ornithine collectively contributing <0.3% to the total pool.

Chapter 5

Table 5.1| The 56 organic compounds identified through GC-MS analysis and assigned as osmolytes, with relative percentage contribution to the total intracellular osmolyte pool in each salinity treatment. Note that for each species the osmolytes are ordered from top to bottom by largest to smallest percentage contribution using the control treatment. Significantly differentially expresses osmolytes in response to salinity (one-way ANOVA: $P \leq 0.05$) are highlighted; blue = downregulated, red = upregulated.

<i>Odontaster validus</i>				<i>Stereichinus neymayeri</i>			
Osmolytes	Control %	Medium %	Low %	Osmolytes	Control %	Medium %	Low %
Glycine	38.13	45.81	48.48	Glycine	55.79	54.38	63.23
Alanine	14.40	14.26	12.27	Alanine	12.08	11.73	6.00
Valine	8.20	3.27	2.26	Valine	5.39	4.93	3.42
Leucine	7.27	3.31	1.63	Leucine	4.38	4.35	2.43
Isoleucine	5.37	2.51	1.55	Glycylglycine	3.06	2.96	1.93
Serine	4.92	3.51	3.95	Isoleucine	3.01	3.25	2.73
Glycylglycine	3.18	3.38	4.26	Cadaverine	2.27	2.40	1.53
Threonine	3.14	1.90	2.54	Homoserine	1.89	2.22	3.62
Cadaverine	2.26	3.02	4.02	Aminomalonate	1.88	2.22	3.62
Taurine	2.20	5.03	4.23	Glutamate	1.84	2.16	2.06
Homoserine	1.98	2.51	2.37	Serine	1.17	1.33	1.52
Aminomalonate	1.98	2.51	2.81	Taurine	1.10	1.09	0.45
Phenylalanine	1.61	1.78	1.54	Hypotaurine	1.03	1.05	1.14
Glutamate	1.02	1.18	1.29	Threonine	0.94	1.12	1.27
L-2-Aminobutyric acid	0.80	0.35	0.33	Proline	0.87	0.96	0.81
Proline	0.64	0.88	0.69	Hexitol	0.54	0.47	0.98
Hypotaurine	0.34	0.39	0.24	Methionine	0.33	0.33	0.19
Glutamic acid	0.32	0.89	1.21	Phenylalanine	0.29	0.31	0.21
Inositol	0.32	0.85	1.22	Myo-Inositol	0.26	0.25	0.41
Tyrosine	0.25	0.29	0.18	Glutamic acid	0.19	0.40	0.28
Aspartic acid	0.18	0.36	0.32	Glycerol-alpha-phosphate	0.15	0.21	0.11
Lysine	0.18	0.22	0.27	Inositol	0.13	0.17	0.36
Tyramine	0.18	0.22	0.27	Tyrosine	0.12	0.13	0.06
Acetyl alanine	0.13	0.35	0.66	Glucose	0.11	0.10	0.10
Myo-inositol delta	0.09	0.24	0.33	Lysine	0.08	0.10	0.05
b-Alanine	0.08	0.07	0.07	Tyramine	0.08	0.10	0.05
Asparagine	0.08	0.05	0.05	3-Aminoisobutyric acid	0.07	0.10	0.18
3-Aminoisobutyric acid	0.07	0.05	0.04	Propylene glycol	0.07	0.06	0.05
1,3-Diaminopropane	0.07	0.09	0.10	L-2-Aminobutyric acid	0.07	0.05	0.03
Ornithine	0.07	0.06	0.07	Dopamine	0.07	0.07	0.07
2-Aminoethanol	0.06	0.06	0.06	Maltose	0.07	0.05	0.05
Glycerol-alpha-phosphate	0.05	0.05	0.05	Asparagine	0.06	0.08	0.06
Methionine	0.05	0.03	0.02	UDP-N-acetylglucosamine	0.06	0.07	0.09
Hexitol	0.04	0.04	0.07	Cysteamine	0.05	0.11	0.05
Myo-Inositol	0.04	0.04	0.05	Aspartic acid	0.05	0.14	0.05
Diglycerol	0.03	0.10	0.06	N-Methylnicotinate	0.05	0.07	0.19
UDP-N-acetylglucosamine	0.03	0.04	0.04	Glutathione	0.05	0.07	0.09
Gamma-Linolenic acid	0.03	0.02	0.04	Myo-inositol delta	0.04	0.04	0.07
Glutathione	0.03	0.05	0.05	Lactic acid	0.04	0.04	0.05
Galactitol	0.02	0.02	0.01	2-Aminoethanol	0.04	0.04	0.05
Niacinamide	0.02	0.02	0.05	b-Alanine	0.03	0.06	0.03
Maltose	0.02	0.01	0.04	Acetyl alanine	0.02	0.03	0.06
Cysteine	0.02	0.03	0.03	Ornithine	0.02	0.02	0.01
Lactic acid	0.02	0.02	0.02	Sarcosine	0.02	0.03	0.10
Glucose	0.01	0.01	0.02	Niacinamide	0.02	0.03	0.04
Creatinine	0.01	0.04	0.01	GABA	0.02	0.01	0.02
Sarcosine	0.01	0.01	0.05	Ciliatine	0.02	0.02	0.02
Propylene glycol	0.01	0.01	0.01	Galactitol	0.01	0.01	0.02
Dopamine	0.01	0.01	0.01	Diglycerol	0.01	0.01	0.03
Ciliatine	0.01	0.02	0.01	Cysteine	0.01	0.01	0.01
GABA	0.01	0.01	0.01	Gamma-Linolenic acid	0.01	0.01	0.01
Cysteamine	0.00	0.00	0.00	1,3-Diaminopropane	0.01	0.01	0.01
2-Aminoadipic acid	0.00	0.03	0.02	Creatinine	0.01	0.01	0.01
N-Methylnicotinate	0.00	0.00	0.00	2-Aminoadipic acid	0.00	0.00	0.00
Fructose 6-phosphate	0.00	0.00	0.00	Fructose 6-phosphate	0.00	0.00	0.00
Kynurenic acid	0.00	0.01	0.00	Kynurenic acid	0.00	0.00	0.00

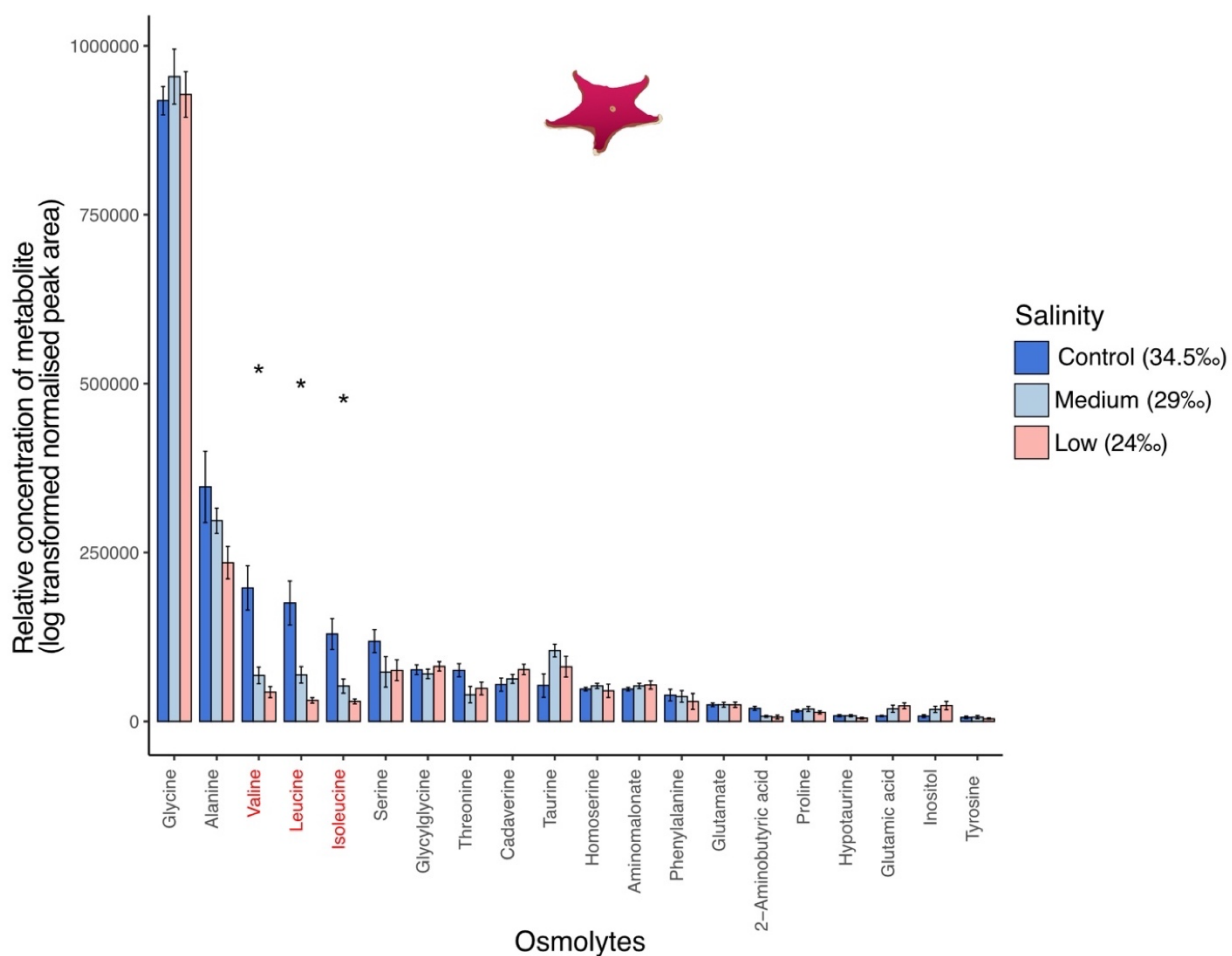


Figure 5.2| Top 20 osmolytes within the total intracellular organic osmolyte pool based on their relative concentration (log transformed peak values) at control salinity (34.5‰) in *Odontaster validus* pyloric caecum tissue. Asterisk and red names specify significant results of one-way ANOVA ($P \leq 0.05$) and FDR *post hoc* test for differences between treatments.

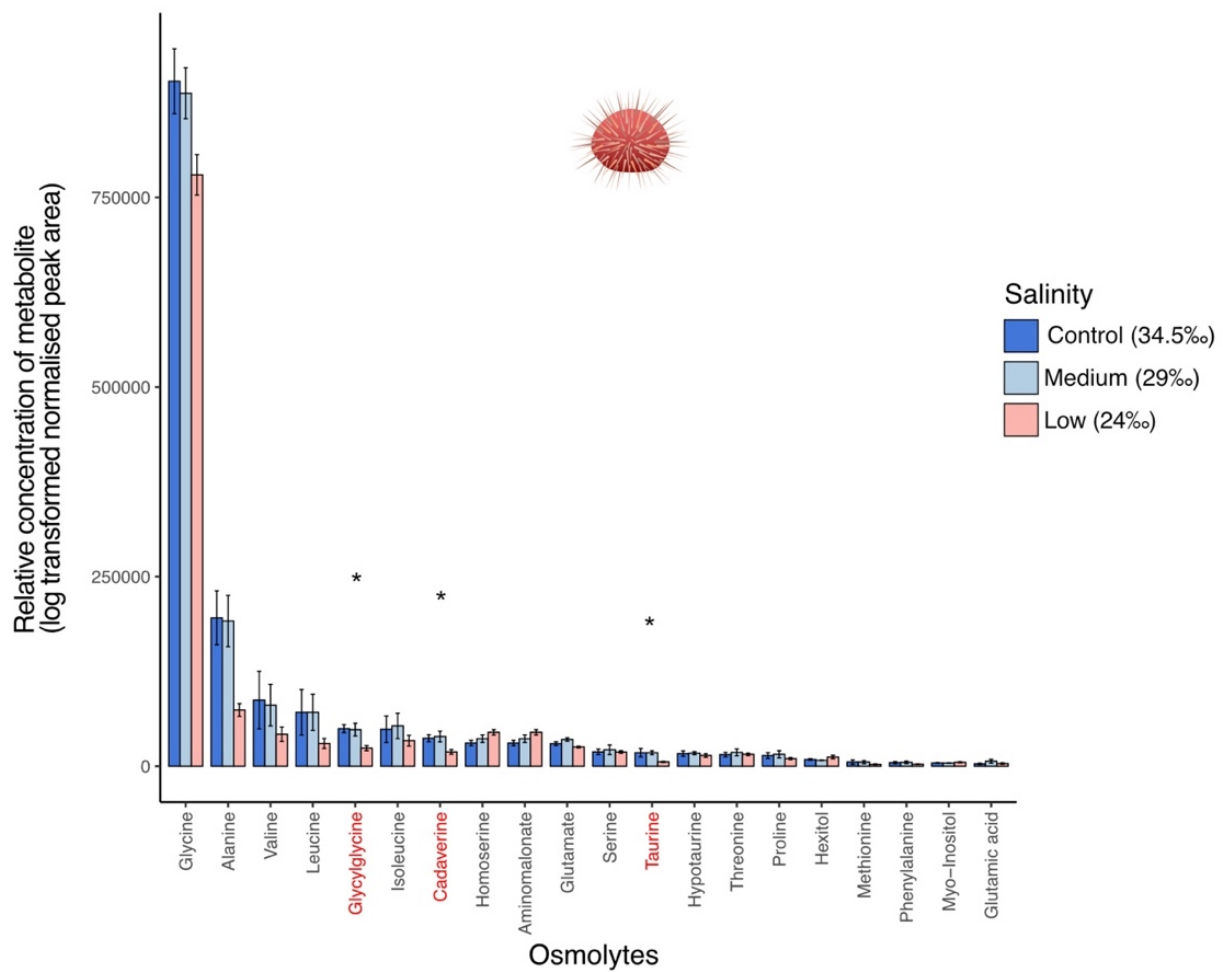


Figure 5.3| Top 20 osmolytes within the total intracellular organic osmolyte pool based on their relative concentration (log transformed peak values) at control salinity in *Sterechnus neumayeri* gut tissue. Asterisk and red names specify significant results of one-way ANOVA ($P \leq 0.05$) and FDR *post hoc* test for differences between treatments.

5.3.4 Total osmolyte pool

The mean sum total of peak values for all osmolytes from each treatment varied significantly with salinity in *O. validus* (One-way ANOVA: $F_{(2,20)}=8.3$, $P < 0.01$) and *S. neumayeri* (One-way ANOVA: $F_{(2,21)}=6$, $P < 0.01$) (Figure 5.4; Table C2). In *O. validus* there were significant decreases in total osmolyte peak values between control and both reduced salinity treatments (Tukey 34.5‰ v 29‰, $P=0.035$; 34.5‰ v 24‰, $P < 0.001$). In *S. neumayeri* there were significant decreases in total osmolyte peak values between control salinity and low salinity, and between medium and low salinity (Tukey 34.5‰ v 24‰, $P=0.02$; 29‰ v 24‰, $P < 0.02$) (Figure 5.4; Table C2).

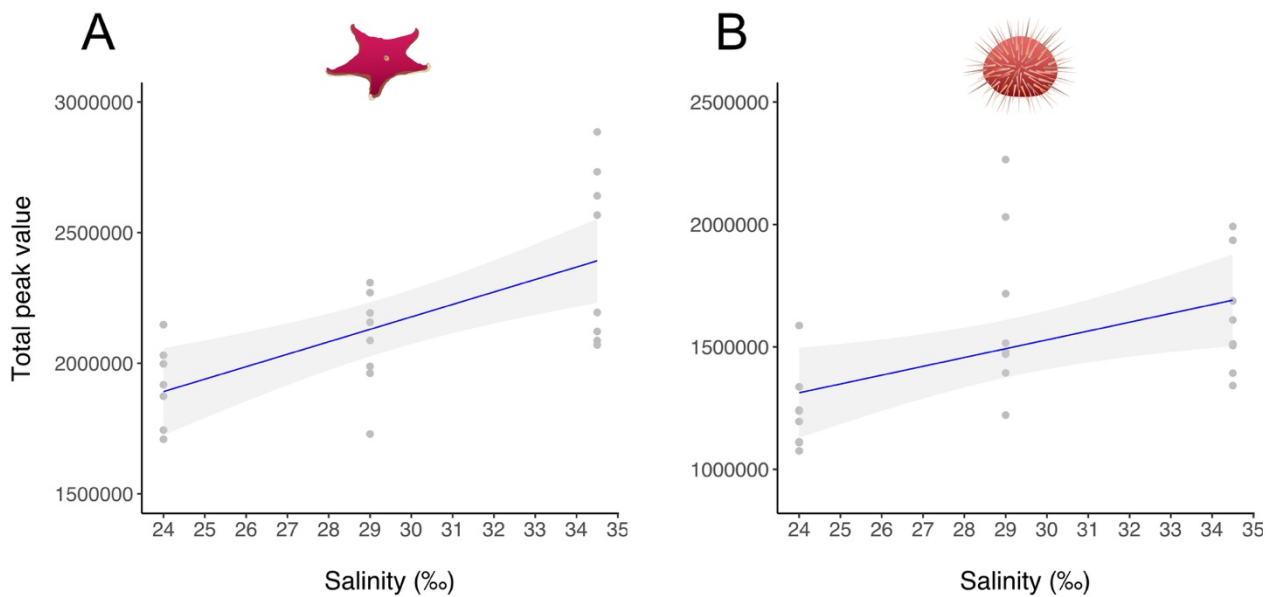


Figure 5.4| Total intracellular osmolyte pool (sum of peak values of all osmolytes) measured at different salinity treatments for A) *Odontaster validus* pyloric caecum tissue and, B) *Stereochinus neumayeri* gut tissue. Grey circles represent each sample ($n=8$, except *O. validus* at 24‰ $n=7$). A linear regression line (blue line) and the standard error of the mean (shaded grey area) were fitted to the data.

5.4 Discussion

5.4.1 Cellular osmoregulation

The total organic osmolyte concentration (total pool) decreased in tissue from both species in response to reduced salinity, suggesting that Antarctic echinoderms utilise organic osmolytes in controlling volume regulation (aim 1). This is in agreement with previous studies on temperate echinoderms, including *Asterias rubens* and *Psammechinus miliaris* and *Strongylocentrotus droebachiensis* (Podbielski et al., 2022b; Schmittmann, 2017). Both species in the current study exhibited similar metabolite profiles concerning those that contributed most to the total osmolyte pool, despite differences in the specific osmolyte used for osmoregulation. For example, the top four osmolytes contributing to the total pool, were the same in both species: glycine, alanine, valine and leucine (see Figures 2 and 3, and Table 1). Similarities in the composition of organic osmolyte pools across temperate echinoderms has been highlighted by Podbielski et al. (2022b), who demonstrated almost identical

osmolyte pool composition between echinoids *P. miliaris* and *S. droebachiensis*, and only a slight difference with the asteroid *A. rubens*. In all three temperate echinoderms, glycine followed by serine were the highest contributing osmolytes to the total pool (Podbielski et al., 2022b), differing from the Antarctic echinoderms in the current study (aim 2).

Cellular volume regulation was driven by valine, leucine and isoleucine in *O. validus*, while in *S. neumayeri* glycylglycine, cadaverine and taurine had the largest effect (aim 3). Interestingly, there were no similarities in the selection of osmolytes between species. The results in both species deviate from previous findings from other echinoderms and marine invertebrates, as none of the free amino acids normally associated with cellular volume regulation were significantly reduced (Podbielski et al., 2022a; Yancey, 2005). Previous studies have demonstrated that glycine is the main osmolyte used in cell volume regulation in temperate echinoderms (Podbielski et al., 2022b; Schmittmann, 2017), and commonly used by many other marine invertebrates such as blue mussels *Mytilus* spp. (Podbielski et al., 2022a; Podbielski et al., 2022b; Sanders, 2018). Other osmolytes of intermediate and minor significance in echinoderms include alanine, aspartate, leucine, serine, and threonine (Podbielski et al., 2022a). The widespread use of glycine across various taxa is consistent with it being the simplest amino acid and least inhibitory to enzyme function (Bowlus and Somero, 1979). The absence of the use of glycine as an osmolyte in volume regulation in the current study is surprising. Interestingly, glycine makes up a substantial proportion of the free amino acid osmolyte pool in both *O. validus* (38%) and *S. neumayeri* (56%). These values are similar to levels recorded in temperate echinoderms at their highest salinity examined, for example in *A. rubens* [~40% of the pool at 23‰ (Podbielski et al., 2022b)] and in *P. miliaris* and *S. droebachiensis* [40-50% of the pool at 28‰ and 30‰ respectively (Podbielski et al., 2022b)]. The absence of its use as an osmoregulatory osmolyte in the current study, may be related to the range of salinity values measured for acclimation. For example, previous studies have examined changes in osmolyte profiles over different ranges of salinities, [e.g., Podbielski et al. (2022b) measured between 29‰ to 15‰ in *P. miliaris* and 30‰ to 17‰ in *S. droebachiensis*]. Podbielski et al. (2022b) demonstrated that, across a wide salinity range (5‰ to 30‰) with multiple osmoconforming marine invertebrates, the specific osmolytes used for volume regulation shifted in response to particular salinity thresholds. In the current study, the lowest acclimated salinity was 24‰, which may have obscured the use of the more commonly identified osmolytes (e.g., glycine and alanine) for volume control at low salinities.

This may also have reduced the statistical power for analysis for differentially expressed osmolytes. For example, in the current study, alanine was the second largest component of the osmolyte pool in both species and showed a mean reduction in response to salinity although this was not statistically significant. Podbielski et al. (2022b) observed that alanine (and other intermediate osmolytes) was mainly implemented as a regulatory osmolyte at low to medium salinities, which may account for the absence of a significant response in the current study.

Previous metabolomic approaches investigating osmolytes in echinoderms (e.g., Podbielski et al., 2022b; Schmittmann, 2017) have used proton nuclear magnetic resonance ($^1\text{H-NMR}$), which differs from the current study which uses GC-MS. Although $^1\text{H-NMR}$ offers detailed molecular structural analysis, it is less sensitive and precise than GC-MS (Bjerrum, 2015) which likely accounts for the higher number of metabolites observed in the current study and made direct comparisons of metabolite profiles challenging.

5.4.2 Osmoregulatory osmolytes in *O. validus*

The three major osmolytes in *O. validus*, valine, leucine and isoleucine have previously not been identified as major osmolytes in echinoderms or other marine invertebrates, although valine and leucine have been identified as minor osmolytes (Podbielski et al., 2022a). Their presence as major osmolytes in *O. validus* is surprising. Previous researchers have hypothesised that solutes with long alkyl chains, which increase their degree of hydrophobicity, would not be selected as osmolytes due to their tendency to bind to nonpolar regions of proteins, increasing the probability of protein unfolding (Arakawa and Timasheff, 1985; Somero and Yancey, 2011). Valine, leucine and isoleucine are the only three branched-chain proteinogenic amino acids (BCAAs) (Singh and Shaner, 1995), and are known for their high hydrophobicity (Taneja and Ahmad, 1994). In contrast, osmolytes like glycine and alanine exhibit negligible to low hydrophobicity, which contributes to protein stability by protecting against thermal denaturation on warming (Arakawa and Timasheff, 1985). However, at lower temperatures, the strength of hydrophobic interactions decreases, due to the increased structuring of water as it approaches freezing (Arakawa et al., 1990). This phenomena is widely believed to be responsible for the concept of ‘cold denaturation’ in proteins (Dias, 2012). For solutes with both hydrophobic and hydrophilic properties, it has been proposed

that they can stabilise proteins at low temperatures and hence act as cryoprotectants (Arakawa et al., 1990). As hydrophobic interactions weaken with falling temperatures, the increased hydrophilic character of the solute helps maintain the protein in its 'native' folded state (Arakawa et al., 1990). For example the organic solute dimethylsulfoniopropionate (DMSP) widely used by marine phytoplankton and bacteria as an osmolyte, has been demonstrated to be both destabilising to protein structure with increasing temperatures, and can act as protein cryoprotectant at lower temperature (6°C) (Nishiguchi and Somero, 1992). The BCAAs also have dual hydrophobic and hydrophilic properties, with the non-polar alkyl groups providing hydrophobic properties while the amino and carboxyl groups are hydrophilic (Holum, 1998). For *O. validus*, which lives at temperatures permanently close to 0°C, the selection of BCAAs as osmolytes may reflect their potential use as protein cryoprotectants. While hydrophobic interactions generally weaken at lower temperatures, the relatively greater hydrophobicity of BCAAs could confer some stability compared to osmolytes with lower hydrophobic characteristics. This contrasts with the typically destabilising effect of hydrophobic side chains on protein structure at higher temperatures—a factor that is unlikely to pose a problem for Antarctic species living in consistently low-temperature environments. However, many amino acids possess greater hydrophilic properties than the BCAAs (Holum, 1998). This observation raises the question as to why cells would preferentially accumulate larger, more metabolically expensive amino acids for volume regulation rather than smaller, less costly alternatives.

An explanation may be attributed to their energy-rich branched side chains, which could serve as an alternative energy source during osmotic acclimation. The catabolism of organic osmolytes is considered one of the methods that marine osmoconformers can use to reduce their osmolyte pool under cellular volume regulation (Gilles, 1987). Rather than reducing solutes through efflux into the coelomic fluid, which might be considered wasteful, the catabolism of BCAAs could support energy metabolism during osmotic stress. In vertebrates, the catabolism of BCAAs during exercise produces intermediates that integrate into the TCA cycle, contributing to ATP production (Shimomura et al., 2004). The selective reduction of BCAAs in *O. validus* after low salinity acclimation, may therefore fulfil the role of cellular volume regulation while also providing a source of energy. The absence of any intermediate BCAA breakdown metabolites under reduced salinity may reflect the stability of the osmolyte profile at the time of sampling (~10 to 12 weeks of exposure to low salinity) as

osmolyte adjustments are likely to take place within the first two weeks (Baginski and Pierce, 1977). Beyond osmotic stress, a common issue faced by Antarctic echinoderms in the winter months is low food abundance (Brockington, 2001; Pearse, 2013). The accumulation of BCAAs within the osmolyte pool may serve as a seasonal energy reserve, where their conversion to simpler osmolytes [e.g., the BCAAs can be converted to alanine (Monirujjaman and Ferdouse, 2014)] provides a valuable energy source while maintaining osmotic balance at time of low food availability. In summary, the high strength hydrophobic properties of the BCAAs may provide a stabilising effect on protein structure at low temperature. This allows them to be accumulated for cell volume regulation and later metabolised as a valuable energy source during osmotic stress or at times of low food abundance (aim 4).

5.4.3 Osmoregulatory osmolytes in *S. neumayeri*

In *S. neumayeri*, the absence of any major osmolytes was unexpected, especially given that intermediate and minor osmolytes contributed only 6.7% to the total osmolyte pool. This suggests that the wider contribution came collectively from minor osmolytes and that there was less specificity in selection. Additionally, the choice of gut tissue for analysis may have been a shortcoming. Animal gut microbiomes play a significant role in amino acid metabolism for the host species while also utilising amino acids as a nitrogen source for themselves (Dai, 2011; Siyuan and Xumei, 2022). This interaction may have contaminated the gut samples, potentially compromising the reliability of the relative analysis of amino acid osmolytes and other compounds, thereby obscuring the effects of salinity acclimation on osmolyte profiles.

