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Micromolecular adaptations in Antarctic echinoderms: Overlapping roles of organic osmolytes in osmoregulation and protein stability under low temperature

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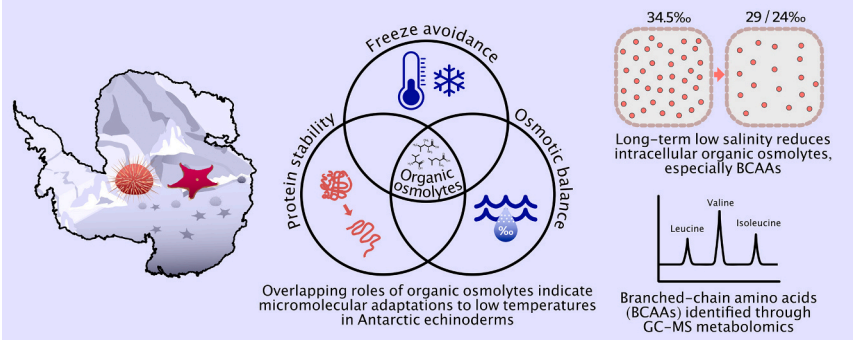
HIGHLIGHTS

- Antarctic echinoderms use organic osmolytes to regulate cell volume under chronic low salinity
- Osmolyte profiles in Antarctic echinoderms appear distinct from temperate species
- The use of branched-chain amino acids (BCAAs) suggests adaptation to Antarctic cold temperatures
- High intracellular BCAAs may help stabilise proteins at low temperatures

GRAPHICAL ABSTRACT



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ABSTRACT

For osmoconforming organisms, prolonged exposure to reduced salinity requires an adjustment to intracellular osmolyte levels to ensure an osmotic balance is maintained between the cell and external sea water. However, osmolytes—low-molecular-mass micromolecules—may also serve overlapping roles in freeze avoidance, desiccation resistance, and protein stabilisation. In Antarctic species living at or below 0 °C, multiple environmental stressors likely shape species-specific osmolyte profiles. Yet, the osmolytes utilised in osmotic acclimation and the broader micromolecular profile of Antarctic marine organisms remain poorly characterised. This study employed gas chromatography–mass spectrometry (GC–MS) to analyse the organic osmolyte composition of two endemic Antarctic echinoderms, the sea star *Odontaster validus* and sea urchin, *Sterechinus neumayeri*, following long-term acclimation (>12 weeks) to reduced salinity levels (29 ‰ and 24 ‰). Significant reductions in total tissue organic solute (osmolyte) concentrations after low salinity exposure indicated active cell volume regulation to reduce intracellular osmotic pressure. The osmolyte metabolic profiles of these Antarctic species appeared distinct from those of temperate echinoderms and other marine osmoconformers, suggesting a specialised adaptive response. Notably, the use of branched-chain amino acids (valine, leucine, and isoleucine) in cell

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volume regulation in *O. validus*, and to a lesser extent in *S. neumayeri*, suggests a micromolecular adaptation tailored to the extreme cold of the Antarctic environment.

1. Introduction

Micromolecules—low-molecular-mass molecules found in biological systems e.g., inorganic ions, small organic molecules (metabolites), protons—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 (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, dimethylsulfoniopropionate (DMSP) etc.) and urea (Yancey, 2005; Somero et al., 2017; Podbielski et al., 2022b). 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; Taneja and Ahmad, 1994; Somero et al., 2017) 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 (Raymond, 1992; Ansart and Vernon, 2003; Lee, 2010; 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 a cryoprotectants has been suggested by some researchers [e.g., taurine, strombine and alanine in the blue mussel *Mytilus edulis* (Williams, 1970; Loomis et al., 1988)].

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, 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 (Fraser et al., 2002), 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 (Stefels, 2000; Somero et al., 2017). 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 (Stefels, 2000; Somero et al., 2017). 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 circumpolar distributions (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, 2022; Haumann et al., 2016). Both species have shown moderate tolerance to acute low salinity (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 (Barrett et al., 2025). 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 (Somero et al., 2017; Podbielski et al., 2022b). 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 *O. validus* and *S. neumayeri* demonstrate supercooling abilities, capable of tolerance down to a median air temperature of ~ −7 °C (Waller et al., 2006). 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. Furthermore, as coastal freshening in the Southern Ocean is projected to intensify under climate change (IPCC, 2022; Haumann et al., 2016), a biochemical, mechanistic understanding of how these endemic species acclimatise to and tolerate reduced salinity will improve our insights into their resilience to environmental change.

