Matrix corrected SIMS *in-situ* oxygen isotope analyses of marine shell aragonite for high resolution seawater temperature measurements

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Key Points:

- Bivalve mollusks were cultured at different temperatures under tightly constrained seawater composition and environmental conditions
- A new matrix correction using Ca abundances increases the accuracy of SIMS *in situ* measurements of $\delta^{18}O$ in biogenic aragonites
- The first high-resolution SIMS-based stable oxygen isotope calibration for determining modern and ancient seawater temperatures is derived

Abstract

Marine mollusk shells continuously incorporate oxygen isotope signatures during growth that are representative of their surrounding environment and thus produce valuable records of seawater temperature and oxygen isotopic composition. These records of past environmental conditions can be measured *in situ* at length-scales down to sub-daily growth increments using high resolution ion microprobes (SIMS). However, the determination of oxygen isotope ratios in aragonite, the most common shell mineral, is hampered by a lack of ideal reference materials, limiting the accuracy of isotope calibrations and temperature reconstructions. Here, we cultured marine *Anadara trapezia* bivalves under controlled environmental conditions at four seawater temperatures ranging from 13 to 28 °C. The start of the growth period was marked in the crossed-lamellar shell ultrastructure using strontium labelling, enabling precise targeting of the SIMS analyses. A novel calibration method, specifically tailored to analyses of biogenic aragonites with organic-inorganic architectures, has been developed to correct matrix biases that affect the accuracy of all such SIMS oxygen isotope analyses. The matrix fractionation bias calibration was achieved by combining two aragonite reference materials in a linear relationship, pinning the SIMS biases to the true composition as a function of calcium abundances. The oxygen isotope calibration provided a novel seawater temperature *versus* seawater-corrected oxygen isotope fractionation relationship of $T\ (°C) = 23.31 \pm 0.34 - 4.31 \cdot (\delta^{18}O_{aragonite}\ [‰\ VPDB] - \delta^{18}O_{seawater}\ [‰\ VSMOW] \pm 0.22)$ that improves the applicability of *in-situ* oxygen isotope-based paleo-environmental reconstructions of marine bio-aragonite proxy archives.

Plain Language Summary:

In this study, we grew marine bivalves in tightly constrained aquaculture conditions at four different seawater temperatures and marked the start of the growth period in the shell structure using the trace element strontium. The newly grown shell material was analyzed between the strontium-labeled shell increment and the shell edge for its oxygen isotope composition. The data was obtained using an *in-situ*, high resolution ion microprobe and a newly developed analytical post-processing strategy specifically designed for biomineral samples with mineral-organic architectures. The strategy involved two reference materials and the calcium content in the shell and the reference materials. The new approach resulted in an accurate and robust model for determining past seawater temperatures from fossil or historic shells based on their oxygen isotope composition at very fine length scales.

1 Introduction:

Marine calcifying organisms, including bivalve mollusks, grow their skeletal hard parts (i.e., shells) from calcium carbonate, with the two most common polymorphs being calcite and aragonite. These occur together with organic phases in different hierarchical architectures (e.g., Boggild, 1930). During growth, shells accumulate and record trace elements and isotope signatures from the marine environment that can be used for paleoclimate or paleoenvironmental reconstructions. Stable oxygen isotope ratios are commonly utilized as a Sea Surface Temperature (SST) proxy from which other parameters, including seasonality and growth rates, are inferred. However, deciphering past environmental conditions encoded in the $^{18}O/^{16}O$ ratios (expressed as $\delta^{18}O$) of growing shells is complicated by a combination of different signals that are recorded in addition to ambient seawater oxygen isotopes ($\delta^{18}O_{sw}$) (Emiliani, 1966; Shackleton, 1967) and the SSTs experienced during shell growth (Epstein et al., 1953). These additionally recorded signals include vital effects involved during shell biomineralization (Pérez-Huerta & Andrus, 2010; Urey, 1947; Weiner & Dove, 2003) and, in the case of fossil shells, post-depositional modification by surrounding pore water (Adams et al., 2023; Cisneros-Lazaro et al., 2022). Hence, $\delta^{18}O$ paleothermometer calibrations have been developed using different approaches, from inorganic precipitation experiments (Kim & O'Neil, 1997; McCrea, 1950; O'Neil et al., 1969; Tarutani et al., 1969; Zhou & Zheng, 2003), through field-based approaches using live or recently live specimens (Aharon, 1991; Böhm et al., 2000; Carré et al., 2005; Chamberlayne et al., 2021; Grossman & Ku, 1986; Horibe, 1972; Rahimpour-Bonab et al., 1997) to highly-constrained species-specific aquaculture experiments (Al-Qattan et al., 2023; Owen et al., 2008; Wanamaker et al., 2006).

A desire for higher-resolution, *in-situ* microanalysis of $\delta^{18}O$ in biomineral archives is driving instrumental development towards obtaining finer scaled paleoclimate and paleoenvironmental reconstructions (e.g., Green et al., 2022; Rollion-Bard et al., 2003; Vetter et al., 2013). Although these applications are becoming increasingly routine for some biomineral systems, quantitative *in-situ* applications to aragonite biominerals are hindered by a lack of suitable micro-analytical reference materials, and of analytical protocols and calibration methods for producing the requisite accuracy for paleotemperature calculations (He et al., 2021; Long et al., 2020; Rollion-Bard et al., 2007).

Here we present the relationship between seawater temperature and SIMS analyses of oxygen isotope composition ($\delta^{18}O$) measured *in situ* on the aragonitic shells produced by the Sydney Cockle *Anadara trapezia* (Deshayes, 1839). We chose this intertidal, semi-endobenthic

species due to its tolerance of a wide range of seawater temperatures and abundance across temperate to subtropical estuarine mudflats and seagrass meadows along Eastern Australia. In the geological past, *A. trapezia* had a wider distribution, extending from New Zealand to Western Australia (Murray-Wallace et al., 2000; Pan et al., 2021; Ryan et al., 2021). We present a carefully monitored and controlled multi-variate set of seawater parameters, including seawater temperatures, salinity, carbonate system parameters, stable oxygen isotopes, and cation chemistry used to culture *A. trapezia* and quantify the influence of seawater temperatures on shell $\delta^{18}O$. We use the shell $\delta^{18}O$ ratios to develop a thermometer regression based on a novel approach combining *in-situ* high-resolution Secondary Ion Mass Spectrometry (SIMS) analyses and Sr pulse-chase labelling (Otter et al., 2019; Otter et al., 2023) that provided precise control over SIMS spot location in the growing shell. Building on previous observations by He et al. (2021) who first presented the relationship between SIMS fractionation bias and Ca mass fractions, we have developed a new matrix calibration strategy that allows for the accurate calibration of residual $\delta^{18}O$ bias of biogenic aragonites, despite the limited availability of suitable reference materials.

2 Materials and methods:

2.1 Aquaculture experiments with living bivalves:

We collected a total of 94 live *A. trapezia* specimens along a 50 km section of the New South Wales coastline (Figure S1) and distributed evenly between four 220 L aquaria at the Australian National University (ANU). Each aquarium was stocked with three sand-filled polyethylene boxes, allowing the bivalves to burrow and for easier handling by moving the boxes instead of the bivalves to reduce handling stress (Otter et al., 2019). After the bivalves acclimated to 22 °C, seawater temperatures were adjusted stepwise at a rate of 1°C day^{-1} using combinations of heaters and chillers until the seawater temperatures reached 13, 18, 23, and 28 °C, respectively. The chosen temperature range was inspired by the natural environment of the animal, which had a 20.2 °C annual mean SST in the previous year (see Table S1), as well as a maximum and minimum of 26.0 and 13.8 °C, respectively (IMOS, 2022).