The osmolyte with the greatest contribution, glycylglycine is a simple dipeptide consisting of two glycine molecules and has previously not been identified as an osmolyte. However, it has been demonstrated to significantly enhance the solubility of recombinant proteins in *in vitro* bacterial systems used for recombinant antigen production (Ghosh et al., 2004; Shawky et al., 2024). Under low temperatures, protein solubility decreases because they are more prone to unfolding which promotes aggregation (Grossmann and McClements, 2023). The presence of glycylglycine in the cytoplasm of both *S. neumayeri* and *O. validus* at similar concentrations (~3.1% of total pool in each) may be an adaptation to cold environmental temperatures, where maintaining protein solubility may be challenging.

A second intermediate osmolyte identified in *S. neumayeri* was cadaverine, which has not previously been recognised as an osmolyte. Cadaverine (also known as 1,5-diaminopentane), a natural polyamine, has been shown to stabilise DNA macromolecules under conditions of molecular crowding caused by increased osmotic pressure—the result of osmolyte accumulation in response to heightened salinity stress (Petraccone et al., 2004). The reduction in cadaverine in *S. neumayeri* may be attributed to decreased osmotic pressure, which leads to reduced molecular crowding, rather than its potential role as an osmolyte.

Taurine was identified as an intermediate osmolyte in *S. neumayeri*. Its use as a regulatory osmolyte is common in many benthic marine invertebrates (Podbielski et al., 2022a; Somero and Bowlus, 1983). Compared to molluscs, which rely heavily on taurine as an osmolyte, its use in echinoderms is considered minimal (Podbielski et al., 2022a), however other studies suggest it plays a significant role in cellular volume regulation in the asteroid *A. rubens* (Jeuniaux et al., 1962; Schmittmann, 2017). Taurine was also present in *O. validus*, however it did not play a role in cellular volume regulation.

5.4.4 Upregulated organic solutes

In both species, niacinamide was significantly upregulated in response to reduced salinity, although its relative concentration was very small (0.05 and 0.04% of the total osmolyte pool at low salinity in *O. validus* and *S. neumayeri*, respectively). As a component of the cofactors NAD and NADP, integral in the oxidative-reduction reaction in multiple metabolic pathways (Cantó and Auwerx, 2013), its upregulation in response to low salinity acclimation may be related to an increased need for NAD production to facilitate an increased metabolic demand for energy and redox balance under osmotic stress. At the time of sampling, oxygen consumption was significantly higher in *S. neumayeri* (see Chapter 4) held at 24‰ salinity, potentially reflecting the rise in niacinamide. Interestingly, increases in oxygen consumption were not observed in *O. validus* at low salinity even though niacinamide was elevated.

5.4.5 Total osmolyte pool and coelomic fluid osmolality

Although the reduction in the total osmolyte pool peak values (total osmolyte pool concentration) in response to salinity was significant, the relative decrease was less

pronounced than anticipated given the observed reduction in mean coelomic fluid osmolality across treatments in both species (see Figure 4.7 in Chapter 4). Intracellular osmolality is expected to follow that of extracellular osmolality after acclimation (Kirschner, 1991; Somero and Yancey, 2011), therefore the change in osmolality should be relative to the change in total osmolyte pool concentration. For example, the difference in mean total peak value from 34.5‰ to 29‰, should be almost the same from 29‰ to 24‰ (with a slightly higher value at 34.5‰ to 29‰ to account for the 0.5‰ difference), because coelomic fluid osmolality was near isosmotic with the treatment salinity at all points over the experimental period (see Chapter 4). However, in *O. validus* the difference in mean total peak values from 34.5‰ to 29‰ was 325719, compared to just 164034 from 29‰ to 24‰ (see Figure 4a; Table C2). In *S. neumayeri* the mean peak value at 34.5‰ was not significantly different to the value at 29‰, which was in fact, higher than at 34.5‰ (see Figure 4b; Table C2). Intracellular hyper-regulation could account for smaller than expected differences in osmolyte concentrations at reduced salinity. Although expected to equilibrate with extracellular osmolality after acclimation (Kirschner, 1991; Somero and Yancey, 2011), intracellular hyper-regulation has been identified in blue mussel *Mytilus* spp. nerve cells at 25‰ (Willmer, 1978), and also at very low salinities (≤ 8 ‰) in whole-body tissue (Podbielski et al., 2022b). However, to maintain a high osmotic gradient between the tissue and coelomic fluids for over two and a half months would require a substantial use of metabolic resources (e.g., Kim et al., 2001; Rivera-Ingraham and Lignot, 2017). This was not evident in the measures of oxygen consumption, which were largely unaffected by salinity in both species for the majority of the experimental period (see Chapter 4). This suggests that the metabolomic analysis was unable to identify the full profile of osmolytes utilised in cell volume regulation, which instead, may have involved the use of inorganic ions (inorganic osmolytes) as observed in other recent studies on marine osmoconforming invertebrates (Podbielski et al., 2022b; Sanders, 2018). Inorganic osmolytes may account for almost half of the total intracellular solute pool (Podbielski et al., 2022a) and are traditionally considered to only be used in critical early stages of cell volume regulation under acute salinity stress (Smith and Pierce, 1987; Somero and Yancey, 2011). However, their role in longer term acclimation to salinity has recently been established in numerous marine invertebrates including echinoderms. For example, Podbielski et al. (2022b) demonstrated that after four weeks of exposure to reduced salinity, both intracellular chloride and sodium concentrations significantly decreased in *A. rubens*, whereas in *P. miliaris*, only chloride levels

showed a significant reduction. In the current study, inorganic osmolytes may have played a major role in cell volume regulation in both species and may account for the small contribution made by organic osmolytes in *S. neumayeri*. By accounting for the total osmolyte pool of inorganic and organic osmolytes, an estimation of intracellular osmolality could be inferred. Comparisons could then be made with coelomic fluid osmolality of *O. validus* and *S. neumayeri* to assess the degree of cell volume regulation under low salinity acclimation. Future Antarctic studies should incorporate inorganic osmolytes into osmolyte analysis, which may account for a substantial part of cell volume regulation in Antarctic marine invertebrates, and would improve identification of compatible osmolytes as has previously been recommended (Podbielski et al., 2022a).

5.4.6 Conclusions

This is, to the best of my knowledge, the first study to evaluate the organic osmolyte profile of Antarctic echinoderm tissue using GC-MS metabolomic profiling. The results demonstrate that *O. validus* and *S. neumayeri* both utilise organic osmolytes for cellular volume regulation in response to low salinity. Both species have similar organic osmolyte pool profiles, however they utilise different osmolytes in cellular volume control. In both species, the osmoregulatory osmolytes differ from those typically found in temperate echinoderms and other osmoconformers, particularly in the absence of glycine. Unusually, the BCCAs (valine, leucine and isoleucine) which have high hydrophobic properties, are major constituents of the total osmolyte pool in both species and were identified as the main osmoregulatory osmolytes in *O. validus*. Their presence and use as osmoregulatory osmolytes, may constitute a micromolecular adaptation to the polar environment, acting as both compatible osmolytes, cryoprotectants and even serving as an energy reserve. One of the osmoregulatory osmolytes utilised in *S. neumayeri*, glycylglycine, also present at a similar level in *O. validus* may offer further protein stability at low temperature. The relatively small contribution of osmoregulatory osmolytes to total osmolyte pool reductions under reduced salinity in *S. neumayeri* may result from interference from the gut microbiomes in the tissue sample. Furthermore, the reduction in total osmolyte pool between salinity treatments was not equivalent to the drop in coelomic fluid osmolality recorded in Chapter 4, suggesting that inorganic osmolytes (which were not measured in this study) may play a large role in Antarctic

echinoderm cellular volume control as observed in other recent studies (Podbielski et al., 2022b). Future work should aim to conduct direct comparisons between temperate and Antarctic echinoderm species utilising the same metabolomics platform over a greater range of salinity variations to improve understanding of osmolyte use, while also measuring inorganic osmolytes.

Overall, this study highlights the use of organic osmolytes as a mechanism underlying cellular volume control in two Antarctic echinoderms. The novel finding that BCAAs are utilised as organic osmolytes which appear to have multiple overlapping roles, suggests this is a micromolecular level adaptation to the polar environment, and should now form the basis of further investigation across multiple taxa to establish whether this a wider phenomenon.

Chapter 6 – Conclusions

6.1 Overview

One of the main aims of ecophysiological research is to develop a mechanistic understanding of how organisms respond to ecological changes (Spicer, 2014), revealing their inherent resilience and potential for survival under the threat of environmental change. Antarctic marine organisms have existed in a low, stable temperature environment, with extreme seasonality for at least 15 million years (Peck, 2018). However, in recent decades, the Southern Ocean has experienced significant changes, including warming, acidification, and freshening (IPCC, 2022a; Schmidt et al., 2014; Swart et al., 2018). While research has extensively examined the impacts of warming and acidification (e.g., Black et al., 2019; Hancock et al., 2020; Peck, 2018; Peck et al., 2014), the response of Antarctic marine organisms to freshening has received less attention. This may be because freshening is primarily a secondary effect of warming (Röthig et al., 2023), and, although there is extensive evidence of increased melting in areas such as the Western Antarctic Peninsula (WAP) (González-Herrero et al., 2024; Silva et al., 2020), localised studies on rates of melting and freshening are less common. However, the rapid acceleration of climate change in recent years (IPCC, 2023), coupled with the uncertainty surrounding tipping point thresholds (Pereira et al., 2023) and the vulnerability of large areas of marine-based ice-sheets to melting and collapse [e.g., Thwaites Glacier, Western Antarctica (Schmidt et al., 2023)], underscores the urgent need to establish a research baseline focused on the impact of freshening on Antarctic marine organisms. This thesis set out to establish the degree of vulnerability faced by echinoderms to climate-change-induced freshening in the Southern Ocean by linking their physiological and behavioural responses to low salinity with underlying cellular and molecular mechanisms. These specific aims were incorporated:

1. Establish a methodology that could identify and differentiate between short- and long-term responses to low salinity.

2. Produce experimental data from non-Antarctic echinoderms that would serve as a template for later Antarctic species comparisons.
3. Assess species-specific responses to acute low salinity in Antarctic echinoderms.
4. Assess the acclimation capacities to low salinity of Antarctic echinoderms.
5. Explore the mechanistic underpinnings of low salinity acclimation in Antarctic echinoderms using cellular and 'omic approaches.
6. Identify knowledge gaps and suggest future research approaches.

6.2 Methodological development and temperate echinoderm comparative analysis

In Chapter 1, a literature review on the impact of low salinity on echinoderms revealed inconsistencies regarding the duration of salinity experiments and the definitions of acute versus chronic responses. Surprisingly, few studies have demonstrated low salinity acclimation using traditional physiological and behavioural metrics (such as respiration rates, feeding behaviour, and activity levels). Moreover, the wide variation in methodologies—such as stepwise dilutions versus direct transfer to low salinity, and fluctuating salinities versus steady-state conditions—further complicates direct comparisons between species or regions. This variability in approaches ultimately made it challenging to draw broad, generalised conclusions. Therefore, aim 1 was to establish a methodology that could identify and differentiate between short- and long-term responses to low salinity. This was covered by Chapter 2 using the temperate echinoid, *Echinus esculentus*, and later applied in Chapter 4 to Antarctic echinoderms. Short-term, 24-hour responses to low salinity immersion were contrasted with those recorded over the course of 25 days exposure, revealing differing responses. For instance, in *E. esculentus* the short-term metabolic response to low salinity immersion was characterised by metabolic depression, with oxygen consumption decreasing linearly as salinity decreases. However, during chronic exposure, oxygen consumption significantly exceeded control levels, suggesting a distinction in mechanisms governing the acute shock response versus long-term acclimation.

Chapter 2 also covered aim 2, which was: to produce experimental data from a non-Antarctic echinoderm that would serve as a template for later Antarctic species comparisons. The chapter demonstrated that *E. esculentus* could successfully acclimate to a moderately reduced salinity with beneficial consequences to further hypoosmotic challenges (i.e.,

improved righting ability and metabolic capacity). The lower salinity limit for acclimation in *E. esculentus* was established to be between 21‰ and 26‰. The data clearly demonstrated a decline in physiological functioning at the lower salinity (21‰) leading to reductions in mass and a deterioration in physical condition, suggesting this was beyond the plasticity threshold for this species to acclimate. These findings established that the experimental approach could generate informative physiological data, providing evidence to assess both the degree of low salinity tolerance and the species' capacity for acclimation. This study also revealed ecological insights, particularly in the context of climate change. Using species salinity tolerance limits, predictions could be inferred about species distribution limits and the potential ecosystem impacts. The study demonstrated that the lower salinity (21‰) likely represented a barrier to their distribution and survival under increased freshening, predicted from heavier rainfall in Scottish loch systems, consequently affecting the growth dynamics of their macroalgae food source.

6.3 Acute responses to low salinity in Antarctic echinoderms

The third aim—to assess species-specific responses to acute low salinity in Antarctic echinoderms—was covered by Chapter 3. Here, the short-term experimental design developed in Chapter 2 was implemented on a variety of Antarctic echinoderms to establish the acute lower lethal limit of salinity, and corresponding behavioural and physiological responses to acute hypoosmotic shock. This revealed unexpected differences between species and within echinoderm class, showing that habitat niche did not correlate with the degree of salinity tolerance observed. For example, the subtidal holothurians, especially *Echinopsolus charcoti* and *Cucumaria georgiana*, exhibited surprisingly high short-term tolerance to low salinity, whereas the echinoid *Sterechinus neumayeri*, which can inhabit much shallower waters, was less tolerant. The chapter also highlighted species particularly vulnerable to acute hypoosmotic events, with the brittle star *Ophionotus victoriae* showing the lowest tolerance to reduced salinity, compounding its already poor ability to withstand warming (Peck et al., 2009a). Chapter 3 also required the development of a novel behavioural metric to assess activity rates in holothurians. The peristaltic rhythmic contractions utilised by many sea cucumbers for locomotion was demonstrated to be quantifiable and repeatable. This offers a simple solution for future researchers aiming to record activity rates in sea

cucumbers, especially for species that exhibit pentaradial symmetry with a lack of identifiable ventral or dorsal surfaces.

6.4 Low salinity acclimation in Antarctic echinoderms

Having established short-term tolerance limits in Chapter 3, Chapter 4 set out to investigate the degree of acclimation capacity to lower salinity, covering aim 4. Logistical constraints limited the number of species that could be practically investigated, so Chapter 4 focused on two of the most conspicuous and ecologically important echinoderms of the shallow Antarctic seas, *Odontaster validus* and *S. neumayeri* (Pearse, 2013; Spota Caputi et al., 2020). Using the methodology developed for acclimation in Chapter 2, long-term experiments demonstrated that both species could successfully fully acclimate to 29‰ and tolerate 24‰ for at least 10 weeks. The long-term exposure data from the temperate urchin in Chapter 2 provided a direct comparison for *S. neumayeri*. The delayed increase in oxygen consumption at low salinity suggests that the inherently low metabolic rate of *S. neumayeri*, due to adaptations to low temperatures, may have allowed the species to survive for extended periods in suboptimal salinity conditions. The lower salinity of 24‰ appeared to be below the physiological limit for the majority of the population in both species, as acclimation was not demonstrated.

6.5 Synthesis of chapters 1 to 4

The following questions provide a synthesis and summary, drawing on the main conclusions from Chapters 1 to 4:

Are there characteristic differences between short- and long-term responses to low salinity in echinoderms?

Underlying response patterns to both short- and long-term exposure to low salinity, are mechanistically different. For example, acute hypoosmotic change drives the establishment of temporary osmotic gradients between the coelomic fluid and seawater, while the rapid release of intracellular inorganic ions is thought to minimise swelling, rescuing cells from

mechanical damage (Castellano et al., 2018; Diehl, 1986; Shumway, 1977; Vidolin et al., 2007). Longer-term changes are thought to involve phenotypic adjustments to modify cell membrane transport proteins, cellular structural changes, and cell volume regulation in addition to modifications to the osmolyte profile to favour more 'compatible' osmolytes (Gilles and Delpire, 1997; Khlebovich, 2017; Podbielski et al., 2022b; Yancey, 2005). Different responses were observed in some metrics between the three comparable salinity treatments (34.5‰, 29‰ and 24‰) in *O. validus* and *S. neumayeri*, when comparing acute and chronic low salinity exposure. For example, in response to acute low salinity, activity coefficient values showed no significant differences between salinities (34.5‰ to 24‰). However, after chronic exposure, significant differences were observed between low (24‰) and higher salinities in both species. This can be explained, in part, by examining coelomic fluid osmolality. At six hours after hypoosmotic exposure to 24‰, coelomic fluids were hyperosmotic compared to external seawater (especially in *S. neumayeri*; see Chapter 3; Figure 3.3). By maintaining an osmotic gradient between the coelomic fluid and the seawater, tissue swelling was likely minimised. This allowed movement to continue, enabling the completion of righting at rates comparable to control. At salinities lower than 24‰, righting was significantly impeded (Chapter 3; Figure 3.2) even though coelomic fluids were hyperosmotic compared to external seawater (Chapter 3; Figure 3.3). This may be the result of swelling in external epithelial tissue (which lacks the coelomic fluid buffer) or even the retraction of podia as an evasive behavioural response [as observed in some holothurians under reduced salinity (Castellano et al., 2018)], impeding movement. Long-term exposure demonstrated coelomic fluids were near isosmotic with external salinity (Chapter 4; Figure 4.7), however, righting took significantly longer than control animals. This was despite the likely completion of full cell volume regulation (as demonstrated in Chapter 5; see Figure 5.4). This suggests that physiological costs associated with maintaining homeostasis at 24‰ were greater than at control, demonstrating that full acclimation had not occurred. Oxygen consumption rates were similar between 34.5‰ to 24‰ over both the short-term and longer-term (weeks 1 to 11) in *S. neumayeri*. It took until week 12 for oxygen consumption to rise significantly at low salinity in *S. neumayeri*. Interestingly, in the temperate urchin *Echinus esculentus*, this pattern was also observed but appeared to be much shorter, with oxygen consumption increasing above control rates within the first week at low salinity. Again, in the acute experiment there were no observable differences in oxygen consumption between low salinity (21‰) and

control, suggesting that different mechanisms were operating over different timeframes. In *O. validus*, acute exposure to 24‰ significantly reduced oxygen consumption. However, after acclimation to 24‰, oxygen consumption was comparable to the control, highlighting the difference between acute and chronic responses.

Based on the patterns observed in both short- and long-term responses to low salinity in echinoderms, it is evident that these responses are mechanistically distinct. The response to acute immersion in low salinity primarily involves rapid physiological adjustments to prevent cellular damage, such as the release of intracellular ions and the establishment of osmotic gradients. These quick adjustments facilitate the maintenance of basic functions such as movement as observed with the ability to right at reduced salinities, although these mechanisms have limitations with a threshold beyond which normal functions are impaired. In contrast, chronic exposure involves more complex processes aimed at acclimation, including cellular restructuring and modifications to cellular osmolyte profiles. Although echinoderms can regulate their coelomic fluid to near isosmotic levels at low salinity, performance metrics like righting ability and oxygen consumption rates indicate that full acclimation is not always achieved. The physiological costs of maintaining homeostasis at low salinity over time become more apparent, as demonstrated by increased oxygen consumption in *S. neumayeri* and *E. esculentus* after extended exposure, indicating that these organisms are still compensating for the stress of the hypoosmotic environment.

The findings from this thesis underscore the complexity of the osmotic response in echinoderms, distinguishing between short-term cellular protective mechanisms and more gradual, longer-term acclimation processes. Furthermore, comparisons between short- and long-term responses show that individual metrics should not be considered in isolation. A multi-response approach, combining whole-animal with cellular or tissue-level metrics, provides a more comprehensive assessment of organismal stress at any given time.

What predictions can be made about Antarctic echinoderms under future coastal freshening?

In shallow Antarctic seas, salinity would need to fall to around 20‰ for 24 hours (or possibly less) before echinoderm populations would be likely to experience significant mortality, with brittle stars being the most vulnerable. For broader impacts on echinoderm populations, salinities would need to drop to between 13‰ and 16‰ for 24 hours (or less). Both these

scenarios are realistic when considering current surface salinities in inner Arctic fjords regularly drop to <15‰ over the summer periods (Sejr et al., 2021), while haloclines results in salinities of ~20‰ at 10m depth (Sejr et al., 2017; Sejr et al., 2022). Marine heatwaves are predicted to increase in their duration, intensity and magnitude throughout the rest of the 21st century under climate change (Oliver et al., 2018). Recent events, such as the 2020 six-day heatwave on the Western Antarctic Peninsula (WAP), where unprecedented mean temperature anomalies of +4.5°C were recorded (González-Herrero et al., 2022), along with record-breaking surface ice melt on the WAP Ice Shelf (Banwell et al., 2021), underscore the potential risk of future hyposalinity events that could negatively impact echinoderm populations and other benthic fauna.

If coastal reductions in salinity were to persist in duration, both species examined for acclimation capacity (*O. validus* and *S. neumayeri*) will likely demonstrate successful acclimatisation down to 29‰. Salinities around 24‰ appear to exceed the physiological limits of both species, suggesting that coastal freshening at this level would compromise survival. Whether the lower salinity survival limits established in Chapter 3 for the other four species can be extrapolated to predict longer term acclimation capacity, would require further investigating. However, some researchers suggest that the tolerance range obtained by acute direct transfer experiments, can be used to predict potential tolerance range (i.e., the widest range of salinity that the organisms can survive through acclimation) (Khlebovich 2017), however this is untested in echinoderms. If this were the case, the results from Chapter 3 suggest that the two most tolerant holothurians, *E. charcoti* and *C. georgiana* may have the capacity to acclimate to 24‰.

If coastal freshening at 24‰ were to reach the shallow waters where *O. validus* and *S. neumayeri* are most abundant (less than 35m depth; Barnes and Brockington, 2003; Pearse, 2013), their migration to deeper, higher-salinity waters or potential demise could have significant implications for benthic food webs and ecosystem dynamics, as both are considered keystone species (McClintock et al., 2008b; Sporta Caputi et al., 2020; Sporta Caputi et al., 2024). Sporta Caputi et al. (2024) demonstrated that *O. validus* and *S. neumayeri* play crucial roles as key connectors in Antarctic food webs. *Odontaster validus* relies heavily on the consumption of *S. neumayeri*, and both species contribute significantly to carbon processing and transfer within the ecosystem. The loss of *S. neumayeri*, in particular, is expected to trigger cascading effects throughout the entire food web (Sporta Caputi et al.,

2020). As both *O. validus* and *S. neumayeri* are inhabitants of shallow seas, and are occasionally found in the intertidal zone, the presence of persistent low salinity at $\leq 24\text{‰}$ in the future will likely cause a change in Antarctic intertidal ecosystems and at shallower subtidal depths.

How does the resilience of Antarctic echinoderms to low salinity compare to that of temperate and tropical species?

Overall, the data from Chapters 3 and 4 suggest that Antarctic echinoderms have comparable levels of resilience to low salinity with temperate and tropical species, with responses to short-term hypoosmotic shock and longer-term acclimation following similar patterns to non-Antarctic species (e.g., Russell, 2013). Indeed, individual species such as *E. charcotti* and *C. georgiana* appear to have exceptional short-term tolerance when viewed through a global lens (Russell, 2013). Perhaps these abilities can be attributed to having evolved in an environment with extreme seasonality, driven by the regular melting and freezing of sea ice and freshwater input from ice shelf melt, which causes salinity variation in shallow seas. Furthermore, innate physiological adaptations in Antarctic echinoderms, such as their extremely low metabolic rates, may enhance resilience to prolonged freshening under climate change, enabling them to endure adverse conditions longer than predicted for temperate species.

Unlike the poor thermal acclimation abilities observed in Antarctic marine species (Peck et al., 2014), echinoderms do not seem to exhibit the same constraints in osmotic acclimation. The long-term low, stable temperatures of Antarctic and Southern Ocean conditions are thought to have led to a significant loss of mechanisms to manage thermal tolerance and acclimation (Peck, 2018; Pörtner et al., 2007). In contrast, the predictable seasonal freeze-thaw cycles of ice, which cause fluctuations in salinity in shallow seas, may have sustained and potentially enhanced osmotic tolerance and acclimation capacity in Antarctic echinoderms. This contrast between thermal and osmotic tolerance suggests that mechanisms for managing osmotic stress are under strong selection pressure and are likely critical for survival in Antarctic shallow sea environments.

6.6 Mechanisms for tolerance and acclimation to low salinity

6.6.1 Metabolomics

Having demonstrated a return to physiological steady state metrics after low salinity exposure in Chapter 4, Chapter 5 was an attempt to reveal mechanisms underlying low salinity acclimation in Antarctic marine echinoderms and fulfil aim 5. Using gas-chromatography mass-spectrometry (GC-MS), quantifiable reductions in tissue total organic solute (osmolyte) concentration after low salinity exposure suggested that cell volume regulation had occurred to reduced intracellular osmotic pressure. The specificity of the osmolyte profile and osmolytes implicated in osmoregulation, appeared to be distinct from previous research on temperate echinoderms and other marine osmoconforming invertebrates. In particular, the use of branched chain amino acids (valine, leucine and isoleucine) in cell volume regulation in *O. validus*, while also present in *S. neumayeri*, hinted at an adaptation at the micromolecule level as a response specifically tailored to the low Antarctic temperatures.

6.6.2 Transcriptomics

Time constraints prevented the inclusion of a complementary Antarctic echinoderm transcriptome analysis for Chapter 5, although samples were taken for later analysis. However, a brief discussion on genetic level responses to low salinity in echinoderms is warranted here to highlight areas that need further investigation.

In general, echinoderms are thought to have poor tolerance to low salinity due to the lack of excretory organs (necessary for removing excess water under low salinity) and an integument which is considered to be highly permeable to water and ions (Binyon, 1972; Russell, 2013; Stickle and Ahokas, 1974). At the molecular level, the role of stress related genes (e.g., heat shock genes) in the response to low salinity in echinoderms has been under-researched. For example, to the best of my knowledge, there are only three studies that have examined genetic level changes under osmotic stress in echinoderms globally (i.e., Arribas et al., 2022; Dong et al., 2008; Meng et al., 2011). Salinity change can impact protein stability causing an increase in denaturing through changes in inorganic ion concentration (discussed in Chapter 5) and pH (Evans and Kültz, 2020). Denatured proteins act as a stimulus for the

cellular stress response and induce the upregulation of stress proteins such as the highly conserved heat shock proteins, HSP70 family (Evans and Kültz, 2020). Acting as molecular chaperones, HSP70 proteins help stabilise and refold denatured proteins, thus offering resilience under stress. For example, in the holothurian *Apostichopus japonicus*, acute low salinity stress triggers the upregulation of *HSP70* genes, but this response is short-lived, returning to baseline levels after ~72 hours (Meng et al., 2011). This brief upregulation coincides with decreased metabolic activity and increased mortality, suggesting that while *HSP70* provides temporary protection against osmotic shock, longer-term regulation may exceed the physiological capacity of *A. japonicus* (Meng et al., 2011). Similarly, in the asteroid *Anasterias minuta*, there was no sustained upregulation of *HSP70* after six weeks of reduced salinity exposure (30‰ from 35‰), which was interpreted as a sign of low salinity tolerance (Arribas et al., 2022). Clearly, more work is needed to evaluate the role of stress proteins in echinoderms under osmotic stress, as called for by Russell (2013). Russell (2013) suggested that HSP expression may reflect the degree of tolerance observed in different echinoderm species, revealing a potential mechanism that some species use to acclimate to low salinity. Whether the short-lived upregulation of stress genes in echinoderms, as suggested by the aforementioned studies, reflects true acclimation and tolerance or alternatively, serves as a barrier to broader euryhalinity in echinoderms requires further investigation.

