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The influence of temperature and seawater carbonate saturation state on ^{13}C–^{18}O bond ordering in bivalve mollusks

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Abstract. The shells of marine mollusks are widely used archives of past climate and ocean chemistry. Whilst the measurement of mollusk $\delta^{18}O$ to develop records of past climate change is a commonly used approach, it has proven challenging to develop reliable independent paleothermometers that can be used to deconvolve the contributions of temperature and fluid composition on molluscan oxygen isotope compositions. Here we investigate the temperature dependence of ^{13}C–^{18}O bond abundance, denoted by the measured parameter Δ_{47}, in shell carbonates of bivalve mollusks and assess its potential to be a useful paleothermometer. We report measurements on cultured specimens spanning a range in water temperatures of 5 to 25 °C, and field collected specimens spanning a range of −1 to 29 °C. In addition we investigate the potential influence of carbonate saturation state on bivalve stable isotope compositions by making measurements on both calcitic and aragonitic specimens that have been cultured in seawater that is either supersaturated or undersaturated with respect to aragonite. We find a robust relationship between Δ_{47} and growth temperature. We also find that the slope of a linear regression through all the Δ_{47} data for bivalves plotted against seawater temperature is significantly shallower than previously published inorganic and biogenic carbonate calibration studies produced in our laboratory and go on to discuss the possible sources of this difference. We find that changing seawater saturation state does not have significant effect on the Δ_{47} of bivalve shell carbonate in two taxa that we examined, and we do not observe significant differences between Δ_{47}-temperature relationships between calcitic and aragonitic taxa.

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1 Introduction

Molluscan carbonate was amongst the first biologically precipitated materials investigated during the development of the oxygen isotope paleotemperature scale (Epstein et al., 1953). Subsequently fossil mollusks have been widely used as an archive of past environmental change and seawater chemistry (e.g., Keith et al., 1964; Killingley and Berger, 1979; Grossman and Ku, 1986; Taviani and Zahn, 1998; Veizer et al., 1999; Tripati et al., 2001; Tripati and Zachos, 2002; Ivany et al., 2008; Wanamaker et al., 2011). However it has proven challenging to develop robust independent paleothermometers in mollusk carbonate; for example, approaches using trace element partitioning (Mg / Ca, Sr / Ca) into mollusk shell carbonate are often hampered by strong biological controls and high inter- and intra-specimen variability (e.g., Dodd, 1965; Lorens and Bender, 1980; Klein et al., 1996; Gillikin et al., 2005; Freitas et al., 2006, 2008, 2009; Heinemann et al., 2011; Wanamaker et al., 2008). Therefore it has not yet been possible to reliably partition the contributions of temperature and seawater $\delta^{18}O$ to bivalve mollusk carbonate $\delta^{18}O$ with a high level of confidence in environments where both parameters could be expected to vary.

"Clumped" isotope paleothermometry is an emerging approach for reconstructing the temperatures of carbonate mineral precipitation (Eiler, 2011). The technique is founded on the principle that rare isotopes of carbon and oxygen have a thermodynamically driven tendency to bond with each other, or "clump", and that this effect increases as temperature decreases (Wang et al., 2004; Schauble et al., 2006). In practice the abundance of ^{13}C–^{18}O bonds in carbonate minerals is measured from the abundance of mass-47 CO_2 (predominantly $^{13}C^{18}O^{16}O$) liberated on phosphoric acid digestion of carbonate minerals (Ghosh et al., 2006). Measured values are compared to a reference frame where isotope abundances from sample gases are compared to reference gases that have been heated to 1000 °C, producing a nearly random distribution of isotopes among all isotopologues (Eiler and Schauble, 2004; Affek and Eiler, 2006; Huntington et al., 2009; Passey et al., 2010). More recently, standardization to CO_2 equilibrated with water at two or more controlled temperatures has been proposed as an "absolute reference frame" in an effort to reduce interlaboratory differences due to mass spectrometric effects such as bond breaking and reordering during sample gas ionization (Dennis et al., 2011). Here we refer to data presented relative to heated gases only as "relative to the stochastic distribution" although this reference frame has also been described as the "Caltech intralab reference frame" as strictly speaking our heated gases do not reach the full stochastic distribution (Ghosh et al., 2006; Huntington et al., 2009). Data presented relative to the newly proposed reference frame is referred to as being on the "absolute reference frame" (Dennis et al., 2011). In both cases, we report data using the Δ_{47} parameter, which expresses the abundance of ^{13}C–^{18}O bonds found in a sample as an enrichment, in per mil, above that expected if isotopes were distributed randomly (Eiler and Schauble, 2004; Huntington et al., 2009).

Following the calibration of the clumped isotope thermometer in inorganically precipitated calcite (Ghosh et al., 2006) detailed calibration studies of foraminifera, coccoliths, tooth bioapatite, and corals from our laboratory have shown that these biologically precipitated materials appear to yield a relationship between Δ_{47} and temperature (Fig. 1) that is very similar to inorganic calcite (Tripati et al., 2010; Eagle et al., 2010; Thiagarajan et al., 2011). The close relationship between the inorganic calcite calibration and Δ_{47} data from foraminifera, coccoliths, and corals – even in taxa that show deviations of up to ~ 4 ‰ from the $\delta^{18}O$ values expected given the temperature and $\delta^{18}O$ of the fluid from which they precipitate – suggests either that inorganic calcite and biogenic carbonates are close to equilibrium or that all exhibit non-equilibrium effects of similar magnitude. In contrast, a study on otoliths and data from a single *Porites* coral specimen exhibit deviations from the inorganic calibration line (Ghosh et al., 2006, 2007). In the case of otoliths this could be explained by uncertainties on the precise formation temperature of the samples, as appears to be also a factor in measurements on thermocline dwelling foraminifera (Tripati et al., 2010), or due to small systematic analytical errors that were likely more common early in the history of Δ_{47} measurements. The difference between *Porites* coral and the inorganic calibration in Ghosh et al. (2006) is relatively large and could be the result of a growth rate related "vital effect" (Saenger et al., 2012).

It is unclear why some biogenic carbonates exhibit relationships between temperature and Δ_{47} that resemble the inorganic calibration of Ghosh et al. (2006) whereas other biogenic materials do not. It is possible that this difference in behavior will shed new light on the long-standing problem concerning the origin of stable isotope "vital effects" (Weiner and Dove, 2003); namely, differences in isotopic composition between biogenic materials and compositions expected for thermodynamic equilibrium with their environment. Various explanations have been advanced for vital effects on the $\delta^{18}O$ of biogenic carbonates, one invoking kinetic isotope effects associated with processes such as the hydration and hydroxylation of CO_2 in solution or crystal growth rate (e.g., McConnaughey, 1989); a second set of explanations invoke an equilibrium isotope fractionation associated with the fractionation of isotopes between species of dissolved inorganic carbon present in an organisms calcifying fluids (i.e., isotope fractionation between CO_3^{2-} and HCO_3^-), which then gets preserved in the solid phase (e.g., Spero et al., 1997; Zeebe, 1999; Adkins et al., 2003; and Tripati et al., 2010). Other models have invoked kinetic effects associated with element partitioning or isotope effects at the surface of a growing crystal, which is influenced by both crystal growth rate and dissolved inorganic carbon (DIC) speciation (Watson, 2004; Tripati et al., 2010). Preliminary predictions suggested a difference in ^{13}C–^{18}O bonding between CO_3^{2-} and HCO_3^- that