After reaching target seawater temperatures, we performed Sr pulse-chase labeling to mark the start of the experimental growth period chemically. Pulse-chase labelling of marine calcifying organisms with trace elements or isotopes is an efficient strategy to mark shell increments within growing calcified hard tissues (e.g., Gorzelak et al., 2011; Houlbreque et al., 2009; Otter et al., 2019; Otter et al., 2023; Winter et al., 2023). The Sr concentration in the seawater was raised to 18x the normal concentration (8 µg g^{-1} at 35 psu salinity) for 72 h by dissolving 96.6 g of $SrCl_2 \cdot 6H_2O$ in 1L of pure water before adding it to the 220 L aquaria. Direct dissolution into the seawater was avoided as this can lead to the immobilization of excess Sr^{2+} via the precipitation of $SrSO_4$, which has a low solubility and is resistant to re-dissolve. After 72 h, shells were returned to normal seawater and grown for 80 days at their respective target temperatures.

Natural seawater sourced from the sample collection site was used throughout the experiment. The aquaria seawater was continuously cleaned and aerated by protein skimmers (Reef Octopus) and completely replaced weekly or when appropriate. Float valves maintained stable salinity levels by automatically adjusting dropping seawater levels by adding pure water to counteract evaporation. Seawater temperatures were recorded at 15-minute intervals throughout

the 80-day growth period using TinyTag (Gemini, United Kingdom) data loggers (Table S2). Salinity measurements were logged (YSI, USA) every 3 days (Table S3). After the 80-day growth period, specimens were frozen, soft tissues removed, the shells rinsed in pure water and air-dried. The bivalves were fed (Shellfish Diet 1800, Reed mariculture Inc, USA) once every three days for the first month of the experiment (including acclimatization). This rate was later increased to every other day and, finally, to daily for the last three weeks of the experiment. Protein skimmers were turned off for 4–6 h after each feeding.

2.2 Seawater characterization:

Seawater samples for pH and alkalinity, $\delta^{18}O_{SW}$, and dissolved cation concentrations were collected every three days from all four aquaria (Tables S4 to S7). Aliquots for pH and alkalinity were filtered (0.45 µm) and analyzed immediately on a USB4000 Fiber Optic Spectrometer (Ocean Optics, Australia) at ANU. The pH values were obtained after pretreatment with 50 µL of meta-cresol purple and reported on the total pH scale (pH_T). Alkalinities were obtained by adding Bromophenol Blue (BPB) dye to a 50 mL flask until the solution reached the endpoint, added to the test cell, and analyzed against seawater without added dye. This approach has an analytical precision of ± 1.5 µmol kg^{-1} for certified reference solutions (Nand & Ellwood, 2018).

Aliquots for cation concentrations were acidified alongside Milli-Q blanks with 4 % HNO_3 and stored at 4 °C. Seawater cation concentrations were analyzed using an Agilent Technologies 5110 Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES) at ANU. Samples were diluted 50x with 2 % HNO_3 and the following spectral lines were chosen: Na330.237, Na568.263, Na568.821, Na588.995, Na589.592, Mg202.582, Mg279.078, Mg280.270, Mg285.213, Mg383.829, K404.721, K693.876, K766.491, K769.897, Ca370.602, Ca422.673, Sr407.771, Sr421.552, and Sr460.733. Each sample was bracketed between an in-house seawater reference solution of a known concentration collected on an *RV Investigator* voyage across the Southern Ocean (IN2020_v08 at 57.977 S, 141.541 E at 10 m depth).

For $\delta^{18}O_{sw}$ measurements, 2 mL glass vials were filled with seawater until overflowing to avoid entrapment of atmospheric air and stored at 4 °C. We used a Picarro L2140-i Cavity Ring-Down Spectrometer (CRDS) at ANU for stable oxygen isotope analyses in high-precision mode set to 7 injections per sample with dry air as the carrier gas. The first 3 injections were used to flush the system and were thus rejected in the final data. In addition, any injections where the median H_2O concentration was outside the ± 1,500 µg g^{-1} range around the run median (around 20,000 µg g^{-1}) were also rejected. Samples were calibrated against a set of commercially available waters used as in-house reference solutions: Fiji (-6.60 ± 0.02 ‰), Smart (-2.69 ± 0.08 ‰), and Kona (0.05 ± 0.03 ‰). These reference solutions were calibrated relative to international reference solutions IAEA SMOW2 and IAEA SLAP2 and reported in ‰ relative to the Vienna Standard Mean Ocean Water (‰ VSMOW). The precision of the $\delta^{18}O_{sw}$ measurements was monitored via repeat analysis of the in-house reference solutions.

2.5 Bivalve shell characterization:

The left shell valve of each specimen was archived, while the right valves were cut twice along the dorso-ventral axis of maximum growth using an IsoMet low-speed saw (Buehler, IL-USA). The resulting 3 mm thin shell slices were further shortened by cutting off the tip of the shell at about 7 mm from the ventral margin using a Dremel power tool (Dremel, Australia). This

produced cross-sections of the shell tips that include the most recently grown shell increments of the outer shell layer, which were then embedded together in epoxy resin mounts. Mounts were lapped and polished following the procedure in Otter et al. (2019) using colloidal silica (0.04 μm, 1 minute) as the final polishing step.

A confocal micro-Raman spectrometer (Horiba Jobin Yvon LabRAM HR Evolution at Macquarie University) was used to verify the aragonitic composition of the shells. Spot measurements were acquired across all shell layers from a polished shell cross-section using a 633 nm laser (10 mW). The acquisition time was 10 seconds per spectral window and 2 accumulations were averaged to eliminate spikes and reduce background noise. A confocal pinhole setting of 100, a grating with 1800 lines per mm, and a 50x objective lens ensured high-resolution and confocal measurement conditions. Data processing involved normalizing each spectrum to its highest intensity and plotting in Origin Pro.

We quantified major and trace element mass fractions of the carbon-coated bivalve shell portions grown in aquaculture via Wavelength Dispersive X-ray Spectroscopy (WDS) using a JEOL JXA-8530FPlus Field Emission Gun-Electron Probe Micro Analyzer (FEG-EPMA) at ANU. Analyses were performed using an acceleration voltage of 15 keV, a beam current of 10 nA, and a spot size of about 20 μm (Table S8). The full calibration procedure is listed in Table S9. Backscattered electron (BSE) images revealed the Sr-labeled growth increment due to its higher Z-contrast compared to increments grown at normal seawater concentrations (Otter et al., 2019; Otter et al., 2023). Based on the BSE images, we selected the shells that had grown >25 μm, combined them into new mounts, and removed the carbon coating (Table S10).

2.6 Microanalytical reference materials for *in-situ* stable isotope analysis:

We used four reference materials for *in-situ* stable isotope analyses, two calcites and two aragonites: in-house calcite reference material S0161 from the Canadian Centre for Isotopic Microanalysis (CCIM), the calcite standard reference material NBS19 (also known as NIST SRM8544), the aragonite reference material VS001/1-A ($\delta^{18}O$ of -12.41 ± 0.03 ‰, He et al., 2021) and the new in-house aragonite reference material S0436 from CCIM ($\delta^{18}O$ of -6.88 ± 0.03 ‰, this study). All carbonate isotope values are expressed in per mil relative to Vienna Pee Dee Belemnite (‰ VPDB). S0436 is speleothem material originating from the Furong Cave, China, previously described in Li et al. (2011). The phase composition of S0436 was verified by powder X-Ray Diffraction (XRD) performed on 20 mg of S0436 crystal fragments crushed in ethanol for about 2 minutes, which were then prepared as smear mounts on glass plates. The XRD analysis was carried out with a Bruker D8 Advance equipped with a Cobalt tube at an acceleration voltage of 35 keV and a 40 mA current. Samples were scanned over a $2\Theta\ \Theta^{-1}$ axis from 3 to 80° at a step size of 0.02° and a scan speed of 1s. The XRD spectrum of S0436 (Figure S2) demonstrates its composition to be at least 97 % aragonite, with calcite as the minor secondary phase. For SIMS analyses, we selected individual grains demonstrated to be aragonitic by micro-Raman spot measurements.