The aim was 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 and protein stability in response to low temperature environments.

2. Methods

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 and 20 m near the British Antarctic Survey's (BAS) Rothera research station, Adelaide Island (67°34'07"S, 68°07°30"W). Following collection, the specimens were moved to the Rothera aquarium and later transported to the UK in a temperature-controlled, containerised aquarium system. Upon arrival in the UK, they were maintained in an aquarium setup at −0.3 ± 0.2 °C and salinity of 34.5 ‰, under a 12:12 h light-dark cycle, for a period of 6–12 months prior to the experiment. Both species were fed weekly with a frozen krill diet.

2.2. Salinity acclimation experiment

Both species were exposed to three salinity treatments: 34.5 ‰ (control), 29 ‰ (medium salinity) and 24 ‰ (low salinity). The two reduced salinity treatments were chosen based on the results of their 24-h short-term tolerance (Barrett et al., 2024b), surface salinity records from the Rothera Time Series [ranging from 28.4 ± to 35.2 ± 0.03 ‰ between December 2012 and January 2021 (Clarke et al., 2022)], approaches from prior salinity studies on Antarctic marine animals (e.g., Navarro et al., 2019; Navarro et al., 2020; Park et al., 2020) and future freshening predictions for Antarctic fjords, which are based on observations from Arctic fjords (e.g., Grange and Smith, 2013; Sejr et al., 2017, 2022). Salinity dilutions were achieved via stepwise reductions at 2 ‰ per day using deionised water until experimental salinities were reached. Experiments were maintained over a 90-day period. For each treatment, 8 animals of each species were isolated in floating trays and split between four replicate tanks (12 tanks in total). Tank temperatures were maintained at −0.42 ± 0.02 °C (mean ± s.e.m., $n = 158$) for the duration of the experiment and monitored daily. All animals were fed weekly on an artificial diet (Vitalis Marine Grazer) which constituted 28 % protein obtained from fish, algae, molluscs and crustacean derivatives. Food was withheld for seven days prior to dissection to allow for gut clearance of digested food. For a detailed methodology of the experimental set-up, see Barrett et al. (2025) (note that the *O. validus* animals used in this experiment were maintained in the same *S. neumayeri* tanks).

2.3. Tank effects

An analysis to identify tank effects on each species determined that there was no significant effect and therefore, each individual was treated as a biological replicate (see Barrett et al., 2025).

2.4. Metabolomics

2.4.1. Tissue extraction

Eight individual replicates per species and treatment were selected for metabolomics 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 h until they reached a constant mass (±0.01 mg). A ~ 30 mg sample was aliquoted into a separate 2 mL homogenization 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 including sample extraction, GC–MS analysis and data processing.

2.5. Sample processing

2.5.1. Extraction

A solvent mixture of 80 % methanol and water was prepared and chilled on dry ice for 1 h. 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 mg/mL to normalise the sample concentrations. Samples were homogenised using a Fisherbrand Bead Mill 24 with two 10-s bursts at 6 m/s, with an 8-s dwell time between bursts. Following homogenization, samples were immediately placed on dry ice and centrifuged at 30,130*g* for 15 min 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 HPLC grade 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 (as detailed in; Dunn et al., 2011; Broadhurst et al., 2018). 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 8 h to lyophilise, and subsequently stored at −80 °C until further processing.

2.5.2. Derivatisation

The lyophilised samples were split into randomized 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.

2.5.3. 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.

2.5.4. Instrument methods

GC–MS instrument calibration and tuning was completed prior to sample analysis (see Table S1 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. For details for the run sequence see Table S2. 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 70 eV 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.

2.5.5. Data processing

A list of software versions is shown in Table S3. 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 min 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 0.75 Da 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 S3).

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 (Sumner et al., 2007), 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-normalisation quality checks. 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.