In some Antarctic echinoderms and other marine organisms, the expression of inducible forms of heat shock proteins after thermal stress appear to have been lost, which has been suggested to result from millions of years adapting to low temperatures and a stable thermal environment (Clark and Peck, 2009). Studies have demonstrated that Antarctic echinoderms including, *S. neumayeri* (González-Aravena et al., 2018), *O. validus* (Clark et al., 2019) and the holothurian *C. georgiana* (Clark et al., 2017) fail to upregulate HSP70 upon acute heat stress; however, a form of HSP70 appears to be constitutively expressed in *S. neumayeri* (González-Aravena et al., 2018). Separately, long-term acclimation of *S. neumayeri* to increased temperature demonstrated a lack of *HSP70* upregulation or any stress-related transcripts (Détrée et al., 2023). The molecular response to heat stress in Antarctic echinoderms indicates differences from non-Antarctic species, suggesting that their response to osmotic stress may also differ, although this has not yet been tested.

A transcriptome study on Antarctic echinoderms was incomplete at the time of writing, however, insights from a related study on the impact of warming and freshening on

Arctic mussels (*Mytilus edulis*), may shed light on the mechanisms of acclimation to low salinity that may be absent in echinoderms. This study, completed during my PhD, but not included in this thesis due to the focus on echinoderms, led to a first-author paper (which is included in Appendix E; Barrett et al., 2022). In this study, a transcriptome analysis revealed a distinct set of heat-shock protein genes from the HSP70 family, *HSPA12* genes, which are regularly expressed in intertidal mussels and other marine bivalves in response to a variety of biotic and abiotic stressors (Cheng et al., 2016; Clark et al., 2021), and were also upregulated in response to osmotic stress. These genes appear to have evolved through duplication events, allowing different *HSPA12* proteins to take on specialised roles, enhancing resilience to a wide range of intertidal stressors (Clark et al., 2021). Barrett et al. (2022) identified 45 *HSPA12* gene transcripts unique to low salinity acclimation in *M. edulis*, suggesting these proteins play an important role in the acclimation process. Furthermore, in mussels exposed to salinities <15‰ in Greenland fjords, *HSPA12* and other stress genes were still upregulated after six days exposure, indicating their continued importance while under persistent osmotic stress and role in the acclimation process. At even lower salinities (5‰), the data suggested the presence of an intracellular osmotic gradient, with mussel tissue being hyperosmotic compared to the haemolymph. At the same time, the upregulation of aquaporins, V-ATPases, and ion exchanger genes hinted at a potential mechanism for dealing with excess intracellular water. Calcino et al. (2019) had previously described the formation of intercellular vesicles or ‘cleavage cavities’ utilised to expel excess water from cells when observing freshwater mussel embryos *in vitro*. The transcriptomic profile of the freshwater quagga mussel *Dreissena rostriformis* was remarkably similar to that of *M. edulis* at 5‰ salinity suggesting a similar process may be operating in marine mussels when exposed to very low salinity (Barrett et al., 2022).

In mussels, such as *M. edulis*, the presence of specialised heat-shock proteins like HSP12A, which remain upregulated under continued reduced salinity, likely enhances protein stability during the cellular restructuring that occurs as part of the acclimation process. Furthermore, the potential use of osmoregulatory mechanisms such as intercellular vesicle formation—an adaptation that is considered a critical step in the exploitation of freshwater ecosystems by bivalves—(Calcino et al., 2019), allows tolerance of very low salinities in marine mussels. These advanced molecular and physiological adaptations may help explain the greater euryhalinity observed in mussels and other bivalves compared to most echinoderms.

Indeed, the high sensitivity of echinoderm embryos and early life stages to reduced salinity (e.g., Cowart et al., 2009) suggests that such adaptive mechanisms are underdeveloped or entirely absent in echinoderms, offering further insight into their inability to colonise freshwater environments and their generally poor tolerance of lowered salinity.

The integration of a transcriptome study on *O. validus* and *S. neumayeri* utilising the same samples taken for the metabolomic analysis, may offer greater insights into mechanisms underlying acclimation and tolerance of low salinity. Further integration of physiology data with both the metabolomics and a transcriptomics through a multi-omics approach, could offer a comprehensive synthesis of the processes that operate in response to osmotic stress. This would better inform our understanding and predictions for how Antarctic echinoderms will respond to future coastal freshening, while more generally provide insights into the molecular mechanism underpinning low salinity acclimation in echinoderms.

6.7 Future research avenues (aim 6)

As discussed at the beginning of this chapter, climate change impacts in the Southern Ocean are far from limited to freshening. Instead, echinoderms and other marine organisms are increasingly experiencing a combination of biotic and abiotic stressors in the Antarctic environment such as warming (Swart et al., 2018), decreases in seawater pH (Metzl et al., 2024b), undersaturation of calcium carbonate (Fabry et al., 2009; Metzl et al., 2024a), lower oxygen availability (Tremblay and Abele, 2016), reduction in sea-ice cover (Cook et al., 2005; Eayrs et al., 2021), increased iceberg scour (Robinson et al., 2021) and increased level of pollution from heavy metals (Webb et al., 2020) to microplastics (Caruso et al., 2022). When organisms are exposed to stressors in combination, there may be interactions that lead to nonlinear outcomes that would be difficult to predict from a single stressor acting alone (Kroeker et al., 2017). For example, when multi-stressors are applied in tandem, the organismal response can be additive, synergistic (a response greater than the sum of individual stressors) or antagonistic (a response which is less than the sum of individual stressors) (Gunderson et al., 2016). This was demonstrated with *M. edulis*, where the combination of warming and low salinity resulted in a synergistic upregulation in genes and gene pathways, pushing the animal to the edge of its tolerance threshold (with increased

mortality) before either stressor alone (Barrett et al., 2022). For Antarctic marine species, the interaction of multiple stressors is considered a key factor in clarifying biological responses to climate change and predicting future biodiversity patterns (Convey and Peck, 2019). Previous physiology studies utilising multi-stressor approaches on Antarctic marine organisms have consistently demonstrated that salinity is a prominent modulator of physiology and behaviour when in combination with other stressors (e.g., Martínez et al., 2021; Navarro et al., 2020; Park et al., 2020). In studies on temperate echinoderms, impacts of freshening combined with other stressors such as temperature can demonstrate greater negative impacts than with each individual stressor. For example larval survivorship in the echinoid, *Heliocidaris crassispina* was lowest when both stressors were combined (Mak and Chan, 2018). Future research on Antarctic echinoderms should explore the combined impact of low salinity and additional stressors, particularly warming. Approaches could involve acclimating echinoderms to low salinity and assessing their interaction with critical thermal maxima, or conversely, a thermal acclimation with an assessment on their response to hypoosmotic shock. A more pressing scenario to investigate would be a combined warming and low salinity acclimation study, as these two stressors are increasingly likely to occur in parallel with climate change (IPCC, 2023).

This thesis focused on adult echinoderms, which are considered to be more tolerant to reduced salinity compared to earlier embryonic life stages in echinoderms (Bressan et al., 1995; Roller and Stickle, 1985; Roller and Stickle, 1993). Chapters 3 and 4 demonstrated that adult *S. neumayeri* are substantially more tolerant than embryonic stages, which have been observed to be extremely sensitive to only small changes in salinity [e.g., a reduction from 34‰ to 30‰ resulted in a 1% development success rate (Coward et al., 2009)]. If this level of sensitivity is mirrored across other Antarctic echinoderm species, this may represent a severe bottleneck on future larval recruitment in the face of increased freshening. Priority should now focus on examining the impact of low salinity on the early-life stages of other Antarctic echinoderm species. Furthermore, research on longer-term adult survival, growth and reproductive capabilities under low salinity would also be beneficial, but logistically challenging.

As discussed in Chapter 3, the impact of short-term freshening events on Antarctic echinoderms appears to be highly species-specific and independent of habitat niche. The brittle star *O. victoriae* demonstrated poor euryhalinity and has previously been observed to

have limited thermal acclimation abilities (Peck et al., 2009a) indicating its vulnerability to rapid climate change. Given that ophiuroids are among the most important Antarctic benthic groups, playing a crucial role in energy transfer between the water column and the sea floor (Ambroso et al., 2016), it would be valuable to investigate whether these traits are consistent across other ophiuroid species, particularly shallow sub-tidal species (<30m) that may encounter low salinity stratified layers. Other echinoderms that deserve more attention and did not feature in this thesis are crinoids. Many Antarctic crinoids have a circum-polar distribution, with the stalked isocrinids (Order Isocrinida) mainly restricted to deeper seas (>100m), while the more mobile feather star crinoids (Order Comatulida), have a much wider depth range and can be regularly found in shallow waters [e.g., common Antarctic species in the genus *Promachocrinus* have a depth range between 10-2000m (McLaughlin et al., 2023; Speel and Dearborn, 1983)]. Due to the rich fossil record of isocrinids, and their previous high abundance in shallow water seas prior to the Mesozoic marine revolution—the increase in shell-crushing predation in shallow benthic seas towards the end of the Triassic, which led to large declines in stalked crinoids—, crinoid fossils are often used as evidence for the existence of previous shallow water benthic ecosystems, subject to low salinity (Aronson and Blake, 2001; García-Penas et al., 2024; Salamon et al., 2021). Yet, there is little evidence of extant crinoids ability to tolerate low salinity, with only one known tolerance study [e.g., Clark (1917) demonstrated tolerance to 75% seawater dilution]. Globally, crinoids are not found in hypo- or hyperosmotic environments, suggesting they have low euryhalinity (Russell, 2013). However, they are abundant in in tropical shallow waters (Terrana et al., 2024) and shallow Antarctic environments (McLaughlin et al., 2023; Speel and Dearborn, 1983), both of which are predicted to experience future freshening under climate-change (Röthig et al., 2023). Thus, future research should focus on investigating crinoid tolerance to low salinity.

One of the major obstacles to researching freshening in the Antarctic, was the paucity of data on regional and fine scale salinity changes, such as within Antarctic fjord systems. Instead, salinity measurements had to be derived from *ad hoc* observations within related papers and non-specific CTD (Conductivity, Temperature, and Depth) data, which made it difficult to assess current conditions and make future predictions. Data from the Arctic, which has been extensively surveyed, reveals that transects along whole bays and fjord systems provide an excellent level of detail for assessing salinity stratification in the water column

(e.g., Sejr et al., 2017; Sejr et al., 2022) and would be highly beneficial for future researchers working on freshening in Antarctica coastal systems.

In summary, these future research avenues are recommended for investigators studying the impact of low salinity on Antarctic echinoderms:

- Assess the use of inorganic osmolytes in cell volume regulation under long-term acclimation.
- Integrate a transcriptomic approach with physiological data to analyse low salinity tolerance and acclimation. This can also be applied to echinoderms globally.
- Include low salinity as a stressor in multi-stressor physiology studies, especially in combination with warming.
- Include early life stages.
- Investigate longer-term (multi-year, multi-generation) adult survival, growth and reproductive capabilities under low salinity.
- Expand species-specific responses to focus on brittle stars (Ophiuroids).
- Investigate the impact of low salinity on crinoids, especially species which inhabit shallow seas. This could also be expanded to include crinoids globally.
- Conduct finer-scale surveys of salinity stratification in Antarctic coastal systems.

Appendices

Appendix A – Supplementary material for Chapter 2

Table A1 | Statistical tests for experiments 1.

Experiment 1. Acute salinity tolerance				
Righting response after 5 days in recovery				
one-way ANOVA (Model: Righting Time~Salinity)	d.f.	mean square	F	P value
(logged data)	2	0.10	0.88	0.43600
Oxygen consumption				
Kruskal-Wallis rank sum test				
(Model: O2 μ mol/hr/gAFDM~Salinity treatment)	d.f.		χ^2	P value
	4		22.678	0.0001469
Post hoc Dunn test (with Bonferroni correction)	comparisons		z	P value
	11‰ v 16‰		-1.349858	0.8853
	11‰ v 21‰		-2.546323	0.0544
	11‰ v 26‰		-4.095593	0.0002
	11‰ v 31‰		-3.666092	0.0012
	16‰ v 21‰		-1.196465	1
	16‰ v 26‰		-2.745734	0.0302
	16‰ v 31‰		-2.316234	0.1027
	21‰ v 26‰		-1.549269	0.6066
	21‰ v 31‰		-1.119768	1
	26‰ v 31‰		0.4295	1
Activity coefficient				
Kruskal-Wallis rank sum test				
(Model: activity coefficient~salinity)	d.f.		χ^2	P value
	4		36.638	0
Post-hoc Dunn test (with Bonferroni correction)	comparisons		z	P value
	11‰ v 16‰		0	0.5
	11‰ v 21‰		-1.295469	0.0976
	11‰ v 26‰		-3.246872	0.0006
	11‰ v 31‰		-4.886706	0
	16‰ v 21‰		-1.295469	0.0976
	16‰ v 26‰		-3.246872	0.0006
	16‰ v 31‰		-4.886706	0
	21‰ v 26‰		-1.951403	0.0255
	21‰ v 31‰		-3.591237	0.0002
	26‰ v 31‰		-1.639834	0.0505
Coelomic fluid osmolality				
Welch's ANOVA (on squared data)				
(Model: CF osmolality~salinity)	d.f.		F	P value
	4		8666.3	2.20E-16
Post-hoc Games-Howell test	comparisons			P value
	11‰ v 16‰			5.34E-05
	11‰ v 21‰			8.25E-07
	11‰ v 26‰			1.06E-06
	11‰ v 31‰			2.22E-16
	16‰ v 21‰			2.22E-16
	16‰ v 26‰			2.22E-16
	16‰ v 31‰			7.92E-07
	21‰ v 26‰			2.22E-16
	21‰ v 31‰			6.92E-07
	26‰ v 31‰			2.39E-06
Coelomic fluid osmolality v tank water				
One-sample t-test	d.f.		t	P value
31‰ treatment, tank water = 951	4		3.2131	0.03249
26‰ treatment, tank water = 794	4		11.752	3.49E-04
21‰ treatment, tank water = 640	4		4.5448	0.01046
16‰ treatment, tank water = 484	4		14.648	0.0001264
11‰ treatment, tank water = 329	4		9.249	0.0007597

Table A2 | Tank effect analysis statistics.

Feeding	Kelp~Tank*Timepoint+Error(Animal/Timepoint)	Variables	d.f.	Mean sq	F	P value
	Control	Tank	2	4.16	0.979	0.4220
		Error: Animal	7	4.25	n/a	n/a
		Timepoint	5	10.43	5.436	0.0008
		Tank:Timepoint	10	2.72	1.421	0.2121
		Error: Animal:Timepoint	35	1.92	n/a	n/a
	Medium	Tank	2	0.66	0.140	0.8710
		Error: Animal	7	4.69	n/a	n/a
		Timepoint	5	1.69	1.239	0.3120
		Tank:Timepoint	10	2.11	1.549	0.1640
		Error: Animal:Timepoint	35	1.36	n/a	n/a
	Low (squared data)	Tank	2	0.10	0.414	0.6760
		Error: Animal	7	0.25	n/a	n/a
		Timepoint	5	0.40	3.986	0.0058
		Tank:Timepoint	10	0.17	1.691	0.1221
		Error: Animal:Timepoint	35	0.10	n/a	n/a
Oxygen consumption	O2~Tank*Timepoint+Error(Animal/Timepoint)	Variables	d.f.	Mean sq	F	P value
	Control	Tank	2	0.53	0.027	0.9740
		Error: Animal	7	19.61	n/a	n/a
		Timepoint	4	6.89	1.716	0.1740
		Tank:Timepoint	8	3.42	0.853	0.5660
		Error: Animal:Timepoint	28	4.02	n/a	n/a
	Medium	Tank	2	5.83	0.191	0.8300
		Error: Animal	7	30.55	n/a	n/a
		Timepoint	4	26.16	2.674	0.0526
		Tank:Timepoint	8	4.63	0.473	0.8647
		Error: Animal:Timepoint	28	9.79	n/a	n/a
	Low	Tank	2	7.07	0.402	0.6840
		Error: Animal	7	17.59	n/a	n/a
		Timepoint	4	98.47	16.222	0.0000
		Tank:Timepoint	8	7.08	1.167	0.3530
		Error: Animal:Timepoint	28	6.07	n/a	n/a
Righting (Activity coefficient)	AC~Tank*Timepoint	Variables	d.f.	Mean sq	F	P value
	Control	Tank	3	5.23	0.350	0.7900
		Timepoint	4	18.16	1.214	0.3300
		Tank:Timepoint	8	7.86	0.525	0.8260
		Error	25	14.96	n/a	n/a
	Medium	Tank	2	10.00	0.718	0.4990
		Timepoint	4	9.57	0.687	0.6090
		Tank:Timepoint	8	3.91	0.281	0.9650
		Error	22	13.92	n/a	n/a
	Low (Logged data)	Tank	2	0.50	0.917	0.4098
		Timepoint	4	3.30	6.031	0.0010
		Tank:Timepoint	8	0.44	0.802	0.6056
		Error	32	0.55	n/a	n/a

Table A3| Statistical tests for experiment 2.

Experiment 2. Low salinity acclimation				
Weight change				
Welch's ANOVA		d.f.	F	P value
(Model: Weight change~Salinity)	2		4.90	0.02107
Post hoc Games-Howell test	comparisons		q value	P value
	Medium-Low		3.63	0.06025
	Control-Low		4.50	0.01903
	Control-Medium		1.71	0.46218
% AFDM (organic mass)				
Kruskal-Wallis		d.f.	X ²	P value
(Model: %AFDM~Salinity)	2		6.86	0.03000
Post hoc Dunn test	comparisons		z	P value
	Medium-Low		-2.60	0.00460
	Control-Low		1.55	0.06060
	Control-Medium		-1.05	0.14590
Oxygen consumption				
two-way ART ANOVA with repeated measures				
(Model: O2μmol_hr_g~Salinity*Timepoint+error(Animal/Timepoint))	Error	d.f.	F	P value
Salinity	Animal	2	7.39	0.00286
Timepoint	Animal	1	0.08	0.77580
Timepoint	Animal:Timepoint	4	4.74	0.00146
Salinity:Timepoint	Animal:Timepoint	8	8.16	0.00000
Oxygen consumption at each timepoint				
one-way ANOVA (Model: O2μmol_hr_g~Salinity)	d.f.	mean square	F	P value
day 1	2	16.4500	1.29	0.29200
day 5	2	46.1100	12.69	0.00014
day 9 (logged data)	2	0.8947	9.62	0.00070
day 17	2	63.4800	10.29	0.00051
day 25 (logged data)	2	0.5903	14.77	0.00005
Post hoc Tukey	comparisons	CI upper	CI lower	P value
	Medium-Low	-6.0932	-1.74	0.00039
	Control-Low	-5.7017	-1.46	0.00078
day 5	Control-Medium	-1.8433	2.51	0.92347
	Medium-Low	-0.8610	-0.86	0.00192
	Control-Low	-0.1750	-0.85	0.00231
day 9	Control-Medium	0.3479	-0.33	0.99718
	Medium-Low	-4.9209	0.75	0.18089
	Control-Low	-7.7776	-2.26	0.00034
day 17	Control-Medium	-5.7686	-0.10	0.04170
	Medium-Low	-0.2514	-0.69	0.00004
	Control-Low	-0.1109	-0.55	0.00258
day 25	Control-Medium	0.3622	-0.08	0.27488
Activity coefficient (AC)				
two-way ANOVA (log transformed data)				
(Model: AC~Salinity*Timepoint)	Error	d.f.	F	P value
	Salinity	2	69.23	0.00000
	Timepoint	4	5.80	0.00028
	Salinity*Timepoint	8	2.16	0.03627
Activity coefficient (AC) at each timepoint				
one-way ANOVA (Model: AC~Salinity)	d.f.	mean square	F	P value
day 1 (Welch's ANOVA)	2	32.66	4.73	0.02160
day 5 (logged data)	2	13.04	21.16	0.00000
day 9 (logged data)	2	4.21	6.78	0.00490
day 17 (logged data)	2	8.48	20.95	0.00000
day 25 (Welch's ANOVA)	2		17.78	0.00034
Post hoc Tukey (except day 5 and 25 Games-Howell test)	comparisons	CI upper	CI lower (q value for day 5 and 25)	P value
	Medium-Low	5.9223	-0.81	0.15748
	Control-Low	-0.6106	-7.65	0.02002
day 1	Control-Medium	2.1422	-5.29	0.54020
	Medium-Low		4.03	0.05723
	Control-Low		-6.43	0.00379
day 5	Control-Medium		-0.84	0.82553
	Medium-Low	2.1812	0.27	0.01064
	Control-Low	-0.2353	-2.09	0.01231
day 9	Control-Medium	1.0172	-0.90	0.98653
day 17	Medium-Low	2.0813	0.57	0.00054

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day 25	Control-Low	-1.0938	-2.55	0.00001
	Control-Medium	0.2757	-1.27	0.26261
	Medium-Low		4.72	0.02826
	Control-Low		4.72	0.00056
	Control-Medium		-1.25	0.65920
Feeding rate				
two-way ART ANOVA with repeated measures				
(Model:				
Kelp_ingested~Salinity*Timepoint+error(Animal/Timepoint)	Error	d.f.	F	P value
Salinity	Animal	2	23.06	0.00000
Timepoint	Animal:Timepoint	5	6.32	0.00003
Salinity:Timepoint	Animal:Timepoint	10	2.96	0.00216
Feeding rate at each timpoint				
Welch's ANOVA (except day 13, one-way ANOVA)				
	d.f.		F	P value
day 1	2		4.41	0.03287
day 5	2		8.23	0.00505
day 9	2		7.60	0.00658
day 13	2		3.70	0.03790
day 17	2		28.97	0.00001
day 25	2		34.53	0.00000
Post hoc Games-Howell (except day 13: Tukey)				
	comparisons	q /Confidence intervals		P value
day 1	Medium-Low	q= 1.784		0.44370
	Control-Low	q = 1.784		0.04140
	Control-Medium	q = 2.398		0.23769
day 5	Medium-Low	q= 1.784		0.00960
	Control-Low	q= 2.555		0.21793
	Control-Medium	q= -2.581		0.19094
day 9	Medium-Low	q = 4.387		0.03008
	Control-Low	q = 3.690		0.06113
	Control-Medium	q= -2.582		0.20258
day 13	Medium-Low	CI upr = 2.7	CI lwr =-0.38	0.16519
	Control-Low	CI upr =3.2	CI lwr = 0.1	0.03475
	Control-Medium	CI upr =2	CI lwr = -1	0.72424
day 17	Medium-Low	q= 8.575		0.00021
	Control-Low	q=7.519		0.00093
	Control-Medium	q=1.677		0.47913
day 25	Medium-Low	q= 11.287		0.00000
	Control-Low	q= 5.351		0.01135
	Control-Medium	q=0.597		0.90735
Coelomic fluid osmolality				
two-way ANOVA				
(Model: osmolality~salinity*timepoint	d.f.	mean squares	F	P value
Salinity	2	577185.00	16312.77	0.00000
Timepoint	4	233.00	6.60	0.00018
Salinity*Timepoint	8	194.00	5.49	0.00003
Post hoc Tukey comparisons all P<0.05				
Coelomic fluid osmolality v tank water at each time point				
One-sample t-test				
Timepoint	Treatment and tank water osmolality (mOsm/kg)	Df	t	P value
day 1	Low (648)	4	1.57	0.19110
	Medium (798)	4	6.88	0.00234
	Control (956)	4	1.21	0.29390
day 5	Low (646)	4	1.59	0.18620
	Medium (794)	4	3.02	0.03898
	Control (937)	4	4.43	0.01145
day 9	Low (649)	4	1.76	0.15230
	Medium (798)	4	1.67	0.17060
	Control (950)	4	2.64	0.05730
day 17	Low (647)	4	3.38	0.02791
	Medium (799)	4	5.25	0.00629
	Control (947)	4	7.74	0.00150
day 25	Low (649)	4	3.93	0.01716
	Medium (800)	4	6.55	0.00281
	Control (971)	4	2.07	0.10710

Table A4 | Statistical tests for experiments 3.

Experiment 3. Hypoosmotic shock on acclimated urchins					
oxygen consumption between treatments					
one-way ANOVA		d.f.	F	P value	
		2	0.65	0.53200	
oxygen consumption - rate on day 25 v hypoosmotic shock (18‰)					
two-sample t-test		d.f.	t	P value	
Control		16	0.28	0.78010	
Medium		15	-2.76	0.01449	
Low		16	2.86	0.01132	
Coelomic fluid osmolality					
Welch's ANOVA		d.f.	F	P value	
Model: osmolality~salinity		2.00	15.82	0.00363	
Post hoc Games-Howell test		comparisons	q value	P value	
		Low v Control	-7.36	0.00751	
		Medium v Control	-4.47	0.03415	
		Medium v Low	4.14	0.08304	
Coelomic fluid osmolality v tank water at each time point					
One-sample t-test		Treatment and tank water (18‰) osmolality (mOsm/kg)	d.f.	t	P value
		Low (551)	4	25.96	0.00001
		Medium (551)	4	8.04	0.00130
		Control (551)	4	7.97	0.00134

Table A5 | Coelomic fluid osmolality (mean $\pm$ s.e.m., $n=6$ biological replicates) at the end of the hypoosmotic shock trial (6 hours in 18‰ salinity water) that was conducted on the acclimated urchins on day 25.

Treatment	Mean osmolality ($\pm$ s.e.m mOsm kg ⁻¹)	Different letter indicates significant differences between groups
Control (31‰)	631 $\pm$ 10	A
Medium (26‰)	595 $\pm$ 6	B
Low (21‰)	579 $\pm$ 1	B
Tank water (18‰)	552	

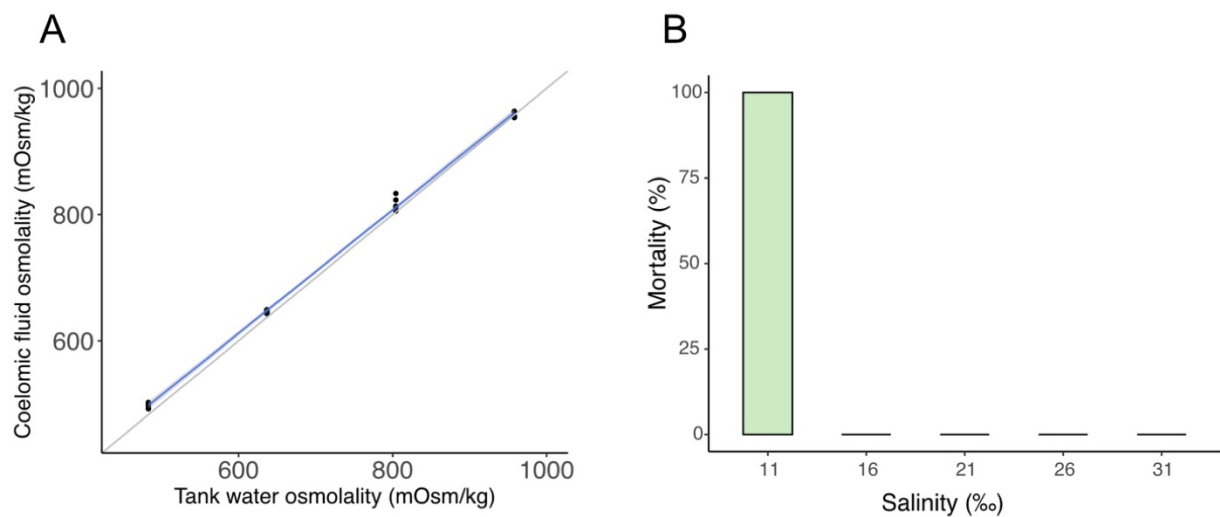


Figure A1| A) Coelomic fluid osmolality of adult *Psammechinus miliaris* 24 hours after transfer from ambient salinity (31‰) and the corresponding tank water. Salinities of tank were 31, 26, 21 and 16 ‰ and corresponds to 958, 804, 637 and 483 mOsm/kg tank water osmolality respectively). A regression line (blue line) and the 95% confidence intervals (shaded grey area) were fitted to the data. The iso-osmotic line is represented by the dashed grey line. **B)** Percentage mortality of adult *P. miliaris* 24 hours after transfer from ambient salinity (31‰).