The stable C and O isotope compositions of S0436 were determined by Isotope Ratio Mass Spectrometry (IRMS) at the Veizer Lab, University of Ottawa. A representative aliquot was crushed to a fine powder and aliquots of 0.6 mg were each dissolved in 0.1 ml phosphoric acid at 25 °C, followed by gas extraction in continuous flow. Measurements were performed on a Delta XP and Gas Bench II (Thermo Finnigan). The analytical precision is ± 0.1 ‰. We used the isotope fractionation correction factors proposed by Kim et al. (2007). The $\delta^{13}C$ yielded +0.09 ±

0.07 ‰ VPDB and $\delta^{18}O$ is -6.88 ± 0.03 ‰ VPDB (n = 6). All analytical precisions are expressed as first standard deviations (1s).

The stable isotope composition of the calcite reference material S0161 was determined by the same methods at the Veizer lab and at the University of Western Ontario, courtesy of F. Longstaffe, where the reaction temperature was 90 °C, and the gas fractionation factor from Rosenbaum and Sheppard (1986) was applied: $\delta^{13}C$ is +0.12 ± 0.04 ‰ VPDB and $\delta^{18}O$ is -5.42 ± 0.03 ‰ VPDB (n = 15) and has been successfully used in previous studies (e.g., Drake et al., 2023; Terwilliger et al., 2023). NBS19 has a certified $\delta^{18}O$ value of -2.20 ‰ VPDB (Friedman et al., 1982).

2.7 *In-situ* and traditional stable isotope analyses of aragonite shells:

The polished epoxy mounts containing shell sections and microanalytical calibration materials were cleaned in petroleum ether, RBS35 detergent, and Millipore water before vacuum drying for at least 12 hours at 60 °C and coated with a ca 10 nm layer of aluminum. Oxygen isotopic compositions were measured *in-situ* over five consecutive days using a Sensitive High Resolution Ion Microprobe-Stable Isotope (SHRIMP-SI) at ANU. Analyses were carried out in multi-collector mode using a 15 keV $^{133}Cs^{+}$ primary beam. The primary beam was focused through a 200 μm aperture to achieve a final spot size of 25 μm. Excess charge at the sample surface was neutralized using a 1.5 keV focused electron beam. A mass resolution of 3,000 at 1 % peak height allowed for the interference-free detection of $^{16}O^{-}$ and $^{18}O^{-}$ with Faraday cups. The electrometers were operated in resistor mode (10^{11} and 10^{12} Ω, respectively). Each SHRIMP analysis took ~6 min (1 min for pre-sputter and baseline measurements, 3 min tuning, and 2 min data acquisition). For more details on SHRIMP-SI see Ávila et al. (2020). All data points were corrected for electrometer baselines and electron-induced secondary ion emission (EISIE) and are expressed in ‰ VPDB. All samples were analyzed in brackets between the four microanalytical reference materials (Table S11).

Lastly, we used a micro mill equipped with 250 and 300 μm diameter drill bits to collect powdered shell samples that were analyzed by IRMS. The micro-milled shell transects, sampled across shell increments grown in the wild, were collected from two specimens and were milled next to previously acquired SIMS and EPMA spots to support a comparison between both bulk-conventional and *in-situ* $\delta^{18}O$ analyses. The milled samples were acid digested at 75 °C in a Thermo Scientific KIEL IV Carbonate Device and analyzed in either a DELTA or a MAT253 IRMS mass spectrometer (Thermo Scientific) at ANU (Table S12). The shell samples were bracketed between three certified microanalytical reference materials NBS18 ($\delta^{18}O$ = -23.00 ‰ VPDB) and NBS19 ($\delta^{18}O$ = -2.20 ‰ VPDB) to normalize the $\delta^{18}O$. IAEA-603 ($\delta^{18}O$ = -2.37 ‰ VPDB) was used for quality control. The average of the IAEA-603 analyzed along with the samples was -2.38 ± 0.04 (n = 6) and -2.36 ± 0.04 ‰ (n = 6) for $\delta^{18}O$ for the MAT253 and DELTA, respectively. Isotope ratios of the shell samples were matrix bias corrected to aragonite using the fractionation factors in Kim et al. (2015). The data were plotted using Origin Pro.

3 Results:

3.1 Growing live *Anadara trapezia* shells in tightly temperature-controlled aquaculture conditions:

The 80-day aquaculture experiments with live *A. trapezia* shells yielded a closely monitored and controlled multi-variate dataset that includes all critical seawater chemistry parameters (Table 1). Target temperatures of 13, 18, 23, and 28 °C were met precisely, with averages measuring 13.0 ± 0.2, 17.9 ± 0.1, 23.0 ± 0.3, and 28.1 ± 0.5 °C, respectively (Tables 1 and S2). Average salinities were 35.8 ± 0.4, 35.6 ± 0.5, 35.3 ± 0.6, and 35.6 ± 0.9 ppt, respectively, for cultures reported in order of increasing temperature (Tables 1 and S3). The seawater pH_T values averaged 8.02 ± 0.06, 8.03 ± 0.05, 8.03 ± 0.05, and 8.02 ± 0.06 and alkalinities averaged 2,332 ± 143, 2,336 ± 143, 2,318 ± 160, and 2,301 ± 198 μmol kg^{-1}, respectively, for cultures listed in order of increasing seawater temperatures (see Tables 1 and S4). Both pH_T and alkalinities reflect those of natural seawater and are critical parameters in characterizing marine seawater carbonate chemistry (Table 1). The in-house reference seawater solution was accurate within an RSD of 6 % for all cations measured by ICP-OES. The aragonite saturation state (Ω_{ara}), which is a measure of the thermodynamic behavior of aragonite to dissolve (Ω <1) or precipitate (Ω >1) was calculated for each culture using mean daily seawater temperatures, salinities, pH_T, and alkalinity values together with the Ca^{2+} concentrations as input parameters and was 2.2 ± 0.3, 2.7 ± 0.4, 3.1 ± 0.4, and 3.7 ± 0.5, respectively (listed in order of increasing seawater temperatures) and thus shows an expected correlation with seawater temperature (see Tables 1 and S5). The Na/Ca, Mg/Ca, K/Ca, and Sr/Ca do not show a significant difference between the temperature-specific cultures (Tables 1 and S6). Lastly, $\delta^{18}O_{SW}$ averaged 0.53 ± 0.15, 0.56 ± 0.16, 0.73 ± 0.21, and 0.87 ± 0.28 ‰ VSMOW, respectively, for cultures listed in order of increasing seawater temperatures (Tables 1 and S7). Analytical precisions are provided as first standard deviations (1s) for all parameters in the multi-variate dataset.

Apart from the temperature-dependent Ω_{ara}, all seawater parameters were well controlled to be identical within analytical precision and do not show a correlation with seawater temperature. Measured seawater temperatures met target conditions with 5 °C intervals between cultures. Carbonate, cation, and oxygen isotope system parameters were controlled by regular water exchanges.

Table 1. Overview of environmental and seawater chemistry parameters for the four temperature-controlled *A. trapezia* aquaculture experiments. The bivalves were grown for 80 days under monitored and controlled conditions. Seawater temperatures were recorded every 15 minutes, while all other data were sampled at 3-day intervals. Salinity was measured via a hand-held logger, free pH_T and total alkalinity were analyzed using a spectrophotometer and aragonite saturation states (Ω_{ara}) were calculated from seawater chemistry. $\delta^{18}O_{SW}$ were acquired on a Picarro CRD spectrometer and Element/Ca ratios by ICP-OES. All data are shown as averages with first standard deviations (1s). See Tables S2 to S7 for full datasets.