2.6. Statistics

The filtered data matrix was $\log_{10}$-transformed and assessed for normality (see Table S4 for original and filtered data matrix). 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 $\leq$0.05 (Volcano plots showing fold change and *t*-test analyses between the low and control salinity treatment groups for each species are provided in the supplementary material; Table S5 and S6; Fig. S1 and S2). 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) from the ANOVA results using the filtered data matrix.

Metabolites identified as possible organic osmolytes were analysed using one-way ANOVA followed by Tukey post-hoc analysis to compare the mean total organic osmolyte content (sum of peak values for each osmolyte) between salinity treatments in each species (Table S7). 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).

3. Results

3.1. Metabolites

In total, 121 annotated metabolites were identified across both species tissue spectra (see Table S4).

3.2. Univariate data

Statistical univariate analysis of these data showed numerous DEMs 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 $\leq$0.05) revealed 8 significant DEMs in *O. validus* tissue and 20 DEMs in *S. neumayeri* tissue (Table S8 and S9). 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 (Fig. 1).

3.3. Organic osmolytes

The 121-metabolite dataset was filtered to remove compounds that were unlikely to function as osmolytes (e.g., alcohols, fatty acids and hydrocarbons etc) resulting in 56 organic compounds considered potential osmolytes. There are hereafter referred to as 'osmolytes' (Table 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