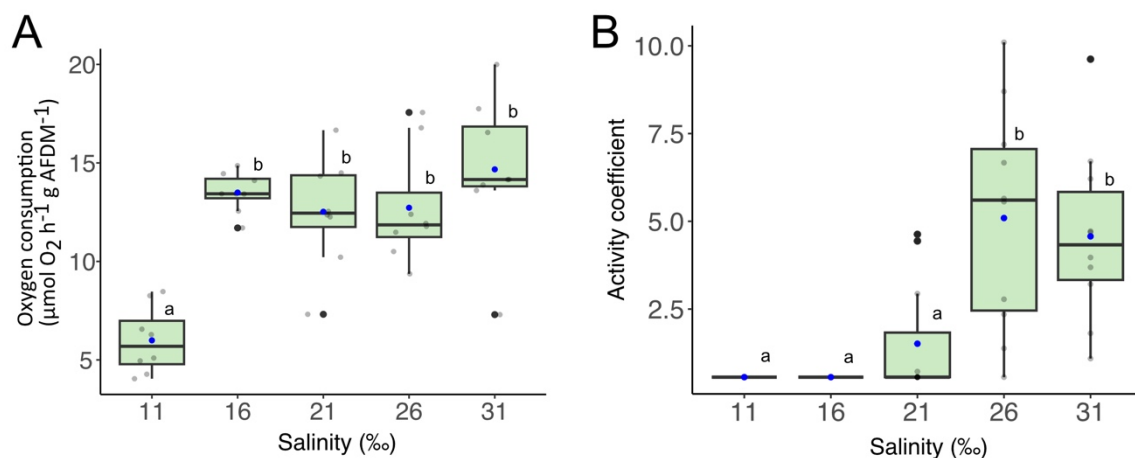


Figure A2| A) Oxygen consumption of *Psammechinus miliaris* two hours after transfer from ambient salinity (31‰). Results are shown for Welch's ANOVA and post-hoc Games-Howell test with different letters indicating significant differences ($P < 0.05$; $n = 8$ biological replicates for each treatment) between treatments. Boxplots show medians (black line), upper and lower quartiles, and maximum and minimum (whiskers). Means are represented by open points and outliers by black points within corresponding box-whisker plots. **B)** Activity coefficient ($AC = 1000/\text{righting time in seconds}$) values of adult *P. miliaris* five hours after transfer from ambient salinity (31‰). Note: a smaller AC value indicates longer righting times. Results are shown for Kruskal-Wallis and post-hoc Dunn test with different letters indicating significant differences ($P < 0.05$; $n = 10$ biological replicates for each treatment) between treatments. Boxplots show medians (black line), upper and lower quartiles, and maximum and minimum (whiskers). Means are represented by open points and outliers by black points within corresponding box-whisker plots.

Appendix B – Supplementary material for Chapter 3

Table B1| Percentage mortality ($n=10$ for each treatment in *Sterechinus neumayeri*, *Odontaster validus* *Ophionotus victoriae*; $n=8$ for each treatment in *Heterocucumis steineni*, *Echinopsolus charcoti*, *Cucumaria georgiana*) and calculated LC_{50} values after 24-hrs in reduced salinities of Antarctic echinoderms.

		Mortality (%)						
Class	Species	Salinity (‰)						LC ₅₀ (‰)
		9	14	19	24	29	34.5	
Echinoidea	<i>Sterechinus neumayeri</i>	x	100	0	0	0	0	16.5
Asteroidea	<i>Odontaster validus</i>	100	40	0	0	0	0	13.4
Ophiuroidea	<i>Ophionotus victoriae</i>	x	100	80	0	0	0	19.9
Holothuroidea	<i>Heterocucumis steineni</i>	100	40	0	0	0	0	13.4
	<i>Echinopsolus charcoti</i>	100	0	0	0	0	0	11.5
	<i>Cucumaria georgiana</i>	100	0	0	0	0	0	11.5

x= not assessed

Table B2| Statistical tests for oxygen consumption and activity rate measurements.

Oxygen consumption – <i>Sterechinus neumayeri</i>				
One way ANOVA				
(Model: treatment)	O ₂ μmol/hr/gAFDM~Salinity	d.f.	F	P value
		4,33	0.538	0.709
				P value
Oxygen consumption – <i>Odontaster validus</i>				
One way ANOVA				
(Model: treatment)	O ₂ μmol/hr/gAFDM~Salinity	d.f.	F	P value
		5, 25	9.103	0.00005
Post hoc Tukey test	comparisons (‰)	CI lower	CI upper	P value
	14 v 9	-2.2988306	-0.6603027	0.00011553
	19 v 9	-1.8672306	-0.2287027	0.0067663
	24 v 9	-1.8568306	-0.2183027	0.00744213
	29 v 9	-1.6320306	0.0064973	0.05272319
	34.5 v 9	-0.9570306	0.6814973	0.99493787
	19 v 14	-0.4240933	1.28729331	0.63416688
	24 v 14	-0.4136933	1.29769331	0.61109016
	29 v 14	-0.1888933	1.52249331	0.19387883
	34.5 v 14	0.48610669	2.19749331	0.00073523
	24 v 19	-0.8452933	0.86609331	0.99999999
	29 v 19	-0.6204933	1.09089331	0.95538933
	34.5 v 19	0.05450669	1.76589331	0.03235883
	29 v 24	-0.6308933	1.08049331	0.9630457
	34.5 v 24	0.04410669	1.75549331	0.03520213
	34.5 v 29	-0.1806933	1.53069331	0.18392033
Oxygen consumption – <i>Ophionotus victoriae</i>				
Welch's ANOVA				
(Model: treatment)	O ₂ μmol/hr/gAFDM~Salinity	d.f.	F	P value
		4, 9.3	2.3429	0.1305
34‰, 29‰, 24‰ only				
One way ANOVA				
(Model: treatment)	O ₂ μmol/hr/gAFDM~Salinity	d.f.	F	P value
		2,12	7.308	0.0084
	comparisons (‰)	CI lower	CI upper	P value
	29 v 24	-0.4160608	1.511661	0.3181659
	34.5 v 24	0.4081392	2.335861	0.0066357
	34.5 v 29	-0.1396608	1.788061	0.0973842
				N/A
				N/A
				N/A
				N/A
				N/A

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N/A
N/A

Oxygen consumption – *Echinopsolus charcoti*

One way ANOVA				
(Model: treatment)	O ₂ μmol/hr/gAFDM~Salinity	d.f.	F	P value
		5, 40	3.645	0.00824
Post hoc Tukey test	comparisons (%)	CI lower	CI upper	P value
	14 v 9	-0.8420633	0.22794752	0.52885518
	19 v 9	-1.1868638	-0.0817614	0.01631956
	24 v 9	-1.1840071	-0.1139963	0.00964147
	29 v 9	-1.0593121	0.01069868	0.05762474
	34.5 v 9	-0.9195585	0.15045232	0.28316005
	19 v 14	-0.8622601	0.20775065	0.4587022
	24 v 14	-0.8588081	0.1749205	0.37157791
	29 v 14	-0.7341132	0.29961548	0.80557389
	34.5 v 14	-0.5943595	0.43936912	0.99753938
	24 v 19	-0.5496945	0.52031633	0.99999943
	29 v 19	-0.4249995	0.64501131	0.98927932
	34.5 v 19	-0.2852458	0.78476495	0.7285864
	29 v 24	-0.3921693	0.64155929	0.97810027
	34.5 v 24	-0.2524157	0.78131294	0.64693169
	34.5 v 29	-0.3771107	0.65661796	0.96418873

Oxygen consumption – *Heterocucumis steineri*

One way ANOVA				
(Model: treatment)	O ₂ μmol/hr/gAFDM~Salinity	d.f.	F	P value
		5,40	9.228	0.000007
Post hoc Tukey test	comparisons (%)	CI lower	CI upper	P value
	14 v 9	-0.1641646	0.41068663	0.792245
	19 v 9	-0.5647315	0.01011974	0.064087
	24 v 9	-0.4185065	0.15634481	0.747295
	29 v 9	-0.0973959	0.47745536	0.372292
	34.5 v 9	-0.0902466	0.48460467	0.332118
	19 v 14	-0.6666713	-0.1344625	0.000757
	24 v 14	-0.5204463	0.0117626	0.068198
	29 v 14	-0.1993357	0.33287316	0.974011
	34.5 v 14	-0.1921864	0.34002246	0.959874
	24 v 19	-0.1198794	0.41232949	0.575219
	29 v 19	0.2012312	0.73344005	0.000073
	34.5 v 19	0.20838051	0.74058936	0.000057
	29 v 24	0.05500613	0.58721498	0.010159
	34.5 v 24	0.06215544	0.59436429	0.008139
	34.5 v 29	-0.2589551	0.27325373	0.999999

Oxygen consumption – *Cucumaria georgiana*

One way ANOVA				
(Model: treatment) on logged data	O ₂ μmol/hr/gAFDM~Salinity	d.f.	F	P value
		5,29	7.33	0.000151
Post hoc Tukey test	comparisons (%)	CI lower	CI upper	P value
	14 v 9	-0.7154026	0.11582011	0.26882994
	19 v 9	-0.6707703	0.1604524	0.43876861
	24 v 9	-0.2574417	0.57378103	0.85164894
	29 v 9	-0.0490644	0.82272934	0.10473523
	34.5 v 9	-0.2195316	0.61169108	0.7042029
	19 v 14	-0.3709791	0.46024363	0.99944404
	24 v 14	0.04234957	0.87357226	0.02426964
	29 v 14	0.25072686	1.12252056	0.00057562
	34.5 v 14	0.08025962	0.9114823	0.01228537
	24 v 19	-0.0022827	0.82893997	0.05191758
	29 v 19	0.20609457	1.07788828	0.00133568
	34.5 v 19	0.03562733	0.86685002	0.02729914
	29 v 24	-0.2072341	0.66455965	0.60559092
	34.5 v 24	-0.3777013	0.45352139	0.99974944
	34.5 v 29	-0.6266496	0.2451441	0.76408863

Activity coefficient – *Stereichinus neumayeri*

Kruskal-Wallis rank sum test			
(Model: activity coefficient~salinity)	d.f.	χ ²	P value
	4	14.58	0.00566

Post-hoc Dunn test	comparisons (%)	z	P value	
	14 v 19	0	0.5	
	14 v 24	-1.8785634	0.03015207	
	19 v 24	-1.8785634	0.03015207	
	14 v 29	-2.0177162	0.02181041	
	19 v 29	-2.0177162	0.02181041	
	24 v 29	-0.1391528	0.44466469	
	14 v 34.5	-3.0613626	0.00110166	
	19 v 34.5	-3.0613626	0.00110166	
	24 v 34.5	-1.1827992	0.11844437	
	29 v 34.5	-1.0436463	0.14832452	
Activity coefficient – <i>Odontaster validus</i>				
Kruskal-Wallis rank sum test				
(Model: activity coefficient~salinity)	d.f.	χ^2	P value	
5		26.29	0.00008	
Post-hoc Tukey test	comparisons (%)	z	P value	
	34.5 v 9	3.6	0.0002	
	29 v 9	3.22	0.0006	
	29 v 34.5	-0.37	0.3539	
	24 v 9	2.17	0.0149	
	24 v 34.5	-1.42	0.0772	
	24 v 29	-1.05	0.147	
	19 v 9	0.56	0.287	
	19 v 34.5	-3.04	0.0012	
	19 v 29	-2.66	0.0039	
	19 v 24	-1.61	0.0535	
	14 v 9	0	0.5	
	14 v 34.5	-3.6	0.0002	
	14 v 29	-3.22	0.0006	
	14 v 24	-2.17	0.0149	
	14 v 19	-0.56	0.287	
Activity coefficient – <i>Ophionotus victoriae</i>				
Kruskal-Wallis rank sum test				
(Model: activity coefficient~salinity)	d.f.	χ^2	P value	
4		21.27	0.00028	
Post-hoc Dunn test	comparisons (%)	z	P value	
	34.5 v 29	-0.19	0.4248	
	34.5 v 24	-2.82	0.0024	
	34.5 v 19	-3.18	0.0007	
	34.5 v 14	-3.18	0.0007	
	29 v 24	-2.63	0.0043	
	29 v 19	-2.99	0.0014	
	29 v 14	-2.99	0.0014	
	24 v 19	-0.36	0.3611	
	24 v 14	-0.36	0.3611	
	19 v 14	0	0.5	
Activity (number of waves)– <i>Heterocucumis steineni</i>				
Poisson				
(Model: Waves ~ Salinity, family = poisson(link = log))				
	Coefficients	SE	z	P value
Intercept	-3.7809	0.71	-5.32	0
Salinity	0.1499	0.02	6.52	0
Exact Poisson test (Two sample Poisson test)				
	comparisons (%)	P value	Bonferroni adjusted P value	
	34.5 v 29	0.18392	1	
	34.5 v 24	0.00256	0.03845	
	34.5 v 19	0	0.00007	
	34.5 v 14	0	0	
	34.5 v 9	0	0	
	29 v 24	0.12208	1	
	29 v 19	0.00149	0.02234	
	29 v 14	0.00001	0.00011	
	29 v 9	0.00005	0.00072	
	24 v 19	0.146	1	
	24 v 14	0.00391	0.05859	
	24 v 9	0.01284	0.19256	
	19 v 14	0.25	1	
	19 v 9	0.26531	1	
	14 v 9	1	1	
Activity (number of waves)– <i>Cucumaria georgiana</i>				

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Poisson (Model: Waves ~ Salinity, family = poisson(link = log))				
	Coefficients	SE	z	P value
Intercept	-1.32637	0.38	-3.46	0.00054
Salinity	0.0981	0.01	7.43	0
Exact Poisson test (Two sample Poisson test)			comparisons (%)	Bonferroni adjusted P value
			P value	
			34.5 v 29	0.07427
			34.5 v 24	0.11425
			34.5 v 19	0.00126
			34.5 v 14	0
			34.5 v 9	0
			29 v 24	0.8852
			29 v 19	0.188
			29 v 14	0.00001
			29 v 9	0
			24 v 19	0.12629
			24 v 14	0
			24 v 9	0
			19 v 14	0.00131
			19 v 9	0.00003
			14 v 9	0.5
Activity (number of waves)– <i>Echinopsolus charcoti</i>				
Poisson (Model: Waves ~ Salinity, family = poisson(link = log))				
	Coefficients	SE	z	P value
Intercept	-1.61883	0.47	-3.42	0.00062
Salinity	0.07915	0.02	4.73	0
Exact Poisson test (Two sample Poisson test)			comparisons (%)	Bonferroni adjusted P value
			P value	
			34.5 v 29	1
			34.5 v 24	1
			34.5 v 19	0.36159
			34.5 v 14	0.00001
			34.5 v 9	0.00005
			29 v 24	1
			29 v 19	0.45826
			29 v 14	0.00002
			29 v 9	0.00009
			24 v 19	0.36159
			24 v 14	0.00001
			24 v 9	0.00005
			19 v 14	0.00049
			19 v 9	0.00186
			14 v 9	1

Table B3| Statistics for coelomic fluid osmolality measurements.

Coelomic fluid osmolality after 6 hours <i>Sterechinus neumayeri</i>							
Kruskal-Wallis rank sum test (Model: CF osmolality~salinity)							
	d.f.	χ^2	P value				
4		17.10	0.00185				
Post-hoc Dunn test	comparisons (%)	z	P value	One-sample t-test	d.f.	t	P value
	34.5 v 29	-1.38	0.08440	34.5‰ treatment, tank water =1073	7	-1.05	0.32900
	34.5 v 24	-2.71	0.00340	29‰ treatment, tank water =864	2	14.08	0.00501
	34.5 v 19	-2.38	0.00870	24‰ treatment, tank water = 716	2	5.22	0.03476
	34.5 v 14	-3.55	0.00020	19‰ treatment, tank water = 565	2	37.34	0.00072
	29 v 24	-1.11	0.13420	14‰ treatment, tank water = 420	2	11.56	0.00740
	29 v 19	-0.83	0.20330				
	29 v 14	-1.80	0.03610				
	24 v 19	0.28	0.39100				
	24 v 14	-0.69	0.24460				
	19 v 14	-0.97	0.16650				
Coelomic fluid osmolality after 6 hours <i>Odontaster validus</i>							

Welch's ANOVA (Model: CF osmolality~salinity)								
		d.f.	<i>f</i>	<i>P</i> value				
	5,6,6		395.73	0.00000				
Post-hoc Games-Howell test	comparisons (%)		<i>q</i>	<i>P</i> value	One-sample t-test	d.f.	<i>t</i>	<i>P</i> value
	34.5 v 29		23.79	0.01032	34.5‰ treatment, tank water =1044	4	2.05	0.10990
	34.5 v 24		58.88	0.00001	29‰ treatment, tank water =879	2	1.71	0.22880
	34.5 v 19		40.14	0.00006	24‰ treatment, tank water = 738	2	13.75	0.07029
	34.5 v 14		13.28	0.00421	19‰ treatment, tank water = 578	2	1.93	0.19360
	34.5 v 9		7.47	0.11712	14‰ treatment, tank water = 413	4	7.15	0.00202
	29 v 24		17.31	0.00141	9‰ treatment, tank water = 267	2	2.98	0.09669
	29 v 19		15.05	0.00040				
	29 v 14		7.36	0.02944				
	29 v 9		5.16	0.22098				
	24 v 19		0.97	0.97744				
	24 v 14		2.38	0.59707				
	24 v 9		3.11	0.47157				
	19 v 14		2.02	0.71546				
	19 v 9		2.97	0.49708				
	14 v 9		2.00	0.72923				
Coelomic fluid osmolality after 6 hours <i>Ophionotus victor</i>								
One way ANOVA (Model: CF osmolality~salinity)								
		d.f.	<i>f</i>	<i>P</i> value				
	3,9		213.00	0.00000				
Post-hoc Tukey test	comparisons (%)	lwr	upr	<i>P</i> value	One-sample t-test	d.f.	<i>t</i>	<i>P</i> value
	24 v 19	-23.057379	57.5573793	0.56501104	34.5‰ treatment, tank water =1067	2	1.15	0.36780
	29 v 19	117.609287	198.224046	3.19E-06	29‰ treatment, tank water =893	2	3.28	0.08153
	34.5 v 19	253.609287	334.224046	1.06E-08	24‰ treatment, tank water = 728	2	5.37	0.03297
	29 v 24	97.5762657	183.757068	1.48E-05	19‰ treatment, tank water = 586	3	33.19	0.00006
	34.5 v 24	233.576266	319.757068	4.00E-08				
	34.5 v 29	92.909599	179.090401	1.96E-05				
Coelomic fluid osmolality after 6 hours <i>Heterocucumis steieni</i>								
Welch's ANOVA (Model: CF osmolality~salinity)								
		d.f.	<i>f</i>	<i>P</i> value				
	5, 4,8		92.23	0.00009				
Post-hoc Games-Howell test	comparisons (%)		<i>q</i>	<i>P</i> value	One-sample t-test	d.f.	<i>t</i>	<i>P</i> value
	34.5 v 29		20.12	0.01441	34.5‰ treatment, tank water =1042	2	2.20	0.15880
	34.5 v 24		12.57	0.04298	29‰ treatment, tank water =873	2	2.93	0.09917
	34.5 v 19		21.82	0.01304	24‰ treatment, tank water = 718	2	3.49	0.07309
	34.5 v 14		4.65	0.26575	19‰ treatment, tank water = 566	2	25.34	0.00155
	34.5 v 9		21.05	0.01448	14‰ treatment, tank water = 405	2	6.87	0.02055
	29 v 24		4.73	0.21510	9‰ treatment, tank water = 276	2	41.46	0.00058
	29 v 19		3.86	0.25104				
	29 v 14		1.47	0.87432				
	29 v 9		5.36	0.11380				
	24 v 19		-2.58	0.56258				
	24 v 14		-0.58	0.99668				
	24 v 9		-1.46	0.88215				
	19 v 14		0.54	0.99715				
	19 v 9		1.73	0.81009				
	14 v 9		-0.06	1.00000				
Coelomic fluid osmolality after 6 hours <i>Cucumaria georgiana</i>								
Welch's ANOVA (Model: CF osmolality~salinity)								

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		d.f.	f	P value				
Post-hoc Howell test	Games- comparisons (%)	5,4	70.44	0.000510				
			q	P value	One-sample t-test	d.f.	t	P value
	34.5 v 29	24.10	0.005167		34.5‰ treatment, tank water =1031	2	2.65	0.118100
	34.5 v 24	5.95	0.174748		29‰ treatment, tank water =864	2	5.28	0.034020
	34.5 v 19	13.63	0.035922		24‰ treatment, tank water = 716	2	2.80	0.107200
	34.5 v 14	20.42	0.015855		19‰ treatment, tank water = 565	2	6.25	0.024640
	34.5 v 9		N/A		14‰ treatment, tank water = 419	2	8.94	0.012270
	29 v 24	1.78	0.792748		9‰ treatment, tank water = 270	2	4.67	0.134300
	29 v 19	7.05	0.110413					
	29 v 14	12.81	0.029038					
	29 v 9		N/A					
	24 v 19	2.43	0.590333					
	24 v 14	5.07	0.151114					
	24 v 9		N/A					
	19 v 14	3.41	0.328950					
	19 v 9		N/A					
	14 v 9		N/A					
Coelomic fluid osmolality after 6 hours <i>Echinopsolus charcoti</i>								
Welch's ANOVA (Model: CF osmolality~salinity)								
Post-hoc Howell test	Games- comparisons (%)	5,5.5	431.33	0.00000				
			q	P value	One-sample t-test	t	P value	
	34.5 v 29	17.41	0.01852		34.5‰ treatment, tank water =1056	-0.66	0.57840	
	34.5 v 24	11.94	0.04686		29‰ treatment, tank water =875	4.57	0.04470	
	34.5 v 19	7.46	0.11641		24‰ treatment, tank water = 718	5.18	0.03536	
	34.5 v 14	62.58	0.00000		19‰ treatment, tank water = 565	2	7.36	0.01797
	34.5 v 9	56.91	0.00002		14‰ treatment, tank water = 413	119.49	0.00007	
	29 v 24	4.08	0.27429		9‰ treatment, tank water = 251	94.18	0.00000	
	29 v 19	2.57	0.57719					
	29 v 14	7.67	0.07698					
	29 v 9	13.92	0.00816					
	24 v 19	-0.13	1.00000					
	24 v 14	-0.94	0.97094					
	24 v 9	2.30	0.65084					
	19 v 14	-0.45	0.99878					
	19 v 9	1.64	0.83064					
	14 v 9	11.65	0.00353					
Osmotic gradient (coelomic fluid compared to tank water) for all species (except <i>O. victoriae</i>) after 24 hours at 14‰								
One way ANOVA (Model: osmolality~species)								
Post hoc Tukey test			d.f.	f	P value			
		4,17		13.73	0.00004			
			CI	CI				
	comparisons		lower	upper	P value			
	<i>E_charcoti</i> - <i>C_georgiana</i>	106.4	284.97	0.00003				
	<i>H_steieni</i> - <i>C_georgiana</i>	-40.6	137.97	0.48338				
	<i>O_validus</i> - <i>C_georgiana</i>	-29.6	130.14	0.34701				
	<i>S_neumayeri</i> - <i>C_georgiana</i>	29.37	177.46	0.00428				
	<i>H_steieni</i> - <i>E_charcoti</i>	-236	-57.70	0.00089				
	<i>O_validus</i> - <i>E_charcoti</i>	-225	-65.53	0.00031				
	<i>S_neumayeri</i> - <i>E_charcoti</i>	-166	-18.21	0.01106				
	<i>O_validus</i> - <i>H_steieni</i>	-78.3	81.47	1.00000				
	<i>S_neumayeri</i> - <i>H_steieni</i>	-19.3	128.79	0.20909				
	<i>S_neumayeri</i> - <i>O_validus</i>	-9.2	115.50	0.11608				

Table B4 Mean osmolality gradients (differences between coelomic fluid and external tank sea water) in six species of Antarctic echinoderms after 6 and 24 hours in different salinities. Values are means $\pm$ s.e.m. osmolality (mOsm/kg).