Target temperature [°C]	Measured temperature [°C]	Salinity [ppt]	pH_T	Alkalinity [µmol kg^{-1}]	Na/Ca [mmol mol^{-1}]	Mg/Ca [mmol mol^{-1}]	K/Ca [mmol mol^{-1}]	Sr/Ca [mmol mol^{-1}]	Ω_{ara} [a]	$\delta^{18}O_{SW}$ [‰, VSMOW]
13	13.0 ± 0.2	35.8 ± 0.4	8.02 ± 0.06	2150 ± 162	46,838 ± 1,880	5,146 ± 13	1,016 ± 66	8.9 ± 0.3	2.2 ± 0.4	0.53 ± 0.15
18	17.9 ± 0.1	35.6 ± 0.5	8.03 ± 0.05	2100 ± 127	45,790 ± 1,656	5,140 ± 10	1,012 ± 58	9.1 ± 0.7	2.7 ± 0.4	0.56 ± 0.16
23	23.0 ± 0.3	35.3 ± 0.6	8.02 ± 0.06	2037 ± 144	46,781 ± 1,960	5,149 ± 15	1,000 ± 62	8.9 ± 0.3	3.0 ± 0.4	0.73 ± 0.21
28	28.1 ± 0.5	35.6 ± 0.9	8.03 ± 0.04	2009 ± 186	45,734 ± 1,870	5,058 ± 71	995 ± 50	8.9 ± 0.3	3.6 ± 0.5	0.87 ± 0.28

[a] Aragonite saturation states calculated using salinity, Ca^{2+} concentrations, total alkalinity, and pH_T as input into the 'CO2Sys MS Excel Macro (Pierrot et al., 2011) using the carbonate species abundances based on the models of Millero et al. (2006) and the solubility product of Morse et al. (1980).

3.2 Composition and growth:

Confocal micro-Raman spectroscopy was used to verify the mineral phase composition of *A. trapezia* shells (Figure 1a). We analyzed a line of spots (n = 31) across a polished shell cross-section perpendicular to the inner shell surface to probe the composition of all three shell layers: the outer crossed-lamellar shell layer (Figure 1b, red spectrum, n = 26) used for SIMS microanalyses, the inner shell layer (Figure 1b, blue spectrum, n=4), and 1 spot was situated on the thin myostracum (Figure 1b, green spectrum) that separates the outer and inner shell layers. In addition, Raman spectra were obtained for the microanalytical reference materials S0436 (Figure 1b, dark gray spectrum, n = 3) and VS001/1-A (Figure 1b, light gray spectrum, n = 1, for more Raman analyses see He et al., 2021). Aragonite was confirmed through peaks related to the lattice mode region, including at 153 and 206 cm^{-1}, and those related to the intrinsic modes of the carbonate anion (CO_3^{2-}), namely the ν_4 internal in-plane bending mode with its characteristic doublet at 701 and 706 cm^{-1} as well as the ν_1 internal symmetric stretching mode at 1085 cm^{-1} (Wehrmeister et al., 2011).

To confirm their growth, we pre-screened cross-sections of randomly chosen shells from each culture by visualizing both the 72-h Sr pulse-chase labeling event and measuring the distance to the inner shell edge for a subset of 45 shells selected across all four groups. This distance quantifies the amount of growth achieved during the 80 days following the Sr label (Figure 1c). The Sr labeled shell increment is visible due to the increased Z-contrast caused by higher Sr mass fractions in the shell, whereas the unlabeled shell portions before and after the label appear as darker greyscale intensities as these shell portions were grown in seawater with lower (i.e., normal marine) Sr concentrations (Otter et al., 2019; Otter et al., 2023). BSE imaging revealed the alternating pattern of crystallographic orientation changes of the first-order lamellae perpendicular to the inner shell surface that were visible as changes in grayscale intensities and are a feature of the crossed-lamellar shell architecture (Agbaje et al., 2017; Böhm et al., 2016).

Shells cultured at 13 °C (n = 12) grew on average 13 ± 11 µm (0.2 ± 0.1 µm day^{-1}), those at 18 °C (n = 14) averaged 29 ± 19 µm (1.0 ± 0.2 µm day^{-1}), shells at 23 °C (n = 14) averaged 27 ± 26 µm (0.3 µm day^{-1}) and lastly those maintained at 28 °C (n = 5) grew on average 15 ± 6 µm (0.2 µm day^{-1}). With 153 µm, specimen At-23-11R yielded the most growth during the experiment, which corresponds to an average growth rate of 2 µm day^{-1}. At group level, shells grown at 18 °C achieved the most growth, followed by the cultures grown at 23, 28, and 13 °C, respectively. Mortality rates were highest in the 28 °C group (1 bivalve survived the 80 day experiment to completion), followed by 18 and 23 °C, which both had 16 dead shells and the lowest rates for the 13 °C experiment (15 dead shells). The high mortality rates demonstrate how challenging it is for this species to withstand prolonged heatwaves, while the significantly downscaled growth rates in the lowest temperature group suggest those individuals were approaching a growth hiatus. Growth rate variation has previously been observed in wild populations, where this effect can challenge crossmatching (Trofimova et al., 2020), as well as those grown in aquaculture experiments (Otter et al., 2019). The high mortality rate observed for the 28 °C group is unsurprising as this temperature is very rarely observed to occur in the natural habitat of *A. trapezia* (compare Table S1) but was included to better understand the resilience and heat tolerance of this species under more extreme future conditions. We selected the shells with the most growth from each group for further *in-situ* stable isotope microanalyses.

Elemental mass fractions of Na, Mg, Ca, P, Cl, and Sr were measured in the shells and the microanalytical oxygen isotope reference materials (Table 2). The shell Ca abundance ranges from 37.1 to 37.8 % m/m, while the stable isotope reference materials have higher values ranging from 39.0 to 40.1 % m/m. Further, the shells have detectable mass fractions of Na, Mg, and Sr, while those elements are mostly below detection limits in the SIMS calibration materials.

Table 2. Quantitative major and minor element mass fractions of shell portions grown in the four tightly temperature-controlled *A. trapezia* aquaculture experiments and the carbonate reference materials measured by WDS FEG-EPMA. Elemental mass fractions are presented as averages with first standard deviations (1s) in % m/m as well as Element/Ca ratios in mmol mol^{-1}. Measurements on bivalve shells were carefully placed between the Sr labeled shell increment and the inner shell surface during live BSE imaging to target only shell material grown during the temperature-controlled aquaculture experiment. S0161 is a calcite reference material candidate, S0436 and VS001/-1A are aragonites. For the full dataset and details on the WDS calibration, see Tables S8 and S9, respectively. Phosphorous, sulfur, and Cl were below detectability in all specimens and are thus not shown.