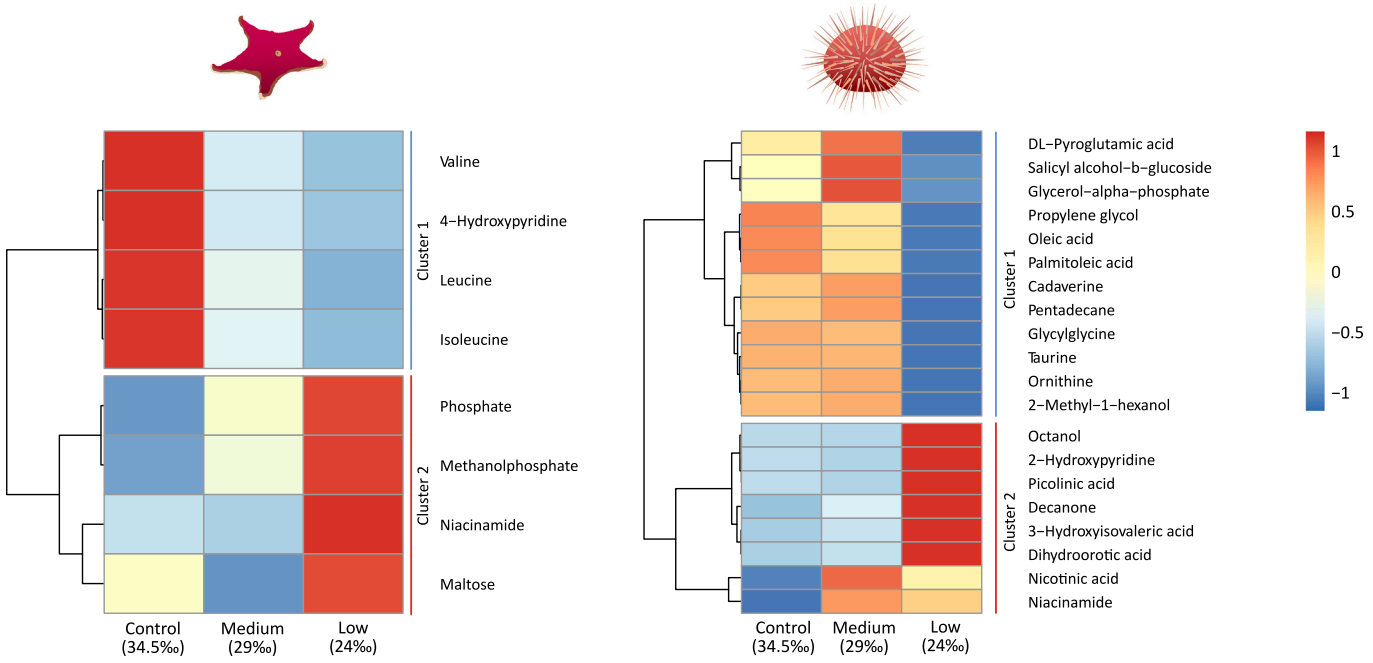

Fig. 1. Heat map showing significantly differentially expressed metabolites in *Odontaster validus* pyloric caecum (left) and *Sterechinus neumayeri* gut tissue (right) among three salinity treatments after acclimation. Columns show the mean normalised peak expression for animals ($n = 8$, except *O. validus* at 24 ‰ $n = 7$) in each salinity treatment, and the rows show each metabolite. Colour scale indicates relative metabolite levels, shown as normalised *Z*-scores. Each metabolite is scaled relative to its own mean across samples. Red (+1) represents values higher than the metabolite's mean (high relative expression), while blue (−1) represents values lower than the mean (low relative 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.

(Table 1). The top 20 constituted 98.3 % and 98.3 % of the total osmolytes pool for *O. validus* and *S. neumayeri* respectively (Figs. 2 and 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 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 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.

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: $F_{(2,21)} = 6$, $P < 0.01$) (Fig. 4; Table S7). 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 ‰, $P < 0.02$) (Fig. 4; Table S7).

4. Discussion

This study aimed to characterise the organic osmolyte profiles of two keystone Antarctic marine echinoderms acclimated to different salinity regimes. The aims were to 1) determine if organic osmolyte concentration decreases during acclimation to low salinity, 2) compare osmolyte profiles of Antarctic echinoderm species with those of non-polar species, 3) identify the specific osmolytes involved in cell volume regulation, and 4) evaluate whether osmolytes perform multiple roles in osmoregulation and protein stability in relation to low temperatures.

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, Psammechinus miliaris* and *Strongylocentrotus droebachiensis* (Schmittmann, 2017; Podbielski et al., 2022a). 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 Figs. 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., 2022a), differing from the Antarctic echinoderms in the current study, which may indicate a difference between polar, low temperate species and temperate echinoderms (aim 2). However, the absence of a directly comparable temperate study, using the same metabolomic platform and comparable salinity range, limits the strength of direct comparisons and highlights the need for such a study in the future.

Cellular volume regulation was driven by valine, leucine and

Table 1
The 56 organic compounds identified through GC–MS analysis and classified as osmolytes, with their relative percentage contribution (based on log-transformed peak values, which are approximations and not absolute quantification of concentration) 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 expressed osmolytes in response to salinity (one-way ANOVA: $P \leq 0.05$) are highlighted; blue = downregulated, red = upregulated.

Odontaster validus				*Sterechinus neymayeri*			
	Contribution to pool (%)				Contribution to pool (%)		
Osmolytes	Control (34.5‰)	Medium (29‰)	Low (24‰)	Osmolytes	Control (34.5‰)	Medium (29‰)	Low (24‰)
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

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 (Yancey, 2005; Podbielski et al., 2022b). Previous studies have demonstrated that glycine is the main osmolyte used in cell volume regulation in temperate echinoderms (Schmittmann, 2017; Podbielski et al., 2022a), and commonly used by many other marine invertebrates such as blue mussels *Mytilus* spp. (Sanders, 2018; Podbielski et al., 2022a, 2022b). Other osmolytes of intermediate and minor significance in echinoderms include alanine, aspartate, leucine, serine, and threonine (Podbielski et al., 2022b). 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., 2022a)] and in *P. miliaris* and *S. droebachiensis* [40–50 % of the pool at 28 ‰ and 30 ‰ respectively (Podbielski et al., 2022a)]. The absence of its use as an