Species	Time in treatment	34.5‰	29‰	24‰	19‰	14‰	9‰
<i>Sterechinus neumayeri</i>	6 hr	1 $\pm$ 1	43 $\pm$ 3**	120 $\pm$ 23*	293 $\pm$ 8***	365 $\pm$ 31**	N/A
<i>Odontaster validus</i>	6 hr	3 $\pm$ 2	16 $\pm$ 9	19 $\pm$ 7	73 $\pm$ 28	270 $\pm$ 38**	282 $\pm$ 95
<i>Ophionotus victoriae</i>	6 hr	7 $\pm$ 6	44 $\pm$ 13	67 $\pm$ 13*	193 $\pm$ 6***	N/A	N/A
<i>Heterocucumus steineni</i>	6 hr	4 $\pm$ 2	29 $\pm$ 10	93 $\pm$ 27	296 $\pm$ 12**	433 $\pm$ 63*	566 $\pm$ 14***
<i>Cucumaria georgiana</i>	6 hr	7 $\pm$ 3	37 $\pm$ 8*	128 $\pm$ 46	186 $\pm$ 30*	236 $\pm$ 27*	492 $\pm$ 106 (n=2)
<i>Echinopsolus charcoti</i>	6 hr	0 $\pm$ 1	48 $\pm$ 11*	127 $\pm$ 25*	285 $\pm$ 39*	459 $\pm$ 4***	553 $\pm$ 6***
<i>Sterechinus neumayeri</i>	24 hr	1 $\pm$ 1	2 $\pm$ 2	39 $\pm$ 4***	92 $\pm$ 7***	152 $\pm$ 12***	N/A
<i>Odontaster validus</i>	24 hr	3 $\pm$ 2	7 $\pm$ 5	18 $\pm$ 3**	30 $\pm$ 7*	99 $\pm$ 21**	133 $\pm$ 24**
<i>Ophionotus victoriae</i>	24 hr	7 $\pm$ 6	3 $\pm$ 3	31 $\pm$ 6*	77 $\pm$ 5***	N/A	N/A
<i>Heterocucumus steineni</i>	24 hr	4 $\pm$ 2	1 $\pm$ 2	35 $\pm$ 9	117 $\pm$ 7**	97 $\pm$ 26	161 $\pm$ 55
<i>Cucumaria georgiana</i>	24 hr	7 $\pm$ 3	1 $\pm$ 1	10 $\pm$ 3	34 $\pm$ 3**	48 $\pm$ 9*	N/A
<i>Echinopsolus charcoti</i>	24 hr	0 $\pm$ 1	12 $\pm$ 3	41 $\pm$ 6*	151 $\pm$ 22*	244 $\pm$ 1***	244 $\pm$ 2***

Asterisk indicates a significant difference (one-sample t-test: *P<0.05, **P<0.01, ***P<0.001)

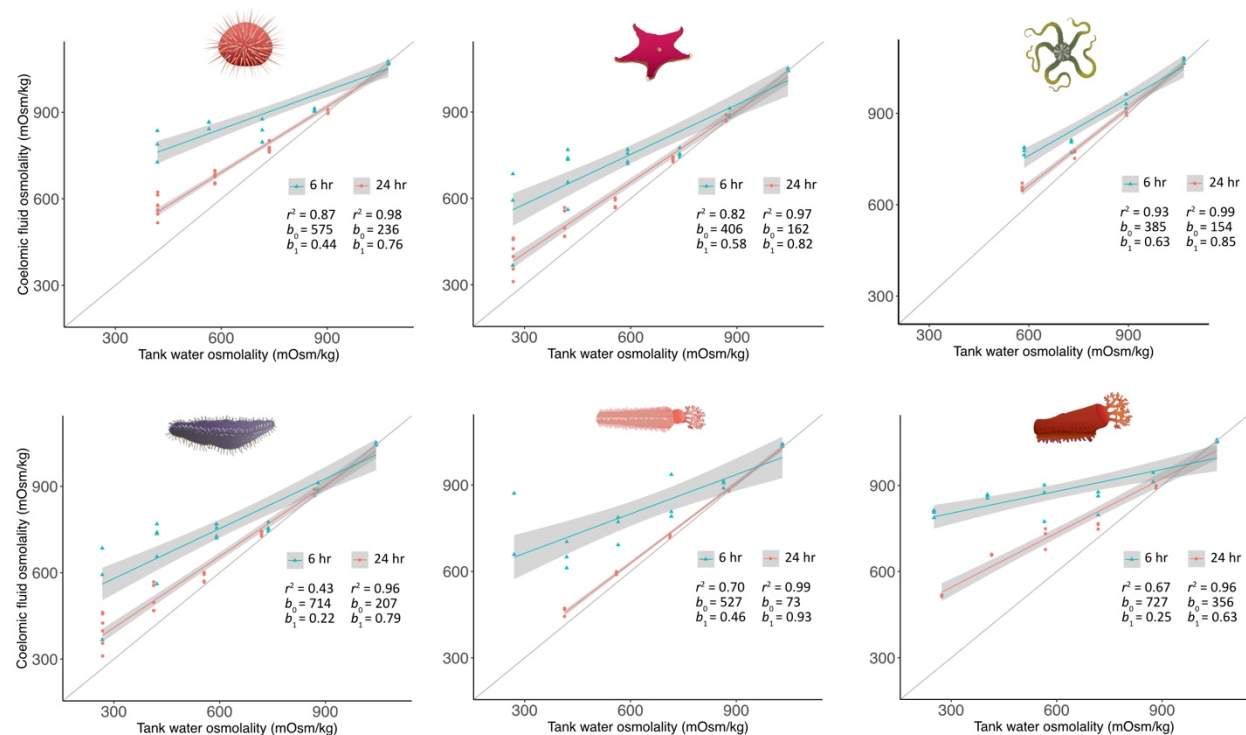


Figure B1 Coelomic fluid osmolality of Antarctic echinoderms at 6 and 24 h after transfer from ambient salinity (34.5‰) versus that of the corresponding tank water. Number of animal replicates at each experimental salinity treatment were *S. neumayeri* at 6 h $n=3$, at 24 h $n=7-8$; *O. validus* at 6 h $n=3-5$, at 24 h $n=5-6$; *H. steineni*, *E. charcoti* and *C. georgiana* at 6 and 24 h $n=3$ (except 9‰ for *C. georgiana* at 6 h, $n=2$). Mean ($\pm$ s.e.m.) tank water osmolality (mOsm kg $^{-1}$) in each salinity experiment was: 34.5‰= 1051 $\pm$ 6, 29‰=876 $\pm$ 3, 24‰=721 $\pm$ 2, 19‰=576 $\pm$ 2, 14‰=414 $\pm$ 2 and 9‰=269 $\pm$ 3. A regression line (blue line) and the standard error of the mean (shaded grey area) were fitted to the data. The iso-osmotic line is represented by a grey line. Regression coefficients and r^2 values are included to the right of the isosmotic line.

Appendix C – Supplementary material for Chapter 4

Table C1| Statistics for *Odontaster validus* measurements.

Feeding rate						
two-way ART ANOVA with repeated measures (data non-normal)						
Model:Food	eaten					P
per(g)AFDM~Salinity*Day+(1 Animal)				d.f.	F	value
Salinity				2256.000	81.068	0.000
				10256.00		
Day				0	2.730	0.000
				20256.00		
Salinity:Day				0	1.760	0.000
Feeding rate at each timepoint						
one way ANOVA (Welch's ANOVA day 29, 43 and 64)	d.f.	Sum Sq	Mean Sq	F	P value	Holm adj P
day -6	2,24	32.200	16.100	0.174	0.841	0.841
day 1 (square root transformed data)	2,24	37.130	18.565	17.370	0.000	0.000
day 8	2,24	2109.000	1054.600	9.601	0.001	0.009
day 15	2,24	2334.000	1166.900	13.020	0.000	0.000
day 22	2,24	1802.000	900.800	7.838	0.002	0.014
day 29 (square root transformed data)	2,14			7.988	0.005	0.020
day 36	2,24	1493.000	746.700	7.111	0.004	0.020
day 43 (logged transformed data)	2,14			7.102	0.007	0.021
day 50	2,24	735.800	367.900	3.605	0.043	0.086
day 57	2,24	1547.000	773.600	10.480	0.001	0.009
day 64 (square root transformed data)	2,14			10.351	0.002	0.014
	Post hoc test	comparisons	estimate	conf.low	conf.high/q	P
					value	value
day 1	Tukey	Medium-Control	-0.255	-1.472	0.962	0.861
		Low-Control	-2.605	-3.822	-1.388	0.000
		Low-Medium	-2.350	-3.568	-1.133	0.000
day 8	Tukey	Medium-Control	-0.004	-12.342	12.334	1.000
		Low-Control	-18.751	-31.089	-6.413	0.002
		Low-Medium	-18.747	-31.085	-6.409	0.002
day 15	Tukey	Medium-Control	-5.065	-16.212	6.081	0.503
		Low-Control	-21.760	-32.907	-10.614	0.000
		Low-Medium	-16.695	-27.842	-5.549	0.003
day 22	Tukey	Medium-Control	0.673	-11.948	13.293	0.990
		Low-Control	-16.982	-29.603	-4.361	0.007
		Low-Medium	-17.655	-30.275	-5.034	0.005
Post hoc Games-Howell test					q value	P
					value	value
day 29	Games-Howell	Medium-Control			0.621	0.900
		Low-Control			-5.630	0.008
		Low-Medium			-5.507	0.006
day 36	Tukey	Medium-Control	-4.530	-16.594	7.533	0.622
		Low-Control	-17.546	-29.609	-5.482	0.004
		Low-Medium	-13.015	-25.079	-0.952	0.033
day 43	Games-Howell	Medium-Control			0.443	0.947
		Low-Control			-5.132	0.012
		Low-Medium			-5.448	0.010
day 50	Tukey	Medium-Control	-3.489	-15.382	8.403	0.747
		Low-Control	-12.398	-24.291	-0.506	0.040
		Low-Medium	-8.909	-20.801	2.983	0.169
day 57	Tukey	Medium-Control	-2.225	-12.338	7.888	0.848
		Low-Control	-17.054	-27.168	-6.941	0.001
		Low-Medium	-14.829	-24.943	-4.716	0.003
day 64	Games-Howell	Medium-Control			3.496	0.064
		Low-Control			-4.822	0.015
		Low-Medium			-6.249	0.004

Tank effects - Feeding						
Mixed model two way ANOVA with repeat measures						
Model:Food eaten~Tank*Day+Error(Animal)	Error	Sum sq	Mean sq	d.f.	F	P value
Control						
Tank	Animal	351	175.400	2,6	0.303	0.749
Day	Within	523	52.350	10,60	0.957	0.489
Tank:Day	Within	783	39.160	20,60	0.716	0.794
Medium						
Tank	Animal	1614	807.100	2,6	1.919	0.227
Day	Within	990.1	99.010	10,60	1.913	0.061
Tank:Day	Within	520.1	26.010	20,60	0.502	0.955
Low						
Tank	Animal	1258	629.000	2,6	1.554	0.286
Day	Within	4076	407.600	10,60	5.315	0.000
Tank:Day	Within	1694	84.700	20,60	1.105	0.369
Righting (Activity coefficient)						
two-way ANOVA with repeated measures						
linear mixed-effects model (LME) fitted by REML						
Model:AC~Salinity*Day+(1 Animal)	Error	Sum sq	Mean sq	d.f.	F	P value
Salinity	Animal	223.94	111.970	2,24	30.768	0.000
				11264.00		
Day	Within	150.76	13.706	0	14.587	0.000
				22264.00		
Salinity:Day	Within	73.308	3.332	0	3.563	0.000
Random effects	Variance	Std.Dev				
Animal	0.225	0.4743				
Residuals	0.9396	0.9693				
ICC value = 0.193 (19% of variance explained by Animal)						
AC at each timepoint						
	d.f.	Sum Sq	Mean Sq	F/ χ^2	P value	Holm adj p
day -6 one way ANOVA	2,24	4.020	2.009	1.320	0.286	0.286
day 1 one way ANOVA	2,24	14.780	7.392	4.412	0.023	0.046
day 8 one way ANOVA	2,24	18.560	9.282	8.607	0.002	0.008
day 15 one way ANOVA	2,24	32.090	16.044	10.160	0.001	0.006
day 22 Welch's ANOVA	2,14			7.191	0.007	0.021
day 29 one way ANOVA	2,24	49.630	24.816	19.320	0.000	0.000
day 36 (logged data) one way ANOVA	2,24	4.615	2.308	8.846	0.001	0.006
day 43 Welch's ANOVA	2,24	18.600	9.298	18.630	0.000	0.000
day 50 (logged data) one way ANOVA	2,24	9.130	4.565	41.250	0.000	0.000
day 57 Welch's ANOVA	2,14			40.573	0.000	0.000
day 64 one way ANOVA	2,24	18.770	9.388	11.900	0.000	0.000
day 71 one way ANOVA	2,24	34.330	17.166	11.250	0.000	0.000
					q value/mean difference	P value
	Post hoc test		conf.low	conf.high		
		Med-Con	-0.352	2.696		0.154
	Tukey	Low-Con	-2.135	0.913		0.583
day 1		Low-Med	-3.307	-0.260		0.020
		Med-Con	-1.633	0.813		0.684
	Tukey	Low-Con	-3.150	-0.705		0.002
day 8		Low-Med	-2.740	-0.295		0.013
		Med-Con	-2.691	0.267		0.123
	Tukey	Low-Con	-4.146	-1.187		0.000
day 15		Low-Med	-2.934	0.025		0.055
		Med-Con			-2.287	0.274
	Games-Howell	Low-Con			-4.727	0.020
day 22		Low-Med			-3.433	0.076
		Med-Con	-1.958	0.711		0.484
	Tukey	Low-Con	-4.471	-1.802		0.000
day 29		Low-Med	-3.848	-1.179		0.000
		Med-Con	-0.857	0.346		0.547
	Tukey	Low-Con	-1.578	-0.375		0.001
day 36		Low-Med	-1.322	-0.120		0.017
		Med-Con			-4.455	0.016
	Games-Howell	Low-Con			-9.035	0.000
day 43		Low-Med			-3.920	0.042
		Med-Con	-0.842	-0.059		0.022
	Tukey	Low-Con	-1.787	-1.004		0.000
day 50		Low-Med	-1.336	-0.553		0.000
		Med-Con			-3.674	0.065
	Tukey	Low-Con			-9.318	0.000
day 57		Low-Med			-11.651	0.000

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		Med-Con	-1.760	0.331		0.223
day 64	Tukey	Low-Con	-3.060	-0.969		0.000
		Low-Med	-2.345	-0.255		0.013
		Med-Con	-0.262	-0.849		0.516
day 71	Tukey	Low-Con	-1.191	-1.778		0.000
		Low-Med	-0.930	-1.517		0.002
Tank effects - Righting (Activity coefficient)						
Mixed model two way Anova with repeat measures						
Model:AC~Tank*Day+Error(Animal)						
Control						
	Error	Sum sq	Mean sq	d.f.	F	P value
Tank	Animal	11.43	5.713	2.000	1.804	0.243
Day	Within	39.25	3.568	11.000	2.326	0.018
Tank:Day	Within	33.91	1.541	22.000	1.005	0.471
Medium						
	Error	Sum sq	Mean sq	d.f.	F	P value
Tank	Animal	13.64	6.822	2.000	1.770	0.249
Day	Within	87.34	7.940	11.000	9.014	0.000
Tank:Day	Within	17.98	0.817	22.000	0.928	0.562
Low						
	Error	Sum sq	Mean sq	d.f.	F	P value
Tank	Animal	1.954	0.977	2.000	0.736	0.736
Day	Within	97.49	8.862	11.000	21.830	0.000
Tank:Day	Within	10.02	0.456	22.000	1.122	0.348
Oxygen consumption						
two-way ANOVA with repeated measures						
linear mixed-effects model (LME) fitted by REML						
	Error	Sum sq	Mean sq	d.f.	F	P value
Model:O2~Salinity*Day+Random(Animal)	Animal	0.2489	0.124	2,24	1.117	0.344
Salinity	Within	0.9381	0.156	6144.000	10.914	0.000
Day				12144.00		
Salinity:Day	Within	0.33656	0.028	0	1.957	0.032
Random effects	Variance	Std.Dev				
Animal		0.08503	0.2916			
Residuals		0.09116	0.3019			
ICC value = 0.483 (48% of variance explained by Animal)						
Oxygen consumption at each timepoint						
Model = one way ANOVA	d.f.	Sum Sq	Mean Sq	F/χ²	P value	Holm adj P
day -6	2,24	0.102	0.051	1.746	0.196	1.00
day 1	2,24	0.600	0.300	1.726	0.199	1.00
day 8 (logged data)	2,24	0.083	0.041	1.522	0.239	1.00
day 15	2,24	0.412	0.206	1.777	0.191	1.00
day 29 (logged data)	2,24	0.059	0.029	0.660	0.526	1.00
day 57	2,24	1.039	0.520	3.210	0.058	0.41
day 71	2,24	0.300	0.150	0.818	0.453	1.00
Tank effects - Oxygen consumption						
Mixed model two way Anova with repeat measures						
Model:AC~Tank*Day+Error(Animal)						
Control						
	Error	Sum sq	Mean sq	d.f.	F	P value
Tank	Animal	0.818	0.409	2.000	0.500	0.630
Day	Within	2.147	0.358	6.000	4.648	0.001
Tank:Day	Within	1.747	0.146	12.000	1.891	0.070
Medium						
	Error	Sum sq	Mean sq	d.f.	F	P value
Tank	Animal	2.837	1.418	2.000	4.074	0.076
Day	Within	4.005	0.668	6.000	7.295	0.000
Tank:Day	Within	2.044	0.170	12.000	1.862	0.074
Low						
	Error	Sum sq	Mean sq	d.f.	F	P value
Tank	Animal	1.344	0.672	2.000	0.901	0.455
Day	Within	1.376	0.229	6.000	3.576	0.007
Tank:Day	Within	0.9608	0.080	12.000	1.248	0.290
Hypoosmotic shock						
Oxygen consumption between acclimated treatments after transfer to 19‰						

Model:O2~Salinity						P value
		d.f.	Sum Sq	Mean Sq	F	
One way ANOVA		2,15	26.770	13.387	7.609	0.005
Post hoc test			conf.low	conf.high	mean difference	P value
		Medium-Control	-2.774	1.205	-0.784	0.573
Tukey		Low-Control	0.115	4.093	2.104	0.038
		Low-Medium	0.899	4.878	2.889	0.005
Oxygen consumption Acclimated (day 71) v Shock						
Two sample t-test				d.f.	T	P value
Control				13.000	4.812	0.000
Medium				13.000	4.407	0.001
Low				13.000	0.026	0.980
AC between acclimated treatments after transfer to 19‰						
Model:AC~Salinity						P value
One way ANOVA		d.f.	Sum Sq	Mean Sq	F	
		2	1.041	0.520	3.285	0.066
AC Acclimated (day 71) v Shock						
Two sample t-test				d.f.	T	P value
Control				8.400	6.800	0.000
Medium (logged data)				13.000	3.800	0.002
Low				9.600	2.000	0.072
Buoyant weight change						
Model:Mass~Salinity						P value
One way ANOVA		d.f.	Sum Sq	Mean Sq	F	
		2,23	0.059	0.029	6.944	0.004
Post hoc test			conf.low	conf.high	mean difference	P value
		Medium-Control	-0.075	0.079	0.002	0.998
Tukey		Low-Control	-0.181	-0.023	-0.102	0.010
		Low-Medium	0.025	0.183	0.104	0.009
Osmolality						
Model: Osmolality~Salinity*Day						P value
Two way ANOVA		d.f.	Sum Sq	Mean Sq	F	
			839088.0			
Salinity		2,30	00	419544	68903.220	0.000
Day		4,30	1680.000	420.000	68.960	0.000
Salinity:Day		8,30	489.000	61.000	10.030	0.000
Tukey All combination of different treatments						0.000
Coelomic fluid osmolality v tank water at each time point						
One-sample t-test						
Timepoint	Treatment and tank water osmolality (mOsm/kg)		Df	t	P value	
1	Control (1061)		2.000	0.655	0.580	
	Medium (887)		2.000	2.795	0.108	
	Low (730)		2.000	1.600	0.251	
	Control (1062)		2.000	3.464	0.074	
8	Medium (882)		2.000	5.261	0.034	
	Low (729)		2.000	1.600	0.251	
	Control (1071)		2.000	1.732	0.225	
	Medium (893)		2.000	-0.277	0.808	
15	Low (733)		2.000	3.000	0.095	
	Control (1070)		2.000	2.600	0.122	
	Medium (877)		2.000	12.124	0.007	
	Low (726)		2.000	3.051	0.093	
35	Control (1065)		2.000	8.718	0.013	
	Medium (900)		2.000	22.000	0.002	
	Low (746)		2.000	1.890	0.199	

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Mortality	Data structure	Control	Medium	Low	
	Mortality	0.000	0.000	3.000	
	Survived	36.000	36.000	33.000	
Pearson's Chi-squared test			d.f.	χ^2	P value
			2.000	6.171	0.046

Table C2| Statistics for *Sterechinus neumayeri* measurements.

Feeding rate						
two-way ART ANOVA with repeated measures						
Model:Food eaten per(g)AFDM~Salinity*Day+(1 Animal)						
			d.f.	F	P value	
Salinity			2,23	63.59	0.000000	
Day			12,252	18.21	0.000000	
Salinity:Day			24,252	7.93	0.000000	
Feeding rate at each timepoint						
Model	d.f.	Sum Sq	Mean Sq	F/ χ^2	P value	Holm adj P
Day -13 one way ANOVA	2,21	227	113.5	0.55	0.588000	1.000000
Day -6 one way ANOVA	2,21	78.4	39.19	0.28	0.759000	1.000000
Day 1 Welch's ANOVA	2,10			17.36	0.000549	0.002745
Day 8 Kruskal-Wallis	2			16.20	0.000304	0.002432
Day 15 Kruskal-Wallis	2			17.24	0.000180	0.001620
Day 22 Kruskal-Wallis	2			15.60	0.000409	0.002454
Day 29 Kruskal-Wallis	2			18.23	0.000110	0.001100
Day 36 Kruskal-Wallis	2			12.63	0.001812	0.005436
Day 50 Welch's ANOVA	2,12			60.03	0.000001	0.000012
Day 57 Kruskal-Wallis	2			14.08	0.000877	0.003508
Day 71 Kruskal-Wallis	2			16.13	0.000315	0.002432
Day 64 one way ANOVA	2,21	6674	3337	15.42	0.000076	0.000836
Day 78 one way ANOVA	2,21	13204	6602	41.45	0.000000	0.000000
	Post hoc test		conf.low	conf.high	q value/mean difference	P value
day 1	Games-Howell	Medium-Control			-2.802	0.153657
		Low-Control			-7.687	0.001916
		Low-Medium			-4.167	0.046047
day 8	Dunn	Medium-Control			0.504268	0.921100
		Low-Control			3.709977	0.000300
		Low-Medium			-3.205708	0.002000
day 15	Dunn	Medium-Control			1.282588	0.299500
		Low-Control			4.06153	0.000100
		Low-Medium			-2.778941	0.008200
day 22	Dunn	Medium-Control			-0.071254	1.000000
		Low-Control			3.384608	0.001100
		Low-Medium			-3.455863	0.000800
day 29	Dunn	Medium-Control			1.512806	0.195500
		Low-Control			4.214246	0.000000
		Low-Medium			-2.701439	0.010400
day 36	Dunn	Medium-Control			1.38947	0.247000
		Low-Control			3.527118	0.000600
		Low-Medium			-2.137647	0.048800
day 50	Games-Howell	Medium-Control			-1.869	0.411805
		Low-Control			-14.9	0.000002
		Low-Medium			-7.419	0.001858
day 57	Dunn	Medium-Control			0.03551	1.000000
		Low-Control			3.266926	0.001600
		Low-Medium			-3.231416	0.001800
day 64	Dunn	Medium-Control			-0.43223	0.998400
		Low-Control			3.241727	0.001800
		Low-Medium			-3.673958	0.000400
day 71	Tukey	Medium-Control	-20.873009	16.2021044		0.946072
		Low-Control	-55.021737	-17.946624		0.000187
		Low-Medium	-52.686285	-15.611171		0.000395
day 78	Tukey	Medium-Control	-18.379882	13.4291487		0.918992
		Low-Control	-66.85307	-35.04404		0.000000
		Low-Medium	-64.377704	-32.568673		0.000001
Tank effects - Feeding						

Mixed model two way Anova with repeat measures

Model:Food eaten~Tank*Day+Error(Animal)	Error	Sum sq	Mean sq	d.f.	F	P value
Control						
Tank	Animal	704	234.7	3,4	0.192000	0.897000
Day	Within	6421	194.6	12,48	3.252000	0.001760
Tank:Day	Within	7005	194.6	36,48	1.183000	0.290560
Medium						
Tank	Animal	5171	1724	3,4	1.291000	0.392000
Day	Within	14633	14633	12,48	4.128000	0.000200
Tank:Day	Within	14179	295.4	36,48	0.849000	0.693800
Low						
Tank	Animal	487	162.3	3,4	0.511000	0.696000
Day	Within	24486	24486	12,48	23.584000	0.000000
Tank:Day	Within	2511	69.7	36,48	0.806000	0.747000

Righting (Activity coefficient)

ANOVA of Aligned rank transformed data

Model:AC~Salinity*Day	d.f.	F	P value
Salinity	2,296	107.43	0.000000
Day	12,296	2.82	0.001142
Salinity:Day	24,296	1.60	0.039848

AC at each timepoint

Model	d.f.	Sum Sq	Mean Sq	F/χ^2	P value	Holm adj P
Day -6 one way ANOVA	2,26	0.196	0.0979	0.14	0.870000	0.87000
Day 1 one way ANOVA	2,20	1.591	0.7953	3.58	0.046700	0.09340
Day 8 Welch's ANOVA	2,14			6.51	0.009830	0.02949
Day 15 Kruskal-Wallis	2			20.35	0.000038	0.00042
Day 22 Welch's ANOVA	2,10			27.64	0.000087	0.00087
Day 29 Kruskal-Wallis	2			14.63	0.000665	0.00399
Day 36 Kruskal-Wallis	2			16.06	0.000326	0.00261
Day 50 Kruskal-Wallis	2			16.76	0.000229	0.00206
Day 57 Welch's ANOVA	2,10			39.71	0.000022	0.00029
Day 64 Kruskal-Wallis	2			13.00	0.001503	0.00752
Day 71 Kruskal-Wallis	2			15.56	0.000417	0.00292
Day 78 Kruskal-Wallis	2			11.40	0.003351	0.01340
Day 85 Welch's ANOVA	2,10			36.35	0.000033	0.00040
						q value/mean difference
		Post hoc test	conf.low	conf.high		P value
day 1	Tukey	Medium-Control	-0.7090366	0.68257408		0.998725
		Low-Control	-1.29151	0.16802357		0.151568
		Low-Medium	-1.1022495	0.00522553		0.052480
day 8	Games-Howell	Medium-Control			3.567	0.067037
		Low-Control			-2.422	0.235001
		Low-Medium			-5.028	0.013069
day 15	Dunn	Medium-Control			0.469975	0.957600
		Low-Control			4.075813	0.000100
		Low-Medium			-3.586647	0.000500
day 22	Games-Howell	Medium-Control			-0.917	0.796349
		Low-Control			-7.629	0.002147
		Low-Medium			-7.972	0.001506
day 29	Dunn	Medium-Control			0.617503	0.805400
		Low-Control			3.577886	0.000500
		Low-Medium			-2.960383	0.004600
day 36	Dunn	Medium-Control			-0.32424	1.000000
		Low-Control			3.2965	0.001500
		Low-Medium			-3.620746	0.000400
day 50	Dunn	Medium-Control			1.002672	0.474000
		Low-Control			3.93907	0.000100
		Low-Medium			-2.936398	0.005000
day 57	Games-Howell	Medium-Control			1.182	0.689254
		Low-Control			-10.598	0.000276
		Low-Medium			-7.738	0.002199
day 64	Dunn	Medium-Control			0.398058	1.000000
		Low-Control			3.255324	0.001700
		Low-Medium			-2.835733	0.006900
day 71	Games-Howell	Medium-Control			0.537265	0.886600
		Low-Control			3.653407	0.000400
		Low-Medium			-3.116142	0.002700
day 78	Games-Howell	Medium-Control			0.267383	1.000000
		Low-Control			3.048166	0.003500
		Low-Medium			-2.780783	0.008100
day 85	Dunn	Medium-Control			2.831	0.150252
		Low-Control			-5.356	0.015862