Sample ID:	n	Na [% m/m]	Mg [% m/m]	Ca [% m/m]	Sr [% m/m]	Na/Ca [mmol mol^{-1}]	Mg/Ca [mmol mol^{-1}]	Sr/Ca [mmol mol^{-1}]
***A. trapezia* shell portions grown at target temperatures:**								
13 °C [a]	14	0.53 ± 0.03	0.05 ± 0.01	37.2 ± 0.4	0.22 ± 0.09	24.9 ± 1.6	2.1 ± 0.6	2.7 ± 1.1
18 °C [b]	15	0.49 ± 0.03	0.04 ± 0.01	37.1 ± 0.7	0.17 ± 0.06	22.9 ± 1.7	1.8 ± 0.6	2.0 ± 0.7
23 °C [c]	14	0.40 ± 0.04	0.04 ± 0.02	37.5 ± 0.6	0.14 ± 0.09	18.5 ± 2.3	1.6 ± 0.7	1.7 ± 1.1
28 °C [d]	15	0.35 ± 0.02	0.02 ± 0.01	37.8 ± 0.3	<0.08	16.2 ± 0.9	1.0 ± 0.5	-
Microanalytical carbonate reference materials:								
S0161	20	<0.01	0.05 ± 0.01	39.7 ± 0.3	<0.08	-	1.9 ± 0.4	-
S0436	20	<0.01	<0.01	40.1 ± 0.3	<0.08	-	-	-
VS001/1-A	9	0.16 ± 0.03	<0.01	39.0 ± 0.2	0.61 ± 0.16	7.1 ± 1.4	-	7.2 ± 1.8
Limits of detection:		0.01	0.01	0.01	0.08	-	-	-

[a] Measurements obtained from 3 shells: At-13-15R, At-13-17R, At-13-19R
[b] Measurements obtained from 3 shells: At-18-07R, At-18-15R, At-18-18R
[c] Measurements obtained from 3 shells: At-23-09R, At-23-11R, At-23-15R
[d] Measurements obtained from 3 shells: At-28-18R, At-28-19R, At-28-22R

3.3 *In-situ* stable oxygen isotope measurements of aragonite bivalve shells:

We collected a total of 586 SIMS analyses on the four microanalytical reference materials, *A. trapezia* shell portions grown under temperature-controlled conditions and from shell portions grown in the wild. We verified all SIMS spot locations on shells and reference materials by post-analysis BSE imaging. Spots situated on topographical features, such as cracks, were excluded from data evaluation. For shell portions grown during controlled aquaculture experiments, we rejected any spots (Figure 1c, red circles) that touched the inner shell edge and thus the epoxy and those that overstepped or fell behind the Sr-labeled shell increment, accepting only spots falling between the Sr label and the inner shell edge (Figure 1c, blue circles). This selection procedure yielded 77 ideally situated shell SIMS analyses (see Table 3). Although the Sr pulse-chase labelling event was performed at target seawater temperatures, we chose to exclude any SIMS analyses that overstepped into the Sr labeled shell portion to avoid potential trace element-induced complications in bias correction as reported in previous studies (Allison & Finch, 2010; Rollion-Bard & Marin-Carbonne, 2011).

During SIMS measurements, we bracketed four carbonate reference materials between the unknowns: two calcites, S0161 used as primary calibration material, the standard reference material NBS19 for quality control, as well as two abiogenic aragonites, VS001/1-A and S0436

(Tables 3 and S11). All four reference materials have been characterized by IRMS (see methods) and have $\delta^{18}O$ values that are acceptably homogeneous at the scale of SIMS analyses.

The internal precision that resulted from averaging 6 individual measurements for each SIMS spot was assessed and the external repeatability that quantifies the analytical variance from repeated measurements on homogeneous calibration materials (Rollion-Bard et al., 2003; Rollion-Bard et al., 2007). The internal precision at a 95 % confidence level is ± 0.15 and ± 0.16 ‰ for NBS19 and S0161, as well as ± 0.14 ‰ for both aragonite reference materials (Table 3). The repeatability, expressed as first standard deviation, was ± 0.20 and ± 0.29 ‰ for NBS19 and S0161 and ± 0.37 ‰ for both aragonite reference materials, respectively (Tables 3 and S11), and are a measure of the individual spot uncertainties when most instrumental effects are propagated.

The $\delta^{18}O$ ratios of aragonite samples obtained by ion microprobe require a two-step correction process addressing both instrumental and matrix-induced fractionation. The first step in quantifying ion microprobe $\delta^{18}O$ ratios is to correct for instrumental mass fractionation by subtracting the difference between the oxygen isotope composition measured on the primary reference material by IRMS and that measured by ion microprobe from all SIMS analyses (see equations provided in Rollion-Bard & Marin-Carbonne, 2011). Secondly, matrix corrections are applied that further correct for the residual bias, i.e., the slight differences caused by composition and structure between the primary reference material and the unknowns. In this context, 'matrix' has a wide definition extending beyond mineralogy and polymorphism and also includes structural and chemical differences, e.g. within solid-solution series (Fayek et al., 2001; Rollion-Bard & Marin-Carbonne, 2011), and the Ca mass fraction variations observed between abiogenic and biogenic aragonites (He et al., 2021) that are also observed in this study (Table 2). Collectively, these factors lead to matrix-specific behaviors in primary ion beam sputtering and secondary ion formation that have to be corrected.

Traditionally, both instrumental and matrix isotope fractionation are corrected by bracketing the unknowns between microanalytical reference materials with matrices matching the samples as closely as possible. However, this approach relies on the availability of suitable microanalytical reference materials that are well characterized and homogeneous in $\delta^{18}O$ as well as available as crystals or crystal fragments. Unfortunately, there are currently no certified aragonite reference materials that meet these criteria. Hence, aragonite samples have only been calibrated using in-house reference materials thought to sufficiently match the unknowns (Rollion-Bard et al., 2003; Rollion-Bard & Marin-Carbonne, 2011; Rollion-Bard et al., 2007) and newly developed potential reference materials (He et al., 2021). However, these materials are limited by a lack of availability or thorough characterization, and insufficient understanding of potential matrix effects arising from the analysis of biogenic aragonite rather than geological/abiotic aragonites. Further, to the best of our knowledge, no unpowdered, homogeneous aragonite reference material of biogenic origin exists. This lack in aragonite reference materials arises from this material growing under naturally variable surface conditions and thus incorporating variable isotope and trace elemental signatures in both biogenic and geological environments.

For matrix correction, we first normalized the SHRIMP $^{18}O/^{16}O$ ratios to the primary calcite reference material (S0161) and refer to these values as $\delta^{18}O_{SHRIMP}$ in the following. In Figure 2, we present the difference between $\delta^{18}O_{SHRIMP}$ (calcite normalized data) and the reference values for the same materials from IRMS expressed as $\delta^{18}O_{SHRIMP-IRMS}$ as a function of Ca mass fractions for the two geological aragonite reference materials, VS001/1-A and S0436,

similar to the observations reported by He et al. (2021). In this study, the straight line in Figure 2 is defined by the two calcite-normalized aragonite reference materials joining these two has a slight positive slope, suggesting that the bias increases with Ca mass fraction, although in our case the slope is lower than that of He et al. (2021),

Equation 1: $y = 0.418 \times Ca\ [\%\ m/m] - 16.079$

To test the strength of this relationship by evaluating it independently using the data on the wild shells for which we have the co-located $\delta^{18}O$ ratios obtained by IRMS (Table S12), in addition to SHRIMP (Table S11) and Ca mass fractions obtained by EPMA (Table S8). In Figure 2, both wild shell portions plot at lower Ca abundances, within error of the extrapolation of the calibration line defined by the geological aragonite reference materials. These results confirm the systematic effects of Ca abundance on SIMS bias shown by He et al. (2021), but as indicated, have a lesser effect in this study.

Having validated the calibration strategy, we applied it to the four temperature-controlled *A. trapezia* cultures (Table 2), using Equation 1 to determine the residual $\delta^{18}O$ bias based upon their measured Ca mass fractions (Tables 3 and 11). This strategy yielded the following bias values: -0.53, -0.57, -0.40, and -0.28, reported in the order of increasing seawater temperature. The y-axis error bars associated with each bias value correspond to SIMS first standard deviations, while the y-error bars of VS001/1-A and S0436 have been propagated from IRMS and ion microprobe standard deviations. Application of our new calibration strategy yields the following final, fully matrix-corrected $\delta^{18}O$ ratios for the four temperature-controlled *A. trapezia* cultures: 2.98 ± 0.31, 1.77 ± 0.39, 0.85 ± 0.18, and 0.02 ± 0.24 ‰, respectively, in order of increasing seawater temperature (Tables 3 and 11).