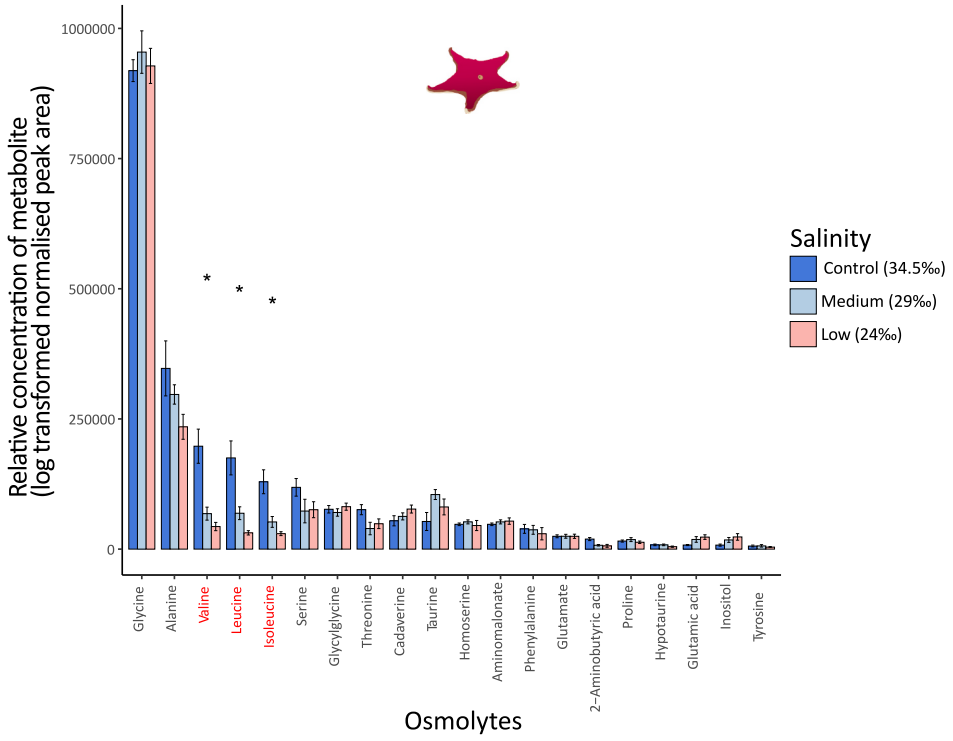

Fig. 2. Relative concentrations of osmolytes at different salinity treatments (using the 20 highest metabolites log transformed peak values at control salinity) within the total intracellular organic osmolyte pool in *Odontaster validus* pyloric caecum tissue. Asterisk and red names specify significant results of one-way ANOVA ($P \leq 0.05$) and FDR post hoc test for differences between treatments. Values are expressed as the mean ± standard error.