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Low-Medium				-11.333	0.000191	
Tank effects - AC						
two way Anova						
Model:AC~Tank*Day						
Control (logged data)						
	Sum sq	Mean sq	d.f.	F	P value	
Tank	0.3774	0.12581	3	0.38	0.769900	
Day	3.3556	0.27964	12	0.84	0.611900	
Tank:Day	5.9869	0.17106	35	0.51	0.980600	
Medium						
	Sum sq	Mean sq	d.f.	F	P value	
Tank	0.0445	0.01483	3	0.05	0.986800	
Day	3.6602	0.30502	12	0.94	0.510200	
Tank:Day	3.6557	0.10155	35	0.31	0.999800	
Low (Kruskal-Wallis on each independent variable)						
Model:AC~Tank/AC~Day			d.f.	χ²	P value	
Tank			4	4.39	0.356300	
Day			12	33.82	0.000719	
Oxygen consumption						
two-way ANOVA with repeated measures						
Mixed effects (Aligned rank transformed data-ART ANOVA)						
Model:O2~Salinity*Day+(1 Animal)			d.f.	F	P value	
Salinity			2,23	0.22	0.80645	
Day			6,126	14.5	0.000000	
Salinity:Day			12,126	4.6	0.000017	
Oxygen consumption at each timepoint						
	d.f.	Sum Sq	Mean Sq	F/χ²	P value	Holm adj P
Day -6 one way ANOVA	2,21	0.409	0.2046	0.58	0.568000	1.000000
Day 1 Kruskal-Wallis	2			2.45	0.294500	1.000000
Day 8 one way ANOVA	2,21	0.966	0.4831	1.15	0.335000	1.000000
Day 15 Welch's ANOVA	2,12			1.35	0.295400	1.000000
Day 29 one way ANOVA	2,21	2.723	1.3616	2.53	0.104000	0.624000
Day 57 one way ANOVA	2,21	2.345	1.1723	2.32	0.123000	0.624000
Day 85 one way ANOVA	2,21	11.78	5.89	6.43	0.00662	0.046340
	Post hoc test		conf.low	conf.high	q value/mean difference	P value
day 85	Tukey	Medium-Control	-0.7714331	1.639183	0.433875	0.64
		Low-Control	0.4489419	2.862558	1.60025	0.01
		Low-Medium	-0.0759331	2.408683	1.166375	0.05
Oxygen consumption - repeat experiment - end point day 78						
	Model	d.f.	Sum Sq	Mean Sq	F/χ²	P value
	one way ANOVA	2,20	7.482	3.741	9.60	0.001190
	Post hoc test		conf.low	conf.high	q value/mean difference	P value
day 78	Tukey	Medium-Control	-0.5538456	1.0251408	0.2356476	0.734060
		Low-Control	-1.9203713	-0.2859651	-1.1031682	0.007390
		Low-Medium	-2.1560189	-0.5216127	-1.3388158	0.001393
Tank effects - Oxygen consumption						
Mixed model two way ANOVA with repeat measures						
Model:AC~Tank*Day+Error(Animal)						
Control						
	Error	Sum sq	Mean sq	d.f.	F	P value
Tank	Animal	7.482	2.494	3	2.30	0.219000
Day	Animal	1.271	0.2119	6	1.27	0.307000
Tank:Day	Animal	4.983	0.2768	18	1.66	0.122000
Medium						
	Error	Sum sq	Mean sq	d.f.	F	P value
Tank	Animal	6.083	2.0276	3	5.51	0.066400
Day	Within	5.804	0.9674	6	5.67	0.000876
Tank:Day	Within	7.648	0.4249	18	2.49	0.019012
Low						
	Error	Sum sq	Mean sq	d.f.	F	P value
Tank	Animal	7.702	2.567	3	1.61	0.321500
Day	Animal	8.948	8.948	1	5.60	0.077200
Tank:Day	Animal	4.102	2.051	2	1.28	0.371200
Day	Within	29.57	4.929	6	29.91	0.000000
Tank:Day	Within	10.94	0.608	18	3.69	0.002550
Faecal production						

two-way ART ANOVA with repeated measures

Model:(mg) AFDM faeces per (mg)AFDM(urchin)~Salinity*Day+(1|Animal)

	d.f.	F	P value
Salinity	2,21	111.49	0.000000
Day	11,231	8.79	0.000000
Salinity:Day	22,231	6.15	0.000000

Faecal rate at each timepoint

Factual rate at each timepoint						
Model	d.f.	Sum Sq	Mean Sq	F/χ^2	P value	Holm adj P
Day -6 one way ANOVA	2,21	14.26	7.131	2.42	0.113000	0.1274
Day 1 one way ANOVA	2,21	136	67.99	3.15	0.063700	0.1274
Day 8 one way ANOVA	2,21	103.8	51.92	6.43	0.006640	0.01992
Day 15 Welch's ANOVA	2,9			21.75	0.000306	0.00153
Day 22 (squared data) Welch's ANOVA	2,10			32.61	0.000042	0.000336
Day 29 Kruskal-Wallis	2			15.76	0.000378	0.00153
Day 36 Welch's ANOVA	2			NaN	0.000010	0.00012
Day 50 Welch's ANOVA	2			NaN	0.000010	0.00012
Day 57 Welch's ANOVA	2			NaN	0.000010	0.00012
Day 64 Kruskal-Wallis	2			18.23	0.000110	0.00066
Day 71 Welch's ANOVA	2			NaN	0.000010	0.00012
Day 78 one way ANOVA	2,21	792.9	396.5	15.80	0.000065	0.000455
		Post hoc test		conf.low	conf.high	q value/mean difference
						P value
day 8	Tukey	Medium-Control	-5.6095437	1.5545437	-2.0275	0.345769
		Low-Control	-8.6437937	-1.4797063	-5.06175	0.005021
		Low-Medium	-6.6162937	0.5477937	-3.03425	0.106785
day 15	Games-Howell	Medium-Control			0.06	0.998996
		Low-Control			-6.709	0.005160
		Low-Medium			-6.945	0.004270
day 22	Games-Howell	Medium-Control			-4.861	0.010508
		Low-Control			-11.204	0.000169
		Low-Medium			4.07	0.050000
day 29	Dunn	Medium-Control			1	0.143175
		Low-Control			3.507789	0.000700
		Low-Medium			-3.364614	0.001100
day 36	Games-Howell	Medium-Control			0.515	0.930046
		Low-Control			-7.673	0.002441
		Low-Medium			-5.022	0.022263
day 50	Games-Howell	Medium-Control			0.016	0.999930
		Low-Control			-59.947	0.000000
		Low-Medium			-71.498	0.000000
day 57	Games-Howell	Medium-Control			-2.495	0.218120
		Low-Control			-28.189	0.000000
		Low-Medium			-20.772	0.000004
day 64	Dunn	Medium-Control			1.512806	0.195500
		Low-Control			4.214246	0.000000
		Low-Medium			-2.701439	0.010400
day 71	Games-Howell	Medium-Control			-1.022	0.755500
		Low-Control			-40.1	0.000000
		Low-Medium			-67.243	0.000000
day 78	Tukey	Medium-Control	-9.3896366	3.23638662	-3.076625	0.450153
		Low-Control	-19.750012	-7.1239884	-13.437	0.000073
		Low-Medium	-16.673387	-4.0473634	-10.360375	0.001307

Hypoosmotic shock

Oxygen consumption between acclimated treatments after transfer to 19‰

Model:O2~Salinity	d.f.	Sum Sq	Mean Sq	F	P value
One-way ANOVA	2,12	1.316	0.658	0.50	0.621000

Oxygen consumption Acclimated (day 85) v Shock

Two sample t-test	d.f.	T	P value
Control	11	-1.40	0.189900
Medium	11	0.090119	0.929800
Low	9	3.5388	0.00627

AC (60 minutes) between acclimated treatments after transfer to 19‰

Model:AC~Salinity	d.f.	χ^2	P value
Kruskal-Wallis	2	2.00	0.367900

AC (90 minutes) between acclimated treatments after transfer to 19‰

Model:AC~Salinity	d.f.	χ^2	P value
Kruskal-Wallis	2	6.90	0.031780
Post hoc test		mean dif	P value
Medium-Control		0	0.500000

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	Dunn	Low-Control		-2.274501	0.011500	
		Low-Medium		2.274501	0.011500	
AC Acclimated (day 85) v Shock						
Two sample t-test			W	d.f.	T	P value
Control				7	4.00	0.005427
Medium				7	8.3	0.000073
Low	Wilcoxon Mann-Whitney		24.5			0.801900
Percentage AFDM						
Model:%AFDM~Salinity		d.f.	Sum Sq	Mean Sq	F	P value
(arcsine transformed percentage data)	One way ANOVA	2,54	0.1536	0.0768	17.35	0.000002
	Post hoc test		conf.low	conf.high	mean difference	P value
	Tukey	Medium-Control	-0.0476148	0.05431734	0.00335127	0.986255
		Low-Control	-0.1658196	-0.0573515	-0.1115856	0.000022
		Low-Medium	0.0631529	0.16672081	0.11493685	0.000006
Osmolality						
Model: Osmolality~Salinity*Day	Two way ANOVA	d.f.	Sum Sq	Mean Sq	F	P value
Salinity		2,50	1341195	670597	44450.57	0.000000
Day		4,50	2443	611	40.48	0.000000
Salinity:Day		8,50	90	11	0.74	0.653000
	Tukey					
	All combination of different treatments					0.000000
Coelomic fluid osmolality v tank water at each time point						
One-sample t-test						
		Treatment and tank water osmolality (mOsm/kg)				
Timepoint			df	t		P value
		Control (1069)	4	2.64		0.057600
		Medium (886)	3	3.38		0.043010
1		Low (732)	4	2.84		0.046880
		Control (1066)	4	2.32		0.081310
		Medium (885)	4	5.36		0.005833
8		Low (730)	3	1.61		0.205100
		Control (1059)	4	2.72		0.052980
		Medium (871)	4	4.83		0.008458
15		Low (723)	4	1.43		0.227200
		Control (1055)	3	4.62		0.019080
		Medium (871)	3	2.66		0.076670
29		Low (720)	3	2.10		0.126500
		Control (1054)	4	3.21		0.032500
		Medium (880)	4	1.72		0.160900
85		Low (723)	4	3.77		0.019550
Coelomocytes - total cells						
Model: Total cells~Salinity	ANOVA	d.f.	Sum Sq	Mean Sq	F	P value
Logged data		2,12	7.989	3.995	3.88	0.050100
Coelomocytes - red cells						
	ANOVA (arcsine transformed data)					
Model: Red cells~Salinity		d.f.	Sum Sq	Mean Sq	F	P value
		2,12	0.01031	0.005155	0.31	0.741000
Coelomocytes - viable cells						
	ANOVA (arcsine transformed data)					
Model: Viable cells~Salinity		d.f.	Sum Sq	Mean Sq	F	P value
		2,12	0.00587	0.002937	0.81	0.466000
Mortality						
	Data structure		Control	Medium	Low	
		Mortality	4	4	9	
		Survived	36	36	31	
Pearson's Chi-squared test			d.f.	χ ²		P value
			2	3.426600		0.180300

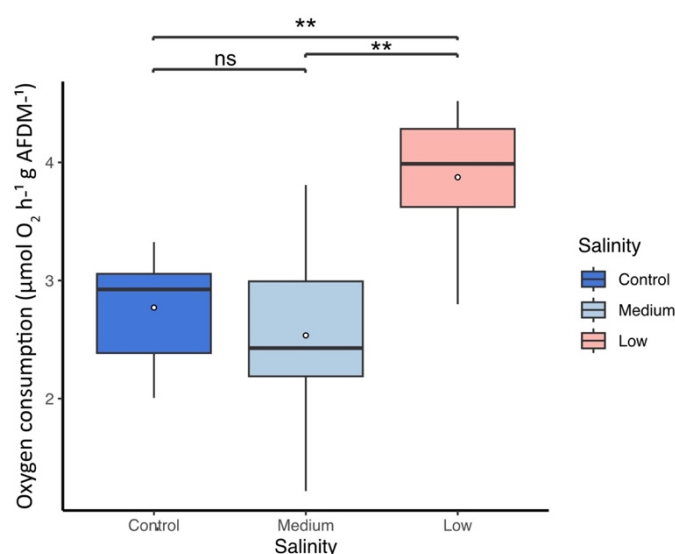


Figure C1 | Oxygen consumption of *Sterechnus neumayeri* exposed to different salinity treatments in the repeat experiment on day 78. Results are shown for one-way ANOVA and post hoc Tukey test between salinity treatments [n.s., not significant; ** $P < 0.01$; control (34.5‰) and medium (29‰) $n=8$, low (24‰) $n=7$].

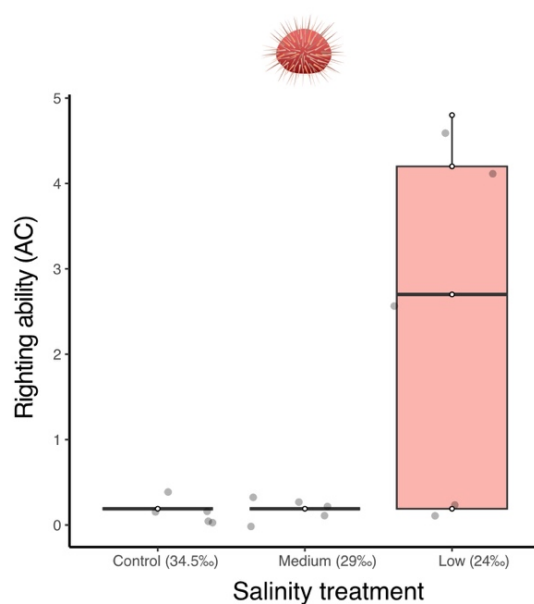


Figure C2 | Righting ability (activity coefficient) of *Sterechnus neumayeri* acclimated to different salinity treatment and then immersion in 19‰ for 6 hours. Maximum righting time was 90 minutes which equals a minimum AC value of 0.19 (1000/5400 s).

Appendix D – Supplementary material for Chapter 5**Table D1|** Equipment list.

Name	Model	Manufacturer
Gas chromatograph	8890	Agilent
ToF-MS	7250	Agilent
Autosampler	PAL3 RTC 120	PAL
GC column	DB-5ms	Agilent
Low pressure drop split/splitless liner	5190-2295	Agilent
Vortex mixer	Wizard	ThermoFisher Scientific
Centrifuge	5430 R	Eppendorf
Heat block	Dri-Block DB3-A	Techne
Vacuum concentrator	Savant SpeedVac SPD130DLX	ThermoFisher Scientific
Vapour trap	Savant RVT5105	ThermoFisher Scientific

Table D2| GC-MS injection sequence.

Injection Number	Sample Type
1	System Suitability Blank Sample
2	System Suitability Sample 1
3	System Conditioning QC sample 1
4	System Conditioning QC sample 2
5	System Conditioning QC sample 3
6	System Conditioning QC sample 4
7	Blank Extraction Sample 1
8	System Conditioning QC sample 5
9	System Conditioning QC sample 6
10	System Conditioning QC sample 7
11	System Conditioning QC sample 8
12	Pooled QC sample 9
13	Pooled QC sample 10
14	Standard Reference Material injection 1
15	Biological sample 1
16	Biological sample 2
17	Biological sample 3
18	Biological sample 4
19	Biological sample 5
20	Pooled QC Sample 11
21	Biological sample 6
22	Biological sample 7
23	Biological sample 8
24	Biological sample 9
25	Biological sample 10
26	Pooled QC Sample 12
27	Biological sample 11
28	Biological sample 12
29	Biological sample 13
30	Biological sample 14
31	Biological sample 15
32	Pooled QC Sample 13
33	Biological sample 16
34	Biological sample 17
35	Biological sample 18
36	Biological sample 19
37	Biological sample 20
38	Pooled QC Sample 16
39	Standard Reference Material injection 2
40	Biological sample 21
41	Biological sample 22
42	Biological sample 23
43	Biological sample 24
44	Biological sample 25
45	Pooled QC Sample 14
46	Biological sample 26
47	Biological sample 27
48	Biological sample 28
49	Biological sample 29
50	Biological sample 30
51	Pooled QC Sample 15
52	Biological sample 31
53	Biological sample 32
54	Biological sample 33
55	Biological sample 34
56	Biological sample 35
57	Pooled QC Sample 16
58	Biological sample 36
59	Biological sample 37
60	Standard Reference Material injection 3
61	Biological sample 38
62	Biological sample 39
63	Biological sample 40
64	Pooled QC Sample 17
65	Pooled QC Sample 18
66	Blank Extraction Sample 2
67	Pooled QC Sample (MS/MS data acquisition 1)
68	Pooled QC Sample (MS/MS data acquisition 2)
69	Pooled QC Sample (MS/MS data acquisition 3)
70	Pooled QC Sample (MS/MS data acquisition 4)
71	Pooled QC Sample (MS/MS data acquisition 5)
72	System Suitability Sample 2

Table D3| Software and libraries used in data processing.

Software	Version	Usage
MassHunter Workstation	10.0	GC-MS instrument control, autosampler control, data acquisition
MassHunter Qualitative Analysis	10.0	Manual data quality checks
ProteoWizard MSConvert	3.0.22286-7583e1c	MS raw file conversion
MS-DIAL	4.9221218	Spectral deconvolution, retention index assignment, peak alignment, library matching, blank subtraction
R/R Studio	4.1.2/2022.02.0 Build 443	Library building, format changes
QCMxP	0.1	Batch correction, normalisation
MetaboAnalyst	6.0	Statistics
Library	Version	
NIST	20	
Fiehn	2013	
MS-DIAL KovatsRI	VS3	

Table D4| Master data matrix containing processed output of the spectral deconvolution, alignment and annotation algorithms from *Odontaster validus* and *Sterechninus neumayeri* tissue samples. Data matrix is available here: https://github.com/njb202/Chapter_5**Table D5|** Statistics data for total osmolyte analysis.

Total osmolytes (peak value)– <i>Odontaster validus</i>				
One way ANOVA				
(Model: Total peak value~Salinity)	resid df	d.f.	F	P value
	20	2.00	8.313	0.002360
Post hoc Tukey test				
	comparisons (%)	CI lower	CI upper	P value
	34.5 v 29	21335.64	630102.4	0.0346776
	34.5 v 24	180262.31	810395.8	0.0020487
	29 v 24	-145456.73	484676.8	0.3790199
Total osmolytes (peak value)– <i>Sterechninus neumayeri</i>				
One way ANOVA				
(Model: Total peak value~Salinity)	resid df	d.f.	F	P value
	21	2.00	5.981	0.008800
Post hoc Tukey test				
	comparisons (%)	CI lower	CI upper	P value
	34.5 v 29	-344799.66	316032.8	0.9933867
	34.5 v 24	54819.26	715651.7	0.0205424
	29 v 24	69202.69	730035.2	0.0161244

Appendix E— Supplementary material for Chapter 6

Link to paper:

Barrett, N.J.; Thyrring, J.; Harper, E.M.; Sejr, M.K.; Sørensen, J.G.; Peck, L.S.; Clark, M.S. Molecular Responses to Thermal and Osmotic Stress in Arctic Intertidal Mussels (*Mytilus edulis*): The Limits of Resilience. *Genes* 2022, 13, 155.

<https://doi.org/10.3390/genes13010155>

During my first two years of PhD study, the pandemic significantly limited my access to the BAS aquarium. As a result, an opportunity arose to collaborate on a molecular study of Arctic mussels subjected to low salinity and warming experiments. The physiology experiments had previously been conducted by Dr. Jakob Thyrring, Prof. Jesper Sørensen, and Prof. Mikael Sejr at Aarhus University, Denmark. Using tissue samples from these experiments, I conducted RNA extractions at BAS, while data analysis was performed with input from Prof. Melody Clarke. I wrote the original draft with guidance from Prof. Melody Clarke, and Prof. Lloyd Peck and Elizabeth Harper provided supervision. All authors contributed to the writing, review, and editing of the final manuscript. The paper is included on the following pages:

Article

Molecular Responses to Thermal and Osmotic Stress in Arctic Intertidal Mussels (*Mytilus edulis*): The Limits of Resilience

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Abstract: Increases in Arctic temperatures have accelerated melting of the Greenland icesheet, exposing intertidal organisms, such as the blue mussel *Mytilus edulis*, to high air temperatures and low salinities in summer. However, the interaction of these combined stressors is poorly described at the transcriptional level. Comparing expression profiles of *M. edulis* from experimentally warmed (30 °C and 33 °C) animals kept at control (23‰) and low salinities (15‰) revealed a significant lack of enrichment for Gene Ontology terms (GO), indicating that similar processes were active under all conditions. However, there was a progressive increase in the abundance of upregulated genes as each stressor was applied, with synergistic increases at 33 °C and 15‰, suggesting combined stressors push the animal towards their tolerance thresholds. Further analyses comparing the effects of salinity alone (23‰, 15‰ and 5‰) showed high expression of stress and osmoregulatory marker genes at the lowest salinity, implying that the cell is carrying out intracellular osmoregulation to maintain the cytosol as hyperosmotic. Identification of aquaporins and vacuolar-type ATPase transcripts suggested the cell may use fluid-filled cavities to excrete excess intracellular water, as previously identified in embryonic freshwater mussels. These results indicate that *M. edulis* has considerable resilience to heat stress and highly efficient mechanisms to acclimatise to lowered salinity in a changing world.

Keywords: blue mussel; cellular stress response; salinity; thermal tolerance; transcriptome; acclimation; freshening; climate change; aquaporins



Citation: Barrett, N.J.; Thyrring, J.; Harper, E.M.; Sejr, M.K.; Sørensen, J.G.; Peck, L.S.; Clark, M.S. Molecular Responses to Thermal and Osmotic Stress in Arctic Intertidal Mussels (*Mytilus edulis*): The Limits of Resilience. *Genes* **2022**, *13*, 155. <https://doi.org/10.3390/genes13010155>

Academic Editor: Joong-Ki Park

Received: 18 December 2021

Accepted: 13 January 2022

Published: 15 January 2022

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1. Introduction

The thermal response of organisms is one of the best quantified in biology (e.g., Clarke and Johnston, 1999; Gillooly et al., 2001 [1,2]) and is essential for predicting the effects of climate change [3,4]. However, knowledge of how additional stressors modify organismal thermal tolerance is still surprisingly limited. In recent years, the Arctic has experienced a rate of warming that is three times higher than the global average [5]. In Greenland, warmer air temperatures have increased melting from glaciers and land ice [6,7], contributing to increased coastal freshwater input and observed declines in sub-surface salinity levels [8]. This has direct consequences for the inhabitants of nearshore ecosystems, especially intertidal organisms, which face the dual challenge of increasingly warmer air temperatures and fresher coastal waters under current and future climate scenarios [9,10]. The blue mussel, *Mytilus edulis*, is a boreal bivalve mollusc that has a distribution stretching from warm temperate to polar latitudes. It is a common inhabitant of the intertidal benthic communities of west Greenland fjords [11] and is exposed to a wide range of air temperatures and salinities. For example, *M. edulis* exhibits freeze-tolerant abilities [12,13], with a lower lethal limit (LLT₅₀) of −13 °C measured in populations from west Greenland [14]. At the opposite end of the scale, high intertidal air temperatures of ~36 °C are becoming more common in the Greenland intertidal zone [15]; however, recent studies have demonstrated a remarkable resilience to high air temperatures in *M. edulis* [14,16,17]. Upper lethal temperatures for *M. edulis* have been observed to be between 25 °C and 38 °C [14,18–20].

This high degree of thermal tolerance in *Mytilus* species is largely dependent on environmental conditions, with acclimation and the predictability of thermal stress being considered key modifiers of thermal resilience [17,21].

With regards to salinity tolerance, *M. edulis* are euryhaline osmoconformers [22], with extracellular fluids being isosmotic with their surrounding medium [23]. Their haline range extends from full-strength seawater (35‰) to very low salinity (~5‰) brackish waters [19,24], although they can tolerate salinities close to zero for limited periods of time [25–27]. Short-term low salinity tolerance in *M. edulis* is initially possible through behavioural mechanisms, which can include closing their shell valves and the exhalant siphon on instant exposure to lowering salinity water [26]. This effectively traps water within the mantle cavity, which can be maintained at a higher osmolality than the external seawater [28]. However, build-up of metabolic waste products, in addition to respiratory and feeding requirements, eventually encourages valve gaping. This brings the mantle tissue in direct contact with external water, initiating osmotic rebalancing of the haemolymph. In turn, this triggers cell volume regulatory changes, which in molluscs constitutes an efflux of intracellular organic osmolytes across the cell membrane [29–31], allowing cells to adjust their osmotic concentration to match that of the extracellular fluid. These cellular adjustments mark the beginning of the acclimatisation process, considered to take one to four weeks in laboratory conditions [32]. Both short and long-term salinity-tolerance strategies come at a considerable metabolic cost, and consequently mussels living in lower salinity environments have higher respiration rates [33], are smaller [34,35], have thinner shells [36] and exhibit depressed shell growth rates [37,38].

Temperature and salinity are considered “master factors” with regard to development, growth and survival of marine animals [39]. As such, the combined effects of temperature and salinity may interact in a complex, non-linear manner, producing responses that would not have been predicted from observing a single variable in isolation [40]. Low salinity has already been demonstrated to significantly depress the temperature at which mortality occur in *M. edulis* from west Greenland [16], suggesting a synergistic interaction between temperature and salinity. At the molecular level, abrupt environmental change often involves the production of stress proteins, such as heat shock proteins (HSPs) [41]. For *Mytilus* species, upregulation of HSPs has been observed in response to heat stress [16,42,43] and osmotic stress [16,42,44]. Previous studies comparing differential gene expression in *Mytilus* species under both high temperature and low salinity conditions observed only a small number of overlapping genes, which showed divergent expression patterns [43,44]. However, Nielsen et al. (2021) [16] observed upregulation of *HSP70* under both low salinity and heat stress.

This study aims to build on findings from a previous ecophysiological study [16] to further explore the molecular responses of *M. edulis* to thermal and osmotic stress by using an RNA-Seq approach. This technique offers detailed molecular insight into the direct responses to environmental stressors by quantifying the presence of transcribed RNA molecules at the point of sampling. A subset of samples generated by Nielsen et al. (2021) [16] where mean mortality was <25% have been used in the RNA-Seq analyses in this study. These samples were partitioned into two separate analyses, with 23‰ at 5 °C used as a control in each experiment:

(1) Low salinity exposure: using samples held at 23‰, 15‰ and 5‰, all at 5 °C. A focus of this analysis was to investigate the physiological mechanisms that mussels employ at very low salinities by analysing the regulation of key marker genes previously identified as important in bivalve osmoregulation including ion channel, aquaporin and osmolyte transporter genes [44,45].

(2) Heat stress and low salinity exposure: the interaction between temperature and salinity using samples held at 23‰ and 15‰, subjected to air exposures of 30 °C and 33 °C. This analysis focused on the interaction between the two stressors and interrogated the extent of the molecular overlap and energy resource allocation.

2. Materials and Methods

2.1. Specimen Collection

Blue mussels (*M. edulis*) were collected from the lower intertidal zone of Kobbefjord south of Nuuk, Greenland (64°10'50.8" N, 51°32'28.1" W) in August 2019. Mussels were kept in seawater obtained from the collection site and held at 5 °C for 3 days before transfer to a holding facility at Aarhus University, Denmark. They were then divided into four aquaria with daily seawater changes, in which water temperature was maintained at 5 °C and salinity at ~23‰, corresponding to the salinity of the collection site. The mussels were exposed to 5 °C air twice a day for 1.5 h at 12 h intervals (simulating a semi-diurnal tide) for one week, prior to starting experimentation to acclimate the animals to the new experimental conditions.

2.2. Salinity and Temperature Experiments

Mussels were distributed among 48, 0.5 L buckets ($n = 10$ per bucket; total mussels $n = 480$). The buckets had holes drilled to allow water to pass through exposing the mussels to air, when

simulating a low tide. The buckets were distributed between 12 aquaria, four for each of the three salinity conditions (5‰, 15‰ and 23‰). Within each salinity treatment, mussels were exposed to experimental air temperatures of 5 °C, 30 °C and 33 °C every 12th hour for 1.5 h over a 6-day period. As *M. edulis* has an upper temperature limit of 36 °C, temperature values close to the upper limit were chosen. Each treatment combination had four replicate buckets, totaling 40 mussels per treatment combination. For a detailed methodological description of the mussel sampling procedure and physiological experimental design, see Nielsen et al. (2021) [16]. At the end of the experiment, mussel length was measured, and gill tissue dissected. Gill samples were flash-frozen and stored at -80 °C until use.

2.3. RNA Extraction and Sequencing

Prior to selecting samples for RNA extraction, tank effects were tested on mortality data using a Fischer's exact test of independence on any tank where mortality was observed (four tanks). There were no tank effects ($p > 0.6$), and hence animals were sampled from each replicate tank for each treatment. A number of treatments from Nielsen et al. (2021) [16] have been excluded. The 36 °C air temperature treatment resulted in 100% mortality, while mortality in mussels exposed to 5‰ salinity and subjected to heat stress (both 30 °C and 33 °C) was >50%, representing a median lethal mortality (LD50). Seven treatments were selected for transcriptomic analysis, based on the previous results of Nielsen et al. (2021) [16], and comprised the following conditions, hereafter referred to by the name in brackets: 23‰ at 5 °C (control), 23‰ at 30 °C (30 °C), 23‰ at 33 °C (33 °C), 15‰ at 5 °C (low salinity), 15‰ at 30 °C (low salinity +30 °C), 15‰ at 33 °C (low salinity +33 °C) and 5‰ at 5 °C (very low salinity) ($n = 5$ per treatment; $n = 35$ samples in total). Mussel shell length was used as the selection criterion for choosing samples to extract RNA. Mussels with shell lengths as near as possible to the mean of all samples (34.7 ± 0.13 mm) were chosen to ensure the animals sampled were of a similar age. This represented one from each of the four tank replicates per treatment plus a fifth based on the nearest suitable size. For each sample, ~30 mg of gill tissue was extracted using the SV total RNA isolation system (Promega, Madison, WI, USA) (which includes a DNase treatment), according to manufacturers' instructions. All samples were checked for RNA purity and concentration on a NanoDrop™ (ThermoFisher, Waltham, MA, USA) spectrophotometer and an Agilent TapeStation™ (Agilent, Santa Clara, CA, USA). Thirty-five individual samples with an RNA integrity number (RIN) ≥ 8 were sent for library preparation and NovaSeq 6000 PE150 (Illumina, San Diego, CA, USA) sequencing at Novogene (Cambridge, UK).