We further assessed how our new SIMS matrix bias calibration strategy compares to other, simpler calibration approaches (Figure 3). For this purpose, we compare the $\delta^{18}O_{IRMS}$ with the calcite-normalized $\delta^{18}O_{SHRIMP}$ obtained from the shell portions grown in the wild (Figure 3). The closest agreement between $\delta^{18}O_{IRMS}$ (Figure 3, red) and $\delta^{18}O_{SHRIMP}$ is observed for our new Ca-corrected matrix bias calibration strategy (Figure 3, blue), followed by the calcite-normalized $\delta^{18}O_{SHRIMP}$ data without further processing (Figure 3, white). Lastly, $\delta^{18}O_{SHRIMP}$ matrix corrected using only one aragonite reference material show the largest deviation from the IRMS value (VS001/1-A: Figure 3, light gray, S0436: dark gray). This comparison demonstrates that the residual $\delta^{18}O$ matrix fractionation bias is best accounted for when using the Ca abundance-dependent calibration strategy (Equation 1, Figure 2).

Table 3. Ion microprobe $\delta^{18}O$ ratios for the shell portions grown in temperature-controlled aquaculture and carbonate calibration materials shown together with corresponding aragonite residual bias values and analytical precision values. The calcite reference material S0161 was used as primary reference material, NBS19 for quality control and the two aragonite reference materials S0436 and VS001/1-A were used together to evaluate the shell samples based on a linear 2-point bias correction method. BSE images obtained after the SHRIMP session ensured that only those spots were used that did not overstep the Sr label. Only shells with >25 µm of growth were analyzed (see Table S10).

Specimen ID:	n	Calcite-normalized $\delta^{18}O$ weighted mean [‰ VPDB]*:	Bias for biogenic aragonites:	Corr. $\delta^{18}O$ weighted mean [‰ VPDB]:	± 1s [‰ VPDB]:	± internal precision (95% conf.)	±Precision estimate [‰ VPDB]
***A. trapezia* shell portions grown at stable temperatures:**							
13 °C [a]	16	2.45	-0.53	2.98	0.31	0.10	0.02
18 °C [b]	35	1.20	-0.57	1.77	0.39	0.10	0.02
23 °C [c]	12	0.45	-0.40	0.85	0.18	0.11	0.03
28 °C [d]	14	-0.26	-0.28	0.02	0.24	0.11	0.03
Microanalytical calibration materials:							
S0161 [f]	111	-5.42	nA	nA	0.29	0.16	0.01
NBS19 [e]	75	-2.34	nA	nA	0.20	0.15	0.01
S0436 [g]	95	-6.19	0.69	-6.88	0.37	0.14	0.01
VS001/1-A [h]	30	-12.18	0.23	-12.41	0.37	0.14	0.02

*Final numbers for calcite materials and calcite-normalized numbers for biogenic aragonites ($\delta^{18}O_{SHRIMP}$). [a] Measurements obtained from 1 specimen: At-13-15R. [b] Measurements obtained from 3 specimens: At-18-07R, At-18-13R, At-18-14R. [c] Measurements obtained from 3 specimens: At-23-09R, At-23-11R, At-23-15R. [d] Measurements obtained from 1 specimen: At-28-22R. [e] Certified $\delta^{18}O$ value for NBS19: -2.20 (Friedman et al., 1982) [f] Known $\delta^{18}O$ value for S0161: -5.42 ± 0.05 (this study) [g] Known $\delta^{18}O$ value for S0436: -6.88 ± 0.05 (this study) [h] Known $\delta^{18}O$ value for VS001/1-A: -12.41 ± 0.03 (He et al., 2021).

3.4 A novel *in-situ* SST *versus* $\delta^{18}O$ thermometer relationship:

Our aquaculture experiments at four tightly controlled seawater temperatures (see 3.1.) and our new $\delta^{18}O$ bias calibration method (see 3.2) allowed us to develop an SST *versus* seawater corrected $\delta^{18}O$ fractionation (as $\delta^{18}O_{arag}$ ‰ VPDB - $\delta^{18}O_{SW}$ ‰ VSMOW) relationship for the aragonitic shells of *A. trapezia*. This relationship has the following linear least squares regression:

Equation 2: $T\ (°C) = (23.31 \pm 0.34) - (4.31 \pm 0.22) \times (\delta^{18}O_{arag}\ [‰\ VPDB] - \delta^{18}O_{SW}\ [‰\ VSMOW])$

$$n = 77,\ R^2 = 0.995$$

The seawater thermometer regression (Equation 2) is visualized in Figure 4 (solid black line). The underpinning data is shown is both averages (black squares, error bars propagated from SIMS $\delta^{18}O_{arag}$ and $\delta^{18}O_{sw}$) and individual data points (black circles) together with the confidence interval (gray shaded envelope). This regression has a slope of 0.23 ‰ $°C^{-1}$ SST for the experimental range of 13 to 28 °C (Figure 4). We used the 95 % confidence level as a reasonable cut-off for identifying statistical outliers, which returned one value in the 18 °C group (Figure 4, red circle). As a single outlier seems negligible and prescreening of each ion microprobe spot was extensive (i.e., based on both optical and electron imaging), we retained this spot in the regression. The degree of remaining scatter observed within the 95 % confidence interval is suggested to result from natural variability within the realm of isotopic equilibrium fractionation.

In Figure S3 we provide an alternative regression that uses the 77 individual data points (Figure S3, blue regression and confidence interval) and compares it to the model based on group averages (Figure S3, gray regression and confidence interval) that has the following equation:

$$T\ (°C) = 23.68 \pm 0.22 - (4.28 \pm 0.14) \times (\delta^{18}O_{arag}\ [‰\ VPDB] - \delta^{18}O_{SW}\ [‰\ VSMOW]),\ R^2 = 0.922,\ n = 77,$$

Equation 3

The average-based thermometer regression (Figure 4, Equation 2) yields a slightly higher R^2 value of 0.995 with a wider confidence interval, while the model based on individual data points (Figure S3, Equation 3) has a narrower and slightly more elongated confidence interval with a lower R^2 value of 0.922. While both equations 2 and 3 provide appropriate models for the data produced in this study, we prefer the application of the average-based model as it eliminates differences in the number of data points (n) and thus counteracts weighting between temperature groups.

4 Discussion:

4.1 Aquaculture conditions with Sr pulse-chase labeling improve the confidence of SIMS $\delta^{18}O$ analyses:

We grew live *A. trapezia* bivalves for 80-days at four tightly controlled (± 0.5 °C) seawater temperatures (13 to 28 °C) under monitored and controlled aquaculture conditions (Tables 1 and S2 to S7). We observe an expected correlation of higher temperatures with Ω_{ara}., while other seawater parameters remained uncoupled from temperature (Table 1). Our comprehensive multi-variate seawater chemistry dataset (Tables 1, and S2 to S7), obtained during our shell growth experiments, ensures a thorough understanding of the growth conditions of the shells and their chemical consistency, which was a crucial foundation for high-resolution ion microprobe analyses.

We pioneered the application of Sr-pulse-chase labelling to mark the start of the growth experiment as a precondition for obtaining well-targeted high-resolution SIMS analyses. This approach played a crucial role in identifying shells with enough growth for SIMS analyses (Table S10) and guiding informed decisions on spot inclusion and rejection within the ion microprobe data. Using both optical microscopy during SIMS sessions and subsequent BSE imaging offered a more confident identification of growth increments compared to previous strategies relying on literature-based growth rates (e.g., Böhm et al., 2000; Carré et al., 2005; Chamberlayne et al., 2021) or shell height measurements (e.g., Wanamaker et al., 2006). As the pulse-chase label is visible in high-resolution BSE images, it even surpasses the imaging resolution of fluorescent markers (e.g., Al-Qattan et al., 2023) and is therefore even better suited for marking narrow growth increments at high resolution as well as for species with inconclusive natural growth banding (e.g., Long et al., 2020).