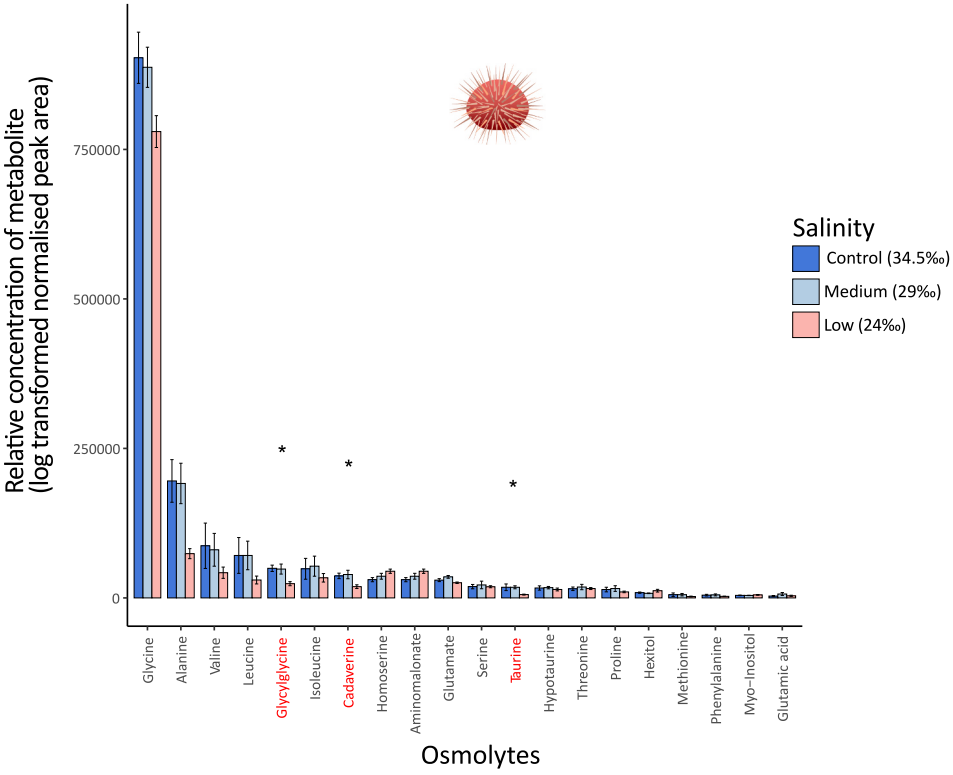

Fig. 3. Relative concentrations of osmolytes at different salinity treatments (based on the highest 20 log-transformed peak values at control salinity) within the total intracellular organic osmolyte pool in *Sterechinus neumayeri* gut tissue. Asterisk and red names specify significant results of one-way ANOVA ($P \leq 0.05$) and FDR post hoc test for differences between treatments. Values are expressed as the mean ± standard error.

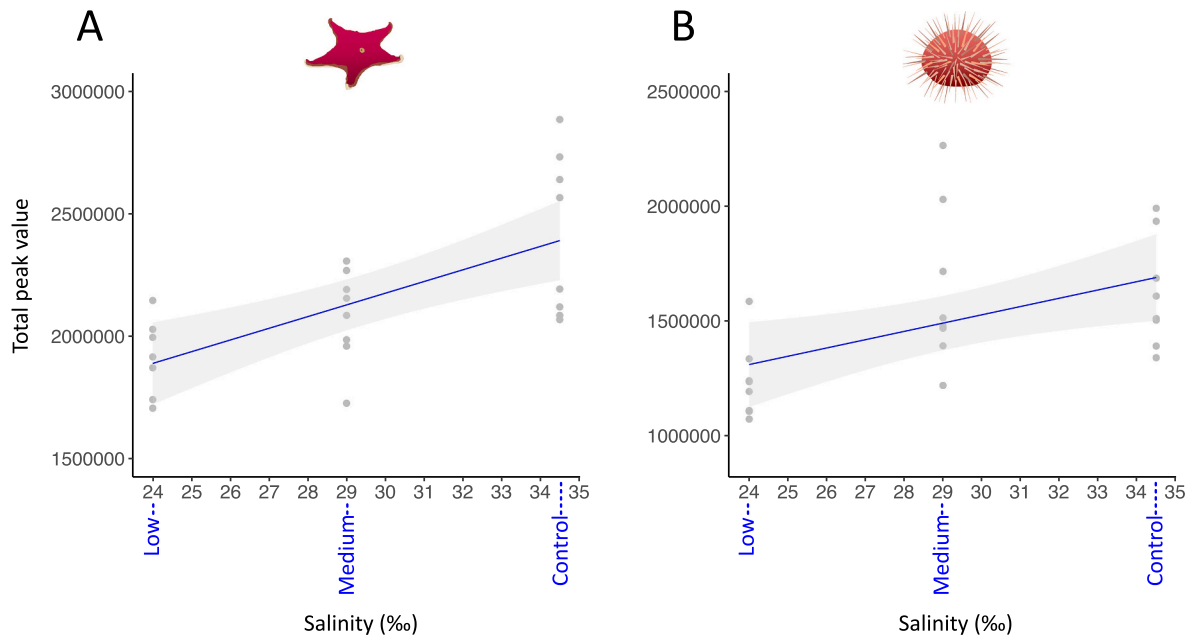

Fig. 4. Total intracellular osmolyte pool (sum of peak values of all osmolytes) measured in different salinity treatments (indicated in blue text) for A) *Odontaster validus* pyloric caecum tissue and, B) *Sterechinus neumayeri* gut tissue. Grey circles represent each sample ($n = 8$, except *O. validus* 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.

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 very 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 consistent 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.

The low number of differentially expressed metabolites across salinity treatment groups was interesting considering that both species demonstrated physiological and behavioural changes within the low salinity treatment (see Barrett et al., 2025). However, this may reflect the substantially lower metabolic rates observed in Antarctic echinoderms compared to temperate and tropical species (Brockington, 2001), resulting in reduced expression of metabolites related to metabolic activity.