2.4. Bioinformatics

The raw sequencing reads from the 35 samples were subjected to quality control, transcript assembly and downstream analysis. This was performed by NovoGene via the following bioinformatics pipeline. Raw-read data were quality-controlled for error rate distribution using Phred score and GC content distribution. Data filtering removed Illumina adaptor sequences, low-quality reads with uncertain nucleotides ($N > 10\%$) and reads with low quality bases that constituted >50% of the read. Transcriptome assembly using the cleaned reads was carried out *de novo* using Trinity 2.6.6 (with parameters $\text{minKmerCov} = 3$ and $\text{min_glue} = 4$), and utilising modules Inchworm, Chrysalis and Butterfly [46]. Hierarchical clustering was performed using Corset 4.6 [47], with parameters set to default (except; $-f$ ture, $-m$ 10), whose groups contigs were based on shared reads, while contigs differentially expressed between treatments were also separated, ultimately removing read redundancy. The longest transcripts from each cluster were selected as unigenes, and these were subjected to gene annotation. Seven gene databases (NR, NT, KEGG, SwissProt, Pfam, GO and KOG; see Table 1) were utilised to achieve functional gene annotation for the unigenes. For each database, the following software and parameters were implemented: NCBI blast 2.9.0 with an e-value threshold of 1×10^{-5} was performed against the NT database [48]. Diamond 0.8.22 [49] with an e-value threshold of 1×10^{-5} was used to blast unigenes against the NR, KOG and Swiss-Prot databases. HMMER 3.1, utilising the hmmscan package with an e-value threshold of 0.01, was used to search the Pfam database [50]. GO annotation was achieved utilising the protein annotation results from NR and Pfam within Blast2GO b2g4pipe_v2.5 [51] software with an e value threshold of 1×10^{-6} . KEGG annotation [52,53] was carried out using KASS (KEGG Automatic Annotation Server) [54] with an e-value threshold of 1×10^{-5} . GO enrichment was performed using Goseq 1.32.0 [55] and topGO 2.32.0 [56] with a corrected p value of <0.05 . KEGG enrichment was performed using KOBAS 3.0 [52] with a corrected p value of <0.05 . For the gene-expression analysis, the *de novo* transcriptome was used as a reference sequence to map reads and quantify expression level using RSEM 1.2.28 (with parameters $-\text{estimate-rspd} -\text{mismatch-rate}$ 0.3) [57] and Bowtie2 2.4.4 [58], outputting a read count of each transcript for each sample and then converting to a FPKM value (Fragments Per Kilobase per Million fragments mapped) [59]. The threshold for expression was set at $\text{FPKM} > 0.3$. The differential expression

analysis was carried out in DESeq2 1.26 [60] (which normalised read counts and estimated an FDR value using BH [61] with an adjusted p value of <0.05), and in edgeR 3.28 [62] with normalisation via TMM and an FDR value estimated using BH [61] with a $\log_2$ fold change >1 and an adjusted p value of <0.005 . The heatmap was generated in R 4.0.5 [63] within R Studio 1.1.456 [64] using the package pheatmap 1.0.12 [65], utilising FPKM values, which were log-transformed to account for zeros (e.g., $\log(\text{count} + 1)$) and z-scored. Transcriptome data validation was undertaken by mapping the transcriptome onto a sample of primers (*HSP70* and *HSP90*) used in Nielsen et al. (2021) [16] in Geneious Prime (2022.0.1) [66].

Specific genes associated with the classical stress response identified in previous Green- land *Mytilus* studies [16,17], and those identified as being associated with osmoregulation in previous studies (e.g., Lockwood and Somero, 2011 [44]; Meng et al., 2013 [45]), were investigated in more detail via datamining of the differentially expressed gene lists. To determine the identity of putative aquaporin transcripts differentially expressed in the low salinity treatments (5‰ vs. control; 15‰ vs. control; 5‰ vs. 15‰), a phylogenetic tree was produced. A maximum likelihood phylogeny was generated in IQ-TREE 1.6.12 [67], using known aquaporin amino acid sequences from human, mouse, cow, fish and mollusc, in combination with the three translated aquaporin sequences from this study. For each representative species, amino acid sequences of *AQP0-12* were downloaded from GenBank, with the exception of *AQP2*, *AQP5* and *AQP6* in the zebrafish *Danio rerio* as tetrapod orthologues were absent; while in the scallop, *Mizuhopecten yessoensis*, aquaporin genes were predicted from genome analysis, so only a single sequence representative from each for the four aquaporin classes has been included (for accession numbers see; Table S1). A 338-length amino acid sequence alignment was created using MAFFT 7.489 [68] using the L-INS-i method and subsequently hand-edited in Geneious Prime 2021.2.2 [66] to remove poorly aligned and divergent regions. Automatic model testing and selection was implemented within IQ-TREE using ModelFinder [69] with the LG + I + G4 model of sequence evolution selected based on the BIC scores. Bootstrap replicates ($\times 1000$) were performed within IQ-TREE using the ultrafast bootstrap [70].

Table 1. Blue mussel (*M. edulis*) transcriptome assembly statistics and gene annotation rates.

Assembly Statistics	
Number of nucleotides (nt)	394,372,995
Number of unigenes	449,638
Unigene minimum length (bp)	301
Unigene mean length (bp)	877
Unigene medium length (bp)	621
Unigene maximum length (bp)	17,583
N50	1104
N90	420
Number of unigenes between 200–500 bp	165,273
Number of unigenes between 500–1k bp	163,324
Number of unigenes between 1k–2k bp	89,465
Annotation Rates	
Database	% annotation
NR (NCBI non-redundant protein sequences)	35.08
NT (NCBI nucleotide sequences)	9.75
Genomes Orthologues)	
Swiss-Prot: (Manually annotated protein sequences)	
Pfam: (Protein domains and families)	12.33
GO: (Gene Ontology)	9.54
KOG: (euKaryotic Orthologous Groups)	7.96
Annotated in all Databases	0.51
Annotated in at least one Database	40.68

3. Results

3.1. Transcriptome Statistics

RNA extracted from gill tissue samples from 35 individual *Mytilus edulis* was sequenced, generating over 913 million raw reads. After removing adaptor sequences and poor-quality reads, the transcriptome generated comprised 449,638 unigenes (Table 1). Trinity transcript and unigene figures were very similar (99.96%); therefore, we have only reported the unigene data. Library coverage across the 35 samples ranged from 18,925,398 to 32,269,107 clean reads with a Phred Q20 score ranging from 96.83% to 98.31% (Table S2). Seven annotation databases were used to provide gene annotation, with the highest rates achieved using the NR (NCBI non-redundant protein sequences) database with a 35.08% annotation rate (Table 1). Due to the lack of availability

of a full *M. edulis* genome for reference, the majority of annotations (>80%) comprised sequence similarities to four bivalve species: *Mytilus galloprovincialis* (27%), *Mizuhopecten yessoensis* (22.9%), *Magallana gigas* (previously reported as *Crassostrea gigas*) (16%), and *Crassostrea virginica* (14.8%), and one gastropod mollusc species (*Lottia gigantea* (1.7%)).

3.2. Differential Expression and GO Enrichment

The results of RNA-Seq analysis revealed high numbers of differentially expressed gene transcripts under all treatment comparisons ranging from 8515 to 17,998 (Table 2). Due to these large numbers, analyses concentrated on the exploitation of GO enrichment data and more detailed analyses of candidate genes putatively involved in osmoregulation and the stress response.

Table 2. Differential gene expression counts in blue mussels (*M. edulis*) subjected to different heat and osmotic stress treatments. Treatment descriptions are as follows: control (23‰ at 5 °C), low salinity (15‰ at 5 °C), very low salinity (5‰ at 5 °C), 30 °C (23‰ at 30 °C), 33 °C (23‰ at 33 °C), low salinity + 30 °C (15‰ at 30 °C), and low salinity + 33 °C (15‰ at 33 °C).

Comparison	Upregulated Genes	Downregulated Genes	Total Genes
Low salinity exposure			
Low salinity vs. control	5004	3768	8772
Very low salinity vs. control	17,267	6824	24,091
Very low salinity vs. low salinity	10,466	5354	15,820
Heat stress + low salinity exposure			
30 °C vs. control	7002	4195	11,197
33 °C vs. control	7033	8824	15,857
33 °C vs. 30 °C	3958	8314	12,272
Low salinity + 30 °C vs. 30 °C	3781	4734	8515
Low salinity + 33 °C vs. 33 °C	6243	2802	9045
Low salinity + 33 °C vs. low salinity + 30 °C	7506	4948	12,454
Low salinity + 30 °C vs. low salinity	4722	4540	9262
Low salinity + 33 °C vs. low salinity	10,796	7202	17,998

3.3. Low Salinity Exposure

These analyses comprised data generated from mussels exposed to different salinities (23‰, 15‰ and 5‰), all held at 5 °C. This was to identify the transcriptional effects of low salinity at the ambient temperature of the fjord, from which the animals were sourced. At 5 °C, differential gene expression increased with decreasing salinity, with the lowest salinity (5‰) revealing a massive upregulation of gene transcripts compared to the control (17,267 gene transcripts). This was reflected in the GO enrichment analysis, which revealed an increase in the number of upregulated enriched categories as salinity decreased (Figure 1; Table S3). Cell Adhesion (Biological Process) was one of the most significantly enriched categories at both low and very low salinity when compared with control samples (Figure 1). Nine genes were shared across both low salinity treatment comparisons (Table S4(i); Spreadsheet S1). These included calcium-dependant cadherins (e.g., *PCDH9* and *FAT4*), which can function as transmembrane proteins connecting the actin cytoskeleton indirectly to neighbouring cells [71], and ependymin-related proteins (e.g., *EPDR1*), a diverse multifunctional gene family, some members of which have been shown to be upregulated in response to environmental stress in bivalves and gastropods [72]. At very low salinity (5‰), five GO enrichment categories were significantly upregulated compared to the control. This included the GO term category Cytoskeleton (Cellular Component), reflecting the most significantly upregulated gene, *Profilin* (log₂fold change 11.57; Adjusted *p* value 6.87×10^{-46}), which encodes an actin-binding protein involved in cell shape changes and cytoskeleton rearrangement [73] (Spreadsheet S1). Directly comparing very low salinity (5‰) vs. low salinity (15‰) revealed five significantly enriched GO categories, with DNA-binding Transcription Factor Activity (Molecular Process) being the most significant, which was also the case in the 5‰ vs. control comparison. Twenty-four genes were shared across both treatment comparisons for DNA-binding Transcription Factor Activity (e.g., *CREBL2* and *Kat6b*) (Table S4(i); Spreadsheet S1).

Genes identified as being specific to osmoregulatory processes revealed high levels of expression at very low salinity (5‰ vs. control) and when very low salinity and low salinity (5‰ vs. 15‰) were directly compared, but there were a much lower number of upregulated gene transcripts for osmoregulatory regulated genes at low salinity (15‰ vs. control) (Table 3; Figure 2; Spreadsheet S2).

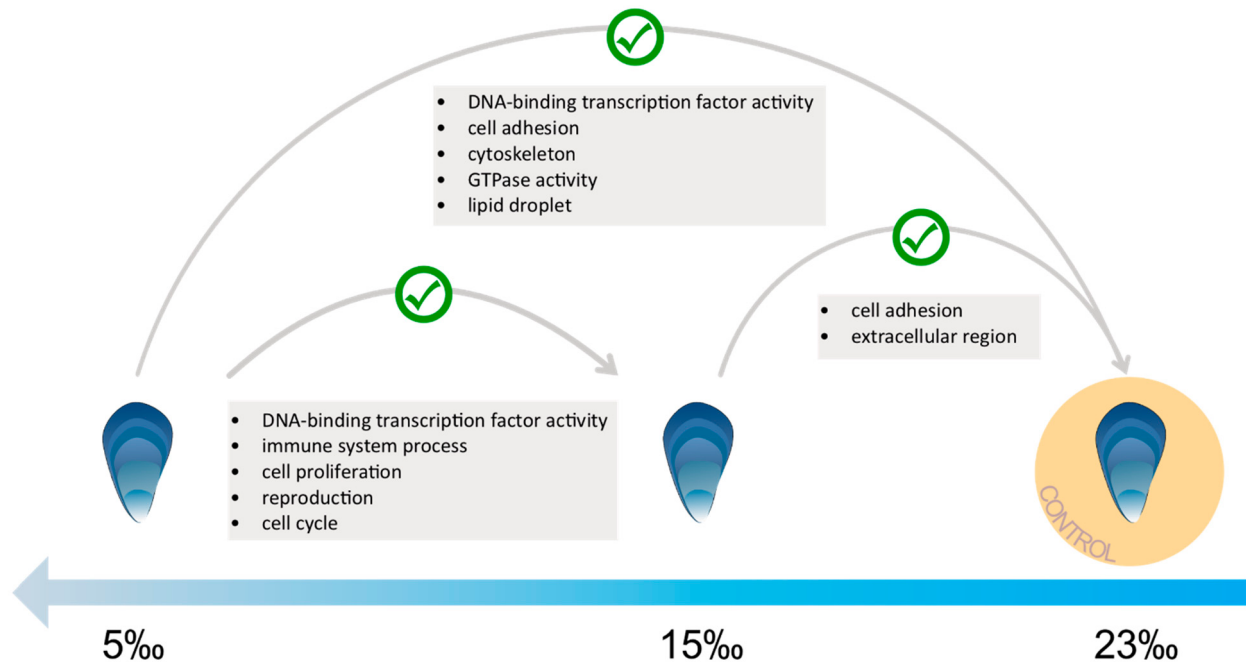


Figure 1. Diagrammatic Gene Ontology term (GO) enrichment results for upregulated gene expression in blue mussels (*M. edulis*) from low salinity exposure treatment comparisons. Green tick represents enrichment between treatments. Lists within grey boxes are significantly enriched GO term categories representing functional profiles of the overexpressed set of genes from the sample comparisons (See Tables S3 and S4(i), Spreadsheet S1).

3.4. Heat Stress and Low Salinity Exposure

These analyses concentrated on mussels exposed to two salinities (23‰ and 15‰), with each subjected to heat shocks of 30 °C and 33 °C during air exposure (control conditions were 23‰ and 5 °C). Differential gene expression patterns under both heat stress treatments (30 °C and 33 °C at 23‰) compared to the control (5 °C at 23‰) revealed similar numbers of upregulated genes (~7000 gene transcripts) at each temperature, while for downregulated genes there was over double the number at 33 °C vs. control than at 30 °C vs. control (Table 2). However, under low salinity exposure (15‰), 4722 gene transcripts were upregulated at 30 °C vs. low salinity (30 °C at 15‰ vs. 5 °C at 15‰), whereas there were 10,796 upregulated at 33 °C vs. low salinity (33 °C at 15‰ vs. 5 °C at 15‰), representing a 129% difference and indicating the synergistic effects of salinity and heat stress (Table 2).

GO term enrichment analysis revealed significant upregulated gene enrichment under both heat stress treatments compared to the control (Figure 3; Table S3). Cell Morphogenesis (Biological Process) was the most significantly enriched GO term, with 40% of genes shared under both conditions (30 °C vs. control and 33 °C vs. control; Table S4(ii)). A closer inspection of the putative genes involved revealed they encoded heat shock proteins/chaperones (e.g., *HSP70*) (Spreadsheet S4). Under the 30 °C treatment, Cell Morphogenesis (Biological Process) was the only significantly enriched category; however, at the higher temperature of 33 °C, eight additional categories were enriched (Figure 3; Table S3). Those defined as Molecular Function GO categories included DNA-binding Transcription Factor Activity, Enzyme Binding and Unfolded Protein Binding, with genes identified as being involved in transcription activity (e.g., *AP-1*, *CREBL2*, *ATF-2*), programmed cell death (e.g., *Ces-2*) and molecular chaperone activities (e.g., *HSP70*, *HSP90*, *CCT3*) (Spreadsheet S4). There was no gene enrichment when heat stress treatments were directly compared at control salinity (33 °C vs. 30 °C both at 23‰), or between the same temperatures at different salinities (Figure 3), indicating that similar processes were upregulated in each treatment.

However, when heat stress treatments were compared at lower salinity (low salinity + 33 °C vs. low salinity + 30 °C), seven GO terms were enriched (Figure 3; Table S3; Spreadsheet S5), further indicating the synergistic effects of combined heat and salinity stress. When exposed to low salinity (15‰), there was a larger number of significantly enriched GO terms for each heat stress treatment compared to control salinity (five categories at low salinity + 30 °C compared to one at 30 °C; 19 categories at low salinity + 33 °C compared to nine at 33 °C), with Cell Morphogenesis (Biological Process) again being the most significant (Figure 3; Table S3). When comparing both 33 °C

treatments (15‰ and 23‰) with their respective 5 °C treatments, nine GO enrichment categories were shared (Figure 3; Table S4(v); Spreadsheet S6), with Cell Morphogenesis (Biological Process) and DNA-binding Transcription Factor Activity (Molecular Function) both being highly significant. Shared genes in the enriched categories relate to stress responses, ubiquitination and protein folding (e.g., *HSP70*, *HSPA5* and *UBC*), transcription activity genes (e.g., *FOSL2*, *CREBL2*) and genes involved in protein syntheses such as elongation factors (e.g., *EEF2*) (Spreadsheet S6). Interestingly, although GO enrichment analysis did not reveal specific terms related to environmental stress, many of the genes underlying the significantly upregulated enriched categories (i.e., Cell Morphogenesis and Unfolded Protein Binding) are classically associated with stress responses (e.g., *HSP70*) (Spreadsheet S6).

Table 3. Osmoregulatory marker genes identified in the differentially expressed profiles from low salinity exposure treatment comparisons, using the SwissProt annotations (see Spreadsheet S2 for gene transcript lists). ↑: represents upregulated genes, ↓: represents downregulated genes.

Gene Type	Comparisons					
	Low salinity vs. control		Very low salinity vs. control		Very low salinity vs. low salinity	
	↑	↓	↑	↓	↑	↓
Ion channel: Potassium channel	2	2	14	0	5	1
Ion channel: Chloride channel	0	0	4	0	2	0
Ion channel: Calcium channel	1	0	6	2	4	1
Ion exchangers	1	1	8	0	4	0
Aquaporins	0	1	1	0	2	0
ATPase pumps (V-type)	0	2	3	2	3	0
ATPase pumps (Other)	0	0	11	1	3	0
Osmolyte transporters (<i>SLC6A5/SLC6A9</i>)	4	0	17	5	7	0

1.1. Stress-Related Genes

Datamining of the differentially expressed gene lists (under the Swiss-Prot and NR database annotations) using terms related to oxidative stress, apoptosis, and osmotic and heat shock stress responses (keywords: caspase, catalase, superoxide dismutase, thioredoxin, glutathione, hypoxia, p38 and heat shock) highlighted the clear induction of candidate heat shock genes under the different treatments (Table 4; Spreadsheet S7). Interestingly, the highest number of heat shock gene transcripts were found in the very low salinity treatment compared to the control (202 transcripts in total). Furthermore, the majority of identified heat shock gene transcripts in each treatment comparison showed highest sequence similarity to *HSP70* family genes, specifically *HSPA12A* and *HSPA12B*. Comparing the differentially expressed gene transcripts (utilising the SwissProt description) for independent effects of heat stress (30 °C and 33 °C vs. control) and independent effects of low salinity exposure (15‰ and 5‰ vs. control) revealed 143 unique *HSPA12A* unigene transcripts and 119 unique *HSPA12B* unigene transcripts (Spreadsheet S8). Less pronounced was the number of transcripts related to classic stress response enzymes associated with oxidative stress and hypoxia (e.g., catalase, superoxide dismutase, thioredoxin and glutathione gene families). Interestingly, there were no upregulated transcripts annotated as p38 mitogen-activated protein kinases, which are considered to play an important role in hypoosmotic stress signaling [74,75]. Gene transcripts related to programmed cell death (e.g., caspase related genes) showed the highest expression in the very low salinity treatment compared to the control (51 transcripts). To validate the transcriptome data, a sample of primers utilised in Nielsen et al. (2021) [16] to amplify stress genes (*HSP70* and *HSP90*) was mapped against the transcriptome generated in this study. These formed strong alignments with a number of gene transcripts (>95% identical sites) (Figure S1). Matching transcripts were checked against the differential expression gene lists in this study and demonstrated similar gene expression patterns, as observed by Nielsen et al. (2021) [16] (Figure S1).

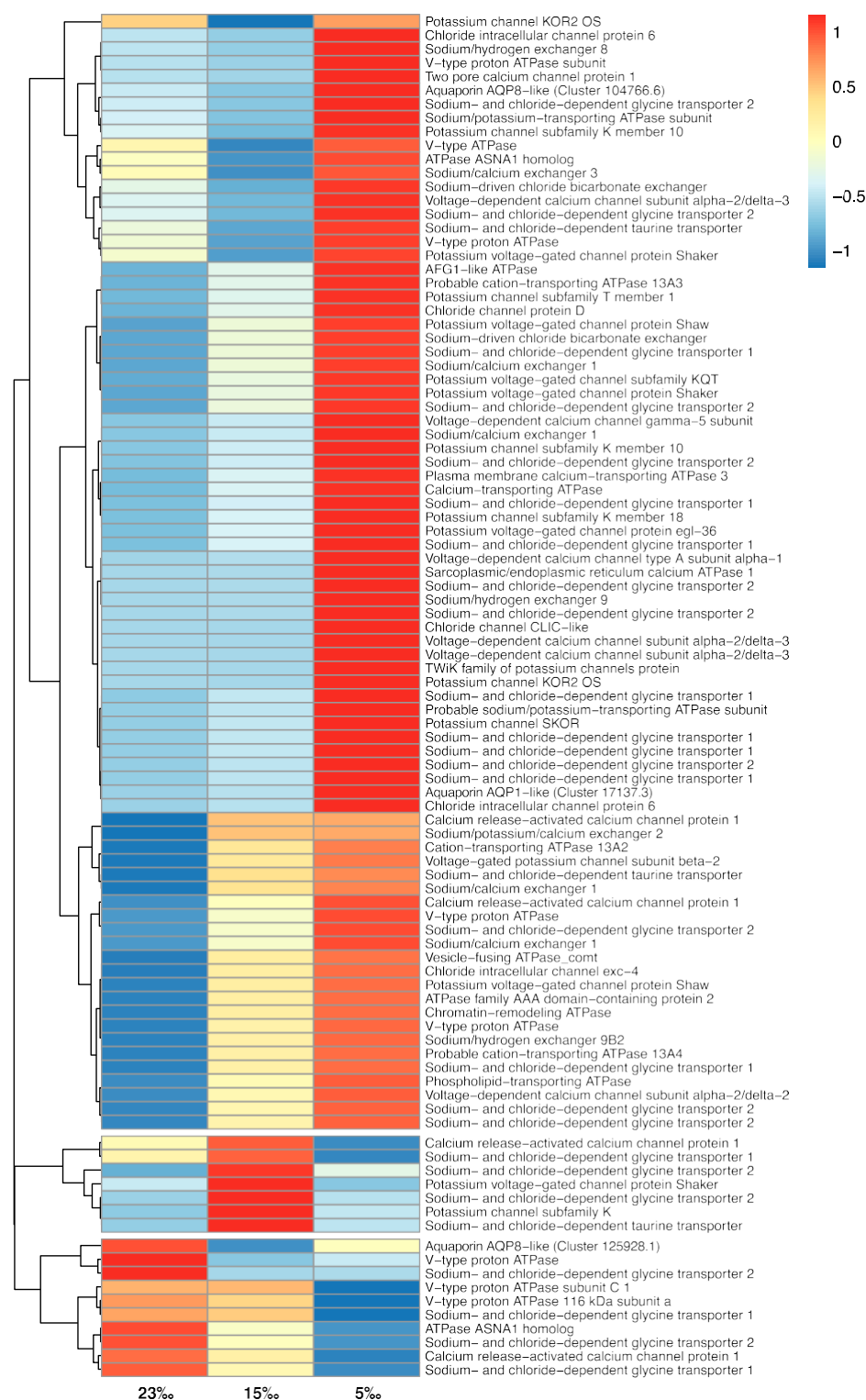


Figure 2. Heatmap showing relative fold change expression of differentially expressed putative osmoregulatory genes in blue mussels (*M. edulis*) from low salinity exposure treatment comparisons, with SwissProt gene transcript descriptions (see Spreadsheet S3 for transcript identification and FPKM values). Colour scale bar indicates relative expression: red = upregulation; blue = downregulation.

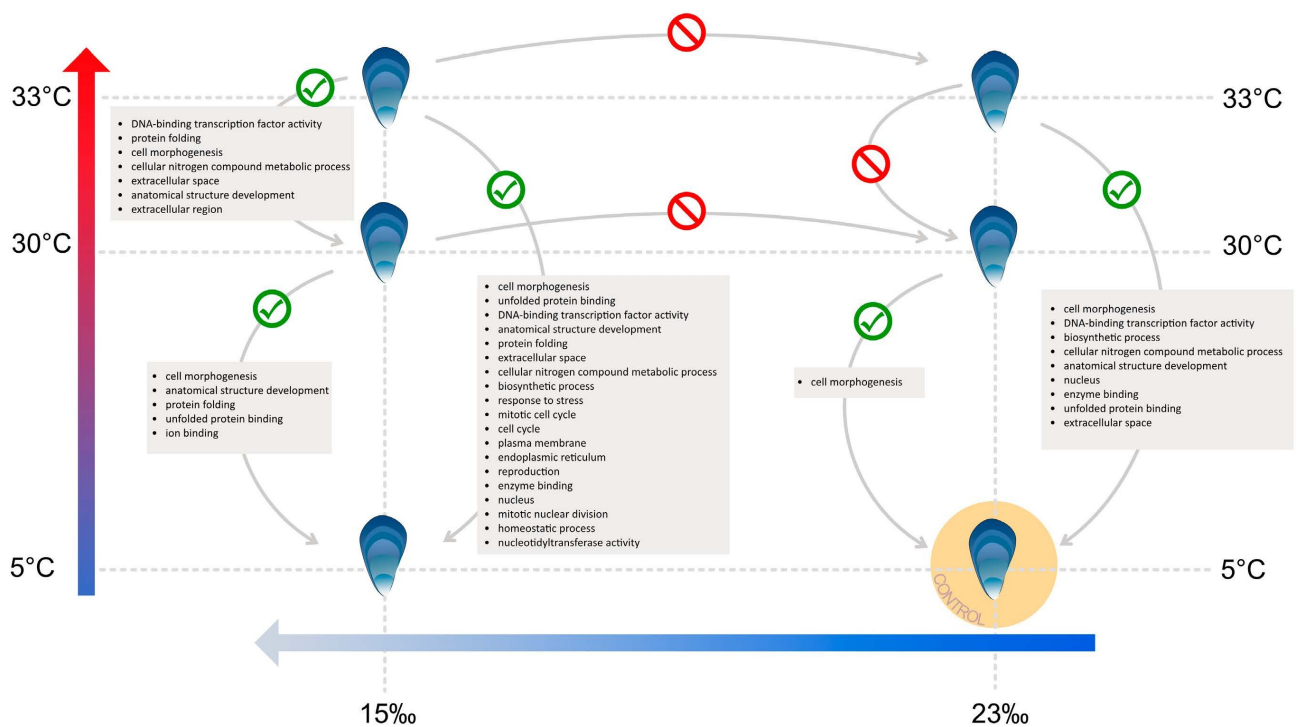


Figure 3. Diagrammatic gene ontology (GO) term enrichment results for upregulated gene expression in blue mussels (*M. edulis*) from heat stress and low salinity exposure treatments. Green tick represents enrichment between treatments. Red no symbol indicates a lack of significant gene enrichment. Lists within grey boxes are significantly enriched GO term categories representing functional profiles of the overexpressed set of genes from the sample comparisons (See Tables S3 and S4(ii–v), Spreadsheet S4–S6).