4.2 Ion microprobe matrix calibration for obtaining quantitative $\delta^{18}O$ from biogenic aragonite with organic-inorganic architectures:

The current study validates the observations of He et al. (2021) who observed a correlation between SIMS bias and Ca mass fractions between biogenic (coral) and geological aragonites. Indeed, this study observes similarly significant differences in Ca abundances between biogenic aragonites (bivalve shells) averaging 37.4 ± 0.6 (n = 58), while the geological

aragonites achieve higher values of 39.0 ± 0.2 (n = 9) for VS and 40.1 ± 0.3 (n = 20) for S0436 (Tables 2 and S8). Building on this observation, we developed a new strategy for correcting SIMS measurements of $\delta^{18}O$ for residual matrix effects in biogenic aragonites using geological aragonite reference materials as a function of their Ca mass fractions. The new approach allows for the accurate isotopic analysis of shell increments that are too narrow for micro-milling and IRMS analyses. The linear relationships differ considerably between both studies, as we report a bias increase of 0.4 ‰/Ca % m/m, while He et al. (2021) report 1.3 ‰/Ca % m/m. Explanations for this are speculative, but it is well-known that instrumental bias is affected by analysis conditions for which these were undoubtedly different owing to the different brands of instruments utilized.

The difference in Ca mass fractions between biogenic and abiogenic aragonites is linked to their distinct formation processes. The abiogenic aragonites used here, VS001/1-A and S0436, formed as vein precipitates (He et al., 2021) and speleothem (this study), with both processes linked to inorganic supersaturation-driven crystallization (e.g., Brown et al., 1962; Riechelmann et al., 2014) although some speleothems reveal a more complex formation process utilizing classical and non-classical crystallization (Frisia et al., 2022). On the other hand, their biogenic counterparts (i.e., shells) are mineral-organic nano-composite materials with fundamentally different crystallization pathways as biominerals are formed by living organisms (Weiner & Dove, 2003). In fact, mineralization follows non-classical pathways via metastable phases, including ACC, which are later transformed to crystalline calcium carbonate (Addadi et al., 2003; Jacob et al., 2008; Yoreo et al., 2015), with the final mineral being aragonite in the case of *A. trapezia* (Figure 1b). Mineralization takes place within, and is orchestrated by, an organic matrix framework with mineral-organic interfaces present at all length scales of the hierarchical 3D arrangements (Wolf et al., 2016). The outer shell layer of *A. trapezia* that was analyzed here consists of a crossed-lamellar shell architecture which is the most common shell architecture found in mollusks (Boggild, 1930) with fossil evidence dating back as far back as the Middle Cambrian (Runnegar, 1985). Also, it is one of the most highly mineralized shell architectures, with <2 % m/m of total organic content (Agbaje et al., 2017; Agbaje et al., 2019; Böhm et al., 2016). It has a plywood-like organization consisting of lamellae with alternatingly tilted crystallographic orientations enveloped in thin organic sheaths (Agbaje et al., 2017; Agbaje et al., 2019; Böhm et al., 2016). These first-order lamellae are composed of successively smaller hierarchical building blocks until organically sheathed nanogranules with sizes ranging from 15 to 150 nm are finally the smallest architectural unit in the shell (Wolf et al., 2016).

4.3 Comparing the *in-situ* SST *versus* $\delta^{18}O$ thermometer relationship to previous models:

In Figure 5, we compare the *in-situ* oxygen isotope-SST relationship (Equation 2, Figure 4) with previously published models (Figure 5, solid black line *versus* colored lines). Our *in-situ* relationship plots closest to models produced from other aragonite biominerals, with the closest relation observed to the mollusk-based version of the model by Grossman and Ku (1986) and the aragonitic shells of *Arthritica helms* bivalves presented by Chamberlayne et al. (2021). Further to the left (i.e. with a lower intercept for the isotope-temperature relationship) plot the aragonitic bivalve larvae shells of *Placopecten magallanes* (Owen et al., 2008) and the aragonitic coralline sponge shown in Böhm et al. (2000) that was extended into colder SSTs by using data from Grossman and Ku (1986). Further away plot the thermometer for aragonitic shells of *Mesodesma donacium* bivalves (Carré et al., 2005) and thermometers based on calcite biominerals based on the prismatic outer shell layer of *Mytilus edulis* (Wanamaker et al., 2006) and the shells of

barnacles (Al-Qattan et al., 2023). The highest degree of difference is observed for inorganically precipitated calcite (Kim & O'Neil, 1997). This is perhaps unsurprising as both systems have significantly different crystallization pathways in addition to the chemical and structural differences between the two polymorphs. While the inorganic precipitates formed by supersaturation and thus by classic monomer-by-monomer crystallization processes, the shells are known to form by non-classical crystallization pathways via transient precursor phases (Addadi et al., 2003; Wolf et al., 2016).

A critical aspect of thermometer relationship comparisons is the slope of the regression as it quantifies the change in °C $‰^{-1}$. The *in-situ* relationship presented in this study yields a slope of 4.31± 0.22 °C $‰^{-1}$ (Equation 2, Figure 4), which is within error to the 4.42 ± 0.10 °C $‰^{-1}$ reported in Böhm et al. (2000) and the 4.43 ± 0.38 °C $‰^{-1}$ of Chamberlayne et al. (2021). These similarities in slope demonstrate the suitability of our SIMS approach for paleoenvironmental reconstructions, relative to traditional methods.

Another important parameter is the sensitivity of a seawater thermometer regression, which in the case of Grossman and Ku (1986) is reported as ± 1.6 °C, and reflects the proximity between measured and calculated SSTs. However, it could be speculated that this number is underestimated given the field-based sampling approach collecting live and recently alive specimens with uncertain growth rates grown in a naturally heterogeneous environment. Here we report a sensitivity of ± 0.3 °C for the calibrated range (Equation 2), which we suggest is linked to a more rigorous procedure of checking every SIMS analysis against the growth interval as visualized in BSE images (Figure 1c) and the tightly constrained growth conditions in our experiments (Tables 1 and S2 to S7). A more thorough comparison between the classic regression by Grossman and Ku (1986) and this study with their statistical errors is provided in Figure S4. Here we demonstrate that both thermometers show a significant overlap within uncertainty for the calibrated range of 13 to 28 °C. The statistical deviation between both models is 1.8 °C on average for the experimental range. The reasons for this deviation are suggested to be a combination of several contributing factors. These factors include profound differences in the crystallization pathways (i.e., classical *versus* non-classical) for inorganic and biogenic minerals, vital effects affecting species and their $CaCO_3$ polymorphs differently, relationships produced from individual species *versus* those produced from several unrelated taxa, as well as the used sampling approach and methodology, e.g., field-based strategies with intrinsically variable environmental parameters and inferred growth rates *versus* aquaculture experiments with precisely determined environmental parameters and growth rates that may explain the higher intercept error of ± 0.6 of the mollusk-based Grossman and Ku model (Figure S4) compared to ± 0.34 (Equation 2) or ± 0.22 (Equation 3) achieved in this study. These aspects and their interplay require more attention in future studies to further improve our understanding of the robustness of paleothermometer relationships.

While some paleothermometer calibrations have been achieved by adjusting salinities to force certain $\delta^{18}O_{SW}$ levels across different seawater temperature groups (e.g., Wanamaker et al., 2006), we consider salinity a less critical variable in our experiments, apart from demonstrating that it fell within the natural habitat range of *A. trapezia* and remained constant during the experiment (Tables 1 and S3). As $\delta^{18}O$-salinity relationships vary significantly on both regional and temporal scales within estuaries, they render any deliberate control on salinity and, thus, $\delta^{18}O_{SW}$ non-unique. Hence, we chose to ensure the broader applicability of our thermometer by using unaltered seawater that also maintained stable Ca and Mg levels while still achieving close

alignment with benchmark aragonite thermometers like the one presented by Grossman and Ku (1986).