Previous metabolomic approaches investigating osmolytes in echinoderms (e.g., Podbielski et al., 2022b; Schmittmann, 2017) have used proton nuclear magnetic resonance (^{1}H NMR), which differs from the current study which uses GC–MS. Although ^{1}H NMR offers detailed molecular structural analysis, it is less sensitive and precise than GC–MS (Bjerrum, 2015) which likely accounts for the high number of metabolites observed in the current study and made direct comparisons of metabolite profiles challenging. By contrast, while ^{1}H NMR is highly quantitative, GC–MS in this study did not use standards for absolute quantification and so the levels reported as percentage of the pool are relative.

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., 2022b). 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 phenomenon 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 suggested 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, the preferential accumulation of large amino acids, such as BCAAs, for volume regulation is likely metabolically costly. One possibility is that their energy-rich branched side chains may also 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. 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. The absence of any intermediate BCAA breakdown metabolites under reduced salinity may reflect the stability of the osmolyte profile at the time of sampling (~12 weeks of exposure to low salinity) as osmolyte adjustments are likely to take place within the first two weeks (Baginski and Pierce, 1977). 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).

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 factor. 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 (even though they were thoroughly washed), potentially compromising the reliability of the relative analysis of amino acid osmolytes and other compounds, thereby obscuring some of 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 due to an increased tendency for 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 (Somero and Bowlus, 1983; Podbielski et al., 2022b). Compared to molluscs, which rely heavily on taurine as an osmolyte, its use in echinoderms is considered minimal (Podbielski et al., 2022b), 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.

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 co-factors 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 support an increased metabolic demand for energy and redox balance under osmotic stress. Prior to sampling, oxygen consumption was significantly higher in *S. neumayeri* (see Barrett et al., 2025) held at 24 ‰ salinity. The observed rise in niacinamide may reflect an increased demand for NAD production to support heightened metabolic activity under osmotic stress.

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 Barrett et al., 2025). 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 very similar to that from 29 ‰ to 24 ‰, because coelomic fluid osmolality was near isosmotic with the treatment salinity at all points over the experimental period (see Barrett et al., 2025). However, in *O. validus* the difference in mean total peak values from 34.5 ‰ to 29 ‰ was 325,719, compared to just 164,034 from 29 ‰ to 24 ‰ (see Fig. 4a; Table S7). In *S. neumayeri*, the mean peak value at 34.5 ‰ was not significantly different from the value at 29 ‰, which was, in fact, higher than at 34.5 ‰ (see Fig. 4b; Table S7). 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., 2022a). However, to maintain a high osmotic gradient between the tissue and coelomic fluids for over 12 weeks 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 Barrett et al.,

2025). 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 (Sanders, 2018; Podbielski et al., 2022a). Inorganic osmolytes may account for almost half of the total intracellular solute pool (Podbielski et al., 2022b) 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., 2022b).

4.6. Conclusions

This is, to the best of our knowledge, the first study to evaluate the organic osmolyte profile of Antarctic echinoderm tissue using GC–MS metabolite profiling. The results demonstrate that *O. validus* and *S. neumayeri* both utilise organic osmolytes for cellular volume regulation in response to low salinity—a condition that is becoming increasingly common in the Southern Ocean due to coastal freshening driven by climate change (Intergovernmental Panel on Climate Change (IPCC), 2022; Haumann et al., 2016). 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 BCAAs (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 represent a micromolecular adaptation to the polar environment, functioning as compatible osmolytes, colligative freezing-point depressants, protein cryoprotectants, and even 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 Barrett et al. (2025), 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., 2022a). 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.

CRediT authorship contribution statement

Nicholas J. Barrett: Writing – original draft, Visualization, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Adam Burke:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Royston Goodacre:** Writing – review & editing, Supervision, Methodology. **Lloyd S. Peck:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Nicholas J Barrett reports financial support was provided by UK Research and Innovation Natural Environment Research Council. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2025.179820.

Data availability

Processed data is publicly available here:
https://github.com/NJ-Barrett/Antarctic_echinoderm_osmolytes
The metabolomics data have been deposited to MetaboLights repository with the study identifier MTBLS12431: https://www.ebi.ac.uk/metabolights/MTBLS12431

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