Table 4. Upregulated stress response genes in blue mussels (*M. edulis*) identified in differentially expressed gene lists from the different treatment comparison, using the SwissProt and NR database annotations.

Gene Family	Family Member	Comparisons								
		30 °C vs.	33 °C vs.	Low Sal. + 30 °C vs.	Low Sal. + 33 °C vs.	Low Sal. + 30 °C vs.	Low Sal. + 33 °C vs.	Low Salinity	Very Low Sal. vs.	Very Low Sal. vs.
		Control	Control	30 °C	33 °C	Low Salinity	Low Salinity	Control	Control	Low Salinity
Catalase		0	0	0	0	0	0	0	0	0
Caspase		16	8	9	13	10	16	14	51	39
Glutathione		12	8	7	13	5	15	4	14	11
Hypoxia		1	0	0	2	0	1	0	2	1
Superoxide dismutase		4	8	5	6	4	11	4	5	1
Thioredoxin		5	4	3	3	2	7	7	11	1
p38 mitogen- activated protein kinases		0	0	0	0	0	0	0	0	0
Heat shock	HSPA12A	27	11	15	21	17	21	18	61	54
	HSPA12B	12	9	20	20	19	22	9	59	48
	small HSP	24	24	0	1	22	23	1	22	13
	HSP68	23	23	1	1	24	22	0	19	16
	HSPA5	1	10	0	0	7	12	0	0	0
	HSP70B2	8	11	2	1	9	10	0	9	9
	HSC70	2	4	1	0	6	5	1	3	7
	HSP90	0	0	0	1	7	10	1	1	3
	HSP110	1	0	0	0	1	1	0	0	0
	HSP70 Family	39	29	4	4	31	36	1	28	27
	HSP total	137	121	43	49	143	162	31	202	177
All stress genes		175	149	67	86	164	212	60	285	230

1.2. Aquaporin Genes and Phylogenetic Analysis

Within the low salinity treatment comparisons (5‰ vs. control; 15‰ vs. control; 5‰ vs. 15‰), three putative aquaporin gene transcripts were identified in the differentially expressed gene lists (Table 3; Figure 2; Spreadsheet S2). Aquaporin channels comprise numerous functionally distinct family members. These have been traditionally divided into four major functional groups, AQP1-like, AQP3-like, AQP8-like and AQP11-like types, based on their distinct evolutionary origin [76]. Previous RNA-Seq studies have successfully used phylogenetic analysis to evaluate the origin of putative aquaporin transcripts identified in a transcriptome (e.g., Calcino et al., 2019 [77]), which provides greater confidence of gene family member identity, compared to blast sequence similarity searches. Phylogenetic analysis utilising the downloaded GenBank aquaporin sequences in combination with the three identified in this study has resolved the four main aquaporin types (AQP1-like, AQP3-like, AQP8-like and AQP11-like) into four well supported clades (Bootstrap values (BS) > 90%). Two of the three putative aquaporin transcripts identified formed a clade with the AQP8-like aquaporins (97% BS), while the third clustered within the AQP1-like aquaporin clade (Figure 4).

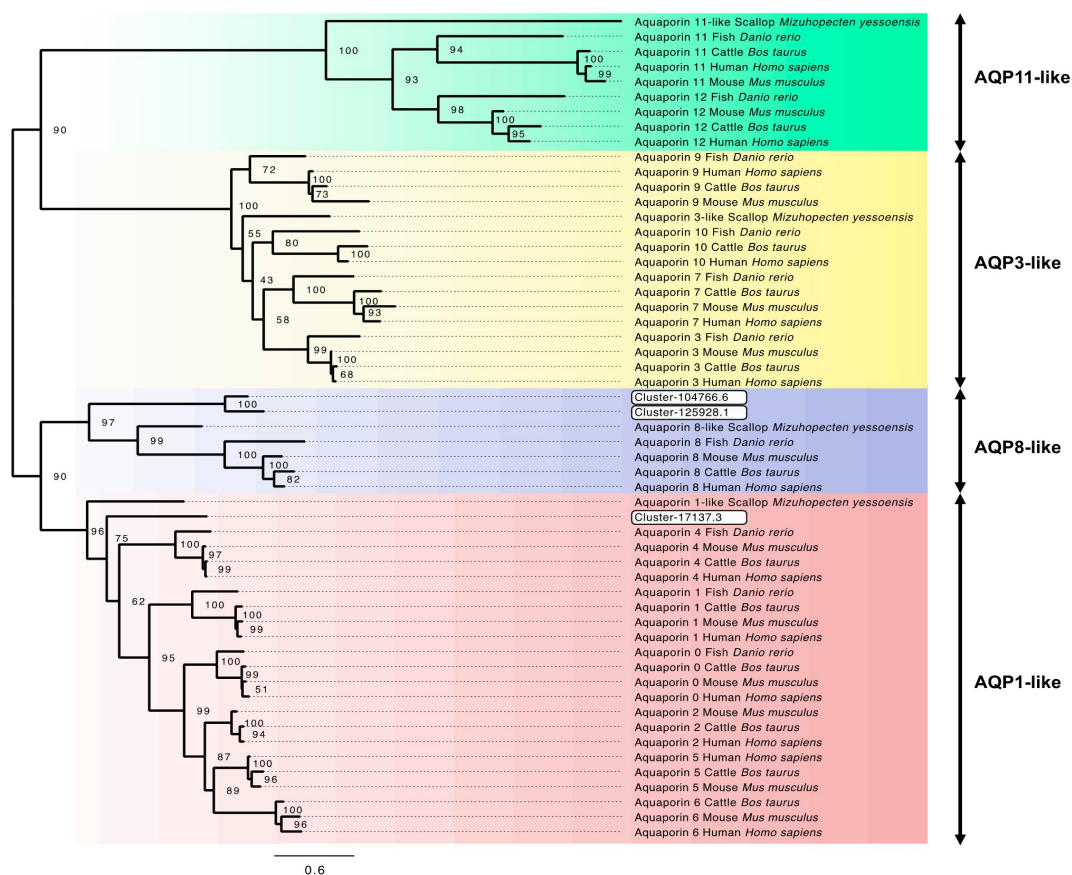


Figure 4. Maximum likelihood unrooted phylogeny of aquaporin genes *AQP0-12* from GenBank including human, mouse, cattle, fish and mollusc (for GenBank accession numbers, see Table S1), and blue mussel (*M. edulis*) putative aquaporins transcripts identified in this study (highlighted in white boxes). Bootstrap values at nodes. *AQP2*, *AQP5* and *AQP6* orthologues are absent in the zebrafish *Danio rerio*. The scallop, *Mizuhopecten yessoensis* aquaporin genes are predicted from genome analysis; therefore, only a single sequence representative from each for the four aquaporin classes has been included.

4. Discussion

Previous work investigating the effects of heat and osmotic stress on *M. edulis* survival demonstrated that exposure to reduced salinity decreased the temperature at which mortal-ity occurred (see Figure 2 in Nielsen et al. (2021) [16]). The molecular results presented here offer insight into the underlying mechanisms that account for these observed outcomes. The results for the effects of increased air temperature on *M. edulis* confirm the high degree of thermal tolerance previously observed by Clark et al. (2021) [17], who examined the effect of temperatures up to 32 °C on *M. edulis* using RNA-Seq. In the current study, significant enrichment for the cellular stress response (CSR), in particular apoptosis-related functions, was not observed. However, the presence of multiple heat shock genes, revealed through differentially expressed datamining

(Table 4) and within enriched GO terms at 33 °C (e.g., Cell Morphogenesis and Unfolded Protein Binding), suggested that stress pathways were clearly active. Furthermore, there was no GO term enrichment when directly comparing the 33 °C and 30 °C treatments. This reflected the lack of mortality observed in Nielsen et al. (2021) [16] in mussels exposed to heat shock at both 30 °C and 33 °C.

The molecular response to low salinity (15‰) was indicative of at least partial acclimation to low salinity, evidenced by the relative low number of upregulated genes (particularly stress and osmoregulatory genes) and low number of enriched GO terms, reflecting the 100% survival rates after 6 days of exposure [16]. *M. edulis* has an extremely wide estuarine and marine distribution [19], and the molecular mechanisms revealed here demonstrate a remarkable degree of efficiency at dealing with osmotic stress, especially considering that the mussels used in this experiment were already acclimated to a salinity (23‰) significantly below full-strength sea water that averages 35‰. This degree of euryhalinity is likely reflective of acclimatisation to the summer environmental conditions experienced by *M. edulis* in the inner Greenland fjords where they were collected [16,78].

In mussels exposed to low salinity, the effects of heat shock increased the overall gene expression (3781 gene transcripts upregulated at 30 °C at 15‰ and 6243 upregulated at 33 °C at 15‰; Table 2), including increased numbers of stress genes (67 upregulated at 30 °C at 15‰ and 86 upregulated at 33 °C at 15‰; Table 4), compared to the effects of heat shock alone (30 °C at 23‰ and 33 °C at 23‰). Furthermore, directly comparing the heat-stressed treatments at low salinity (low salinity + 33 °C vs. low salinity + 30 °C) revealed seven significantly enriched categories, while there was no enrichment when directly comparing 33 °C and 30 °C at control salinity. The mortality data for these treatments (low salinity + 30 °C/33 °C) from Nielsen et al. (2021) [16] show a small increase in mortality at 30 °C increasing to around 20% of the sample population at 33 °C. Taken together, this indicates that accommodating both environmental stressors represents a tipping point in the mussels' ability to cope with stress. At very low salinity (5‰), that threshold has clearly been breached, evidenced by the dramatic rise in mortality as heat stress was applied [16].

When multiple environmental stressors are applied at the same time, they may interact in complex, non-additive ways (e.g., synergistic or antagonistic) resulting in outcomes that are difficult to predict from one stressor alone [79]. Additionally, each environmental stressor may impact differing molecular and physiological pathways of an organism, resulting in an energy allocation trade-off between competing demands for metabolic functions [40]. For example, cell volume regulation in response to reduced salinity is metabolically costly [31]. At the same time, organisms remodel the composition of membrane phospholipids in response to salinity and temperature changes using different mechanisms [80,81], while the energy required to maintain basal metabolic rate, on average, increases with temperature [1,2,4]. Therefore, when both stressors are applied in combination, metabolic resources may become limiting, and a trade-off occurs. Previous studies comparing differential gene expression in *Mytilus* species under both high temperature and low salinity observed only a small number of overlapping genes, which responded in divergent expression patterns [43,44]. In contrast, in the current study, directly comparing differentially expressed gene transcripts between low salinity compared to control (15‰ at 5 °C vs. 23‰ at 5 °C) and 30 °C heat stress compared to control (30 °C at 23‰ vs. 5 °C at 23‰) revealed 1148 upregulated and 1072 downregulated shared gene transcripts (Spreadsheet S9), suggesting that there was a substantial number of molecular responses shared by each independent stressor. It is important to note that Lockwood and Somero (2011) [44] measured the acute response to hypo-osmotic shock (over a four-hour immersion), while the reduction in salinity was from 35‰ to 29.75‰. In addition, the use of microarrays in their experiment constrained the number of genes examined, potentially obscuring differentially expressed genes, particularly from closely related gene family members. These are all factors likely accounting for the discrepancy in outcomes between Lockwood and Somero (2011) [44] and the current study. Our study clearly showed that the combination of stressors appeared to work in a synergistic fashion on a gene expression level, with gene upregulation more than doubling at the highest stressor combination (low salinity + 33 °C vs. low salinity) compared to the effects of the slightly lower heat stress (low salinity + 30 °C vs. low salinity). As competition for resource allocation between osmoregulation and heat stress was stretched, additional stress responses appeared to be triggered, perhaps in a positive feedback-type scenario. Ultimately, the drain on energy resources appeared to reduce the capacity for *M. edulis* to tolerate heat stress. However, the large overlap in genes responsible for salinity tolerance and heat shock suggested that both stressors stimulate similar components of the CSR. The level of macromolecular damage rather than the specificity of the abiotic stressor is considered to be the trigger for the CSR [82,83], meaning that pre-conditioning to one stressor may confer an increased tolerance to another, a phenomenon known as cross-tolerance [84–86]. Pre-conditioning was not a component of the current study; however, testing the hypothesis that an osmotic shock and subsequent recovery may offer increased tolerance to heat shock in *M.*

edulis would be an interesting avenue for future researchers.

1.3. HSPA12 Genes

An interesting finding was the lack of GO term enrichment for HSPs or the CSR when heat stress treatments were compared at different salinities (Figure 3). However, there was high expression of a set of HSP70 family genes, *HSPA12*, upregulated under every treatment. *HSPA12* genes are structurally and functionally distinct from the classical HSP70 family members, with phylogenetic analysis implying that they have highly divergent origins from other *HSP70s* [87–89]. Clark et al. (2021) [17] found a similar expansion of *HSPA12* genes and a concurrent lack of enrichment for a cellular stress response in *M. edulis* upon heat shock. Clark et al. (2021) [17] suggested that through the process of gene duplication events, multiple copies of *HSPA12* proteins have taken on subtly differing roles and collectively act as intertidal stress regulators, offering substantial thermal tolerance. Enhanced expression of *HSPA12* genes in other marine bivalves suggests that particular subsets of *HSPA12* are stressor-specific, with variations in expression levels demonstrated in response to toxic shock and heavy-metal contamination [90,91]. Indeed, in the current study large numbers of unique *HSPA12* gene transcripts were identified under both heat stress and hypoosmotic exposure. For example, out of 143 identified *HSPA12A* gene transcripts, 23 were uniquely differentially expressed as a response to heat stress, 45 for hypoosmotic stress and 25 were shared between stressors (Spreadsheet S8). These results highlight the specific role that *HSPA12* may undertake in response to osmotic stress and imply that their stressor specific sub-functionalisation is a key molecular adaptation enabling stress tolerance in extreme environments.

1.4. Molecular Response to Low Salinity Exposure

The finding that low salinity reduces the thermal resilience of *M. edulis* focused the current analyses on the molecular responses to osmotic stress. The molecular data from *M. edulis* exposed to low salinity at 5 °C demonstrated changes for processes involved in osmoregulation and cell volume regulation. At the lowest salinity, there was significant upregulation of genes putatively involved in active membrane transport such as ATPase pumps (e.g., *ATP2B3* and *nkb-3*), potassium and chloride ion membrane channels (e.g., *KOR2*, *Sh*, *Kcnk10*, *CLIC6*, *CLCD*) and ion exchangers (e.g., *SLC9B2* and *SLC8A1*), indicating a change in osmoregulatory response (Table 3; Figure 2; Spreadsheet S2). In addition, there was also significant upregulation of organic osmolyte transporter genes (e.g., *SLC6A5* and *SLC6A9*), suggesting an increased demand to balance solute concentrations. As an osmoconformer, *M. edulis* is considered to have little ability to regulate extracellular fluid osmolality when exposed to decreasing salinity [23,92]. Instead, to balance homeostasis within the cell, mussels reduce cytosol solute concentrations to match that of the external environment, primarily through adjustments in the intracellular concentration of free amino acids and other small organic osmolytes [30,93]. The inorganic ion proportion of intracellular osmolality (e.g., K^+ , Na^+ , Cl^-) is suggested to remain at a relatively constant level (~500–600 mOsm kg^{-1} within marine animals [94]). In the 5‰ treatment, the cytosol of *M. edulis* was likely to be highly hyperosmotic in comparison to the external haemolymph, which would be expected to be near isosmotic with the external low salinity seawater due to the lack of extracellular osmoregulatory abilities [23,92]. To maintain the minimum level of ions and organic osmolytes necessary for cellular functions, active uptake from the extracellular fluids is necessary. This was reflected by the increased up-regulation at 5‰ (5‰ vs. control) of ATPase genes including *ATP2B3* ($\log_2$ fold change 6.12; Adjusted p value 2.75×10^{-4}), and osmolyte transporter genes including sodium- and chloride-dependent glycine transporter gene, *SLC6A5* ($\log_2$ fold change 6.15; Adjusted p value 7.33×10^{-6}), which had been previously identified as a marker gene for the effects of low salinity [45]. Lockwood and Somero (2011) [44] investigated low salinity stress in two *Mytilus* species, *M. galloprovincialis* and *M. trossulus*, and observed upregulation of ion channels but downregulation of ion and amino acid transporters in response to an acute low salinity (29.75‰) exposure over four hours. This response was considered to reflect the need to allow ion concentrations to equalise, while reducing active transport of ions and osmolytes into the cell, which would be hyperosmotic to the external medium. In contrast, the duration of osmotic stress in our study was six days, akin to the beginning of an acclimation process, which has been proposed to take between one and four weeks in temperate marine animals [32]. In addition, the *Mytilus* species, *M. galloprovincialis* and *M. trossulus* in Lockwood and Somero (2011) [44], were originally acclimated to 35‰ and then exposed to only a four-hour immersion at ~30‰ prior to sampling, representing a 15% change in osmotic pressure. *M. edulis* in the current study was already acclimated to 23‰ in the natural environment and then experienced a ~35% and ~78% change in osmotic pressure when exposed for six days at 15‰ and 5‰ salinity respectively. Therefore, it is not surprising to find considerable differences in transcriptomic response between these two sets of low salinity experiments, with the former assessing the acute response, while this study is a longer-term acclimation experiment, with larger

changes in external salinity. Previous investigations into the long-term acclimation to salinity stress in the blue crab, *Callinectes sapidus*, demonstrated an increase in the activity and synthesis of ATPase pumps in posterior gill tissue [95]. In the current experiment, upregulation of a number of ATPase pumps at 5‰ salinity is consistent with these findings.

After six days of exposure to 15‰ salinity, there was relatively little differential expression of genes related to ion channels (15‰ vs. control) in comparison to 5‰ salinity (5‰ vs. control) (Table 3). At 15‰ salinity (15‰ vs. control), one copy of a potassium voltage-gated channel gene (*Sh*) and one potassium channel gene (*KOR2*) were downregulated; however, one calcium-activated potassium channel gene (*orai1*) and one potassium channel gene (*SK*) were upregulated (Figure 2; Spreadsheet S2). Interestingly, the same potassium voltage-gated channel gene, *Sh*, that was downregulated at 15‰ salinity (15‰ vs. control) was significantly upregulated in the 5‰ vs. 15‰ salinity treatment. The activation of voltage-gated channels is considered to occur within milliseconds upon membrane depolarisation [96], allowing for rapid osmotic re-balancing. The downregulation of a potassium voltage-gated channel after six days exposure at 15‰ suggests that membrane depolarisation has led to a stable change in osmotic balance. Meng et al. (2013) [45] observed a similar expression pattern in the Pacific oyster, *Magallana gigas*, after seven daysexposure to 15‰ salinity. As 15‰ salinity is a value comparable to the ionic osmolality of the cell cytosol (~ 500–600 mOsm kg⁻¹ [94]), presumably adjustments to balance the osmotic gradient may have already taken place over a shorter time frame, and at the point of sampling there was no requirement for an osmoregulatory adjustment. This is further validated by the relatively small number of classical stress response genes upregulated at 15‰ (15‰ vs. control) in this study, indicating that *M. edulis* has partially acclimated or is near to acclimating to the lower salinity.

At 5‰ salinity (5‰ vs. control), the increased upregulation of potassium and chloride channel proteins in addition to ion exchangers and osmolyte transporters likely reflected the ongoing need to maintain the intracellular environment as hyperosmotic to the haemolymph. At the same time, water would be osmotically drawn into the cytosol. Aquaporins are membrane channel proteins that allow for the passive transport of water and uncharged solutes [97,98] and are considered to play an essential role in osmoregulation in molluscs [76]. Interestingly, there was significant upregulation of an aquaporin transcript at 5‰ salinity (5‰ vs. control) and two when 5‰ and 15‰ treatments were compared. Increased aquaporin production within the external cellular membrane would enable passive water flow into the cell, which would appear counterproductive, considering the cell is likely already dealing with excess intracellular water. In the freshwater mussel, *Dreissena rostriformis*, recent evidence suggests that during embryogenesis, aquaporins, vacuolar-type ATPase (V-ATPase) and sodium/hydrogen exchangers are involved in osmoregulation in low salinity environments, and were likely a key evolutionary step in the transition from marine to freshwater tolerance [77]. Calcino et al. (2019) [77] described the formation of embryonic ‘cleavage cavities’, which fill with excess cellular water that is later discharged outside the cell. These membrane-bound cavities are acidified by V-ATPase pumps, creating an internal solute concentration higher than the rest of the cell cytosol. Aquaporin water channels facilitate the movement of water down osmotic gradients into the swelling cavity, which is later expelled at the cell membrane, possibly in a manner similar to contractile vacuoles in protists [99,100]. Phylogenetic analysis of the putative aquaporin transcripts identified in the low salinity exposure analysis from the current study showed clustering within both AQP1-like and AQP8-like clades for the upregulated aquaporins. AQP1-like aquaporins are considered to be classical aquaporins, allowing for selective uptake of water molecules, while AQP8-like aquaporins are ammonia-selective and have been termed aquaamoniaporins [76]. Interestingly, in vertebrate hepatocytes it has been demonstrated that AQP8 plays a significant role in water transport during bile secretion, where it is localised in intracellular vesicles [100]. Upon cAMP signaling, AQP8-associated vesicles can be rapidly inserted into the cell membrane between cells, facilitating the movement of water to form a fluid-filled structure called the canaliculus, which has been suggested to be very similar in form to the cleavage cavities observed in *D. rostriformis* [77]. At 15‰ salinity (15‰ vs. control), there was downregulation of a separate aquaporin identified by the phylogeny as ATP8-like, which interestingly showed neutral expression at 5‰ (5‰ vs. control). This implies that these particular aquaporins were located in the external cell membrane. By reducing their synthesis while exposed to low salinity (15‰), the cell minimises passive water movement into the cytosol to prevent excess swelling, and cell volume regulation is undertaken. Very similar RNA-Seq results were observed in the Pacific oyster, *M. gigas*, after seven days exposure to low salinity, where the lowest expression for three aquaporin mRNA was at 15‰ salinity [45].

In this study, there was upregulation of putative V-ATPase transcripts (e.g., *Atp6v0a1*, *ATP6AP1*) at 5‰ salinity (5‰ vs. control), and three more (*VhaAC39-1*, *Vha26*, *Atp6v0a1*) when 5‰ and 15‰ treatments were compared. In addition, sodium/hydrogen and sodium/calcium exchangers were upregulated at 5‰ salinity (5‰ vs. control) (e.g., *SLC9B2* and *SLC8A1*), suggesting the need to recover sodium from the extracellular fluid. Together, the finding of increased

expression of aquaporins, V-ATPase and ion exchangers at very low salinity suggested that *M. edulis* may use a similar method for coping with the constant influx of osmotically driven water at very low salinities as observed in embryonic freshwater bivalves and fluid transport in more complex adult vertebrate organs [77,100].

1.5. Cytoskeleton

The actin cytoskeleton is considered to play an essential role in cell volume regulation under osmotic stress [101]. For cells in a hypotonic environment, as in the case of very low salinity, the influx of osmotically drawn water causes the cell to swell, which can result in extensive re-organisation of the actin cytoskeleton [101]. Within the current study, many significantly upregulated genes (including *Profilin*) and a number of GO terms (Cytoskeleton and Cell Adhesion) relating to the actin cytoskeleton were identified. This suggests that the long-term effects of low salinity exposure induced structural changes to the cell cytoskeleton to counter the effects of maintaining homeostasis in a hypotonic environment. Previous studies have identified upregulation of *Profilin* in response to osmotic stress in annelids [102], in addition to both oxidative and heavy-metal stress in *M. galloprovincialis* [103,104]. Osmotic stress is also associated with specific heat shock proteins, in particular small heat shock proteins (sHSPs), which are known to colocalise with the cell cytoskeleton when under osmotic stress [105]. Our data show significant upregulation in sHSPs under very low salinity (5‰ vs. control), while there was almost none at 15‰, compared to control salinity. Previous studies on renal and ocular physiology have shown that sHSPs are synthesised on exposure to high NaCl concentrations and remain at high levels as long as cells are exposed to osmotic stress [106,107]. Beck et al. (2000) [108] suggested that it is the tonicity not the osmolality that is responsible for the presence of sHSPs, which likely play a role in acclimation to differing salinities by their involvement in cytoskeleton modulation. It is therefore possible that the high expression of sHSPs at very low salinity (5‰) in our data suggests the cell is under constant cellular stress as a result of swelling due to the hypotonic environment, while their almost complete absence at 15‰ implies that the cell osmolality is in balance with the external medium and therefore acclimation is near completion.

6. Conclusions

The molecular data within this study confirm that *M. edulis* has considerable resilience to heat stress and highly efficient mechanisms to acclimatise to lowered salinity. Thermal and osmotic stress tolerance appear to be regulated by stressor-specific sets of *HSPA12* genes, which may function as intertidal stress regulators, as previously suggested [17]. However, although both stressors also induce several shared molecular responses, when they are applied in combination their synergistic effects can push the animal to the edge of their tolerance thresholds before either factor alone. This implies that, although acclimation to lower salinity (15‰) is possible, the underlying energetic cost for maintaining homeostasis appears to be greater than at pre-acclimation levels, which may help account for the observed impacts on growth and morphology in *M. edulis* living in lower salinity environments [34,36,38]. At very low salinities, the molecular data suggested *M. edulis* is in a constant state of stress and appeared to be activating an osmoregulatory response to maintain a hyperosmotic intracellular environment while also implementing structural changes to the cell cytoskeleton. We suggest that *M. edulis* may use a similar mechanism to excrete excess intracellular water to that found in embryonic freshwater mussels and more complex adult vertebrate organs [77,100].

The current study highlights the importance of combined stressor studies for predicting the future range abundance and distribution of Arctic intertidal species. Furthermore, it is also of clear value for the mussel aquaculture industry when identifying future sites for commercial ventures in view of predicted environmental changes. Overall, these results inform our understanding of how *M. edulis* responds at the molecular level to the effects of heat stress, freshening, and their combined interaction, offering insights into the ability of intertidal mussels to resist future climate changes, and a measure of their physiological limitations.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/genes13010155/s1>.

Data Availability Statement: RNA-Seq data has been deposited in the ArrayExpress database at EMBL-EBI (<https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-11178>) (accessed on 17 December 2021) under accession number E-MTAB-11178. The assembled transcriptome has been deposited in the European Nucleotide Archive (ENA) under accession number ERZ4783392 accessible via <https://www.ebi.ac.uk/ena/browser/view/PRJEB48959> (accessed on 17 December 2021).

References: See online version <https://doi.org/10.3390/genes13010155>

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