Altogether, we demonstrated that high-resolution ion microprobes are indeed capable of producing paleothermometer relationships of similar if not improved sensitivity and confidence compared to traditional approaches, as long as rigorous calibration procedures and sampling strategies are applied with care.

5 Conclusions

The main objective of this study was to test whether *in-situ* ion microprobe analyses can be used to produce a $\delta^{18}O$ *versus* SST relationship for aragonite marine shells, despite the analytical challenges posed by this mineral in terms of matrix bias correction. For this purpose, we developed a novel approach for obtaining accurate, high-resolution *in-situ* stable oxygen isotope ratios from a suite of well-characterized *Anadara trapezia* bivalve shells grown under tightly controlled aquaculture conditions (Table 1). We marked the start of the growth period using Sr pulse-chase labelling visualized using BSE imaging that ensured the selection of shells with the highest growth rates and precise control over SIMS analyses locations. The *in-situ* seawater-corrected oxygen isotope fractionation *versus* seawater temperature relationship produced here is shown to plot within uncertainties to published aragonite regressions (Figure S4, grey shaded error envelopes). The similarity demonstrated between our *in-situ* thermometer and previously published ones produced by the traditional stable isotope analyses approach (and particularly the similarity for the isotope-temperature slope relationship) demonstrates the utility of ion microprobe analyses. In addition, our *in-situ* approach offers the advantage of measuring $\delta^{18}O$ with a spot size of down to 25 µm (Figure 1c), making it ideal for high-resolution SST reconstruction applications at small scales, including daily to sub-daily growth increments. Future studies may explore the applicability of this thermometer calibration to reconstruct seawater temperatures from the skeletal hard parts of different aragonite marine calcifying organisms.

A precondition for establishing the *in-situ* oxygen isotope-thermometer relationship was the implementation of a new bias calibration method for SIMS $\delta^{18}O$. This method effectively minimized the residual matrix-effects between the pure geological aragonites and their biogenic equivalents with organic-inorganic composite material properties. We achieved this by combining geological aragonitic reference materials in a linear relationship and expressed their $\delta^{18}O$ bias as a function of Ca mass fractions obtained by EPMA (Figure 2) that shows an improved accuracy compared to other approaches (Figure 3). We solved the calibration by using the Ca mass fractions for the shell increments grown under controlled aquaculture conditions to obtain bias correction values that were used to correct the shell data. Assessing this approach on shell increments grown in the wild allowed us to compare different SIMS calibration strategies with corresponding IRMS measurements, with the best agreement achieved for the new calibration strategy. We anticipate that this approach may apply to other aragonite biominerals and their organic-inorganic ultrastructures like corals and fish otoliths, provided that the Ca mass fractions of the sample is known.

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Data Availability Statement: The supporting data used in this study including aquaculture experiment parameters and shell compositions (Medd et al., 2024) are available at https://data.mendeley.com/datasets/bywms32stn/1 with a Creative Commons Attribution 4.0 International licence.

Figure captions

Figure 1. *Anadara trapezia* bivalve shells grown under controlled aquaculture conditions for quantitative *in-situ* stable oxygen isotope analyses. (a) The exterior shell surface partly covered by an organic, brown periostracum layer, revealing the white, highly mineralized carbonate layer underneath. (b) Micro-Raman spectra taken across all shell layers of *A. trapezia* and the aragonite reference materials VS001/1-A and S0436. (c) Representative BSE image of a polished shell cross-section (specimen At-28-22R) showing the Sr-labeled increment as brighter greyscales running parallel to the inner shell surface, while surrounding shell portions grown at natural seawater composition appear darker gray. The alternating stripped pattern in the shell oriented perpendicular to the Sr label results from crystallographic orientation changes of the crossed-lamellar architecture. Dark round spots resulted from SIMS spot analyses indicating which analyses were accepted (blue circles) and rejected (red circles). We only accepted spots located between the Sr label and the inner shell surface for our $\delta^{18}O$ and SST calibration across the four temperature-specific cultures.

Figure 2. Calcite-normalized $\delta^{18}O_{SHRIMP}$ fractionation *versus* Ca mass fraction-dependent calibration method for obtaining accurate *in-situ* $\delta^{18}O$ from biogenic aragonites. The assumed linear relationship shows the instrumental fractionation biases ($\delta^{18}O_{SHRIMP}$ - $\delta^{18}O_{IRMS}$) of both aragonitic reference materials, VS001/1-A (black circle) and S0436 (black square) as a function of their Ca mass fractions (Table 2). The primary reference material S0161 was used to normalize the data (white star). Their y-error bars are propagated 1s from SIMS and IRMS, while their x-errors show 1s of Ca mass fractions. This relationship was then applied to the Ca mass fractions of aquaculture-grown shell portions (Table 2) to determine their residual bias values (red, yellow, green, and blue triangles). These bias values were used to correct all $\delta^{18}O$ SIMS analyses for aquaculture grown shell portions (Table 3). For biogenic aragonites, y-errors show 1s of SIMS analyses as IRMS measurements were not obtainable. The gray diamonds show measurements obtained from shell portions grown in the wild and show their fractionation biases ($\delta^{18}O_{SHRIMP}$ - $\delta^{18}O_{IRMS}$) as a function of Ca mass fractions plotting amidst the bias values of aquaculture-grown increments, demonstrating that our new calibration produces results that are consistent with the traditional approach.

Figure 3. Box and whisker charts comparing the new $\delta^{18}O$ calibration for biogenic aragonites. For this proof-of-concept, we analyzed shell portions grown in the wild of two randomly selected *A. trapezia* specimens for which we collected co-located SHRIMP, EPMA and IRMS data (Tables S8, S11, and S12). We chose to do this comparison using wild shell portions as those grown in aquaculture were not wide enough for micro-milling and IRMS. a) compares IRMS and SHRIMP from specimen At-28-22R (IRMS n = 7; SHRIMP n = 8) and b) from At-13-17R (IRMS n = 8; SHRIMP n = 9). Whiskers show the full data range, boxes outline the second and third quartiles, solid lines denote the median, and squares represent the means. SIMS analyses calibrated using the new calibration with two pinned geological aragonites match the IRMS data most accurately, visible as overlapping interquartile ranges, while the calcite-normalized data and calibration strategies using one geological aragonite reference material diverge more severely from the IRMS data. Test portions for IRMS range from 93 to 127 µg, while those obtained by SIMS are about 4 ng.

Figure 4. SST *versus* seawater-corrected *in-situ* oxygen isotope fractionation relationship for *A. trapezia*. This relationship shows the four temperature-controlled cultures (black circles within 95 % confidence interval, red circle outside), while black squares represent the averages of the four cultures. The x-errors are $\delta^{18}O$ 1s propagated from SIMS and seawater and y-errors show 1s for the temperature-controlled seawater. The solid black line represents a least squares regression ($R^2 = 0.995$), with the gray shaded area visualizing the confidence interval.

Figure 5. Comparison of the SST *versus* seawater-corrected oxygen isotope fractionation relationship obtained by SIMS to previously published thermometer relationships. The linear regression presented in this study (solid black line) is shown together with previous studies (dashed and/or dotted lines) for different marine calcifying organisms as well as inorganic carbonate precipitates. Aragonite data plot more to the right and calcite to the left, with inorganic calcite precipitates farthest from the regression presented in this study. Note: This study is the only regression using *in-situ* stable isotope microanalyses.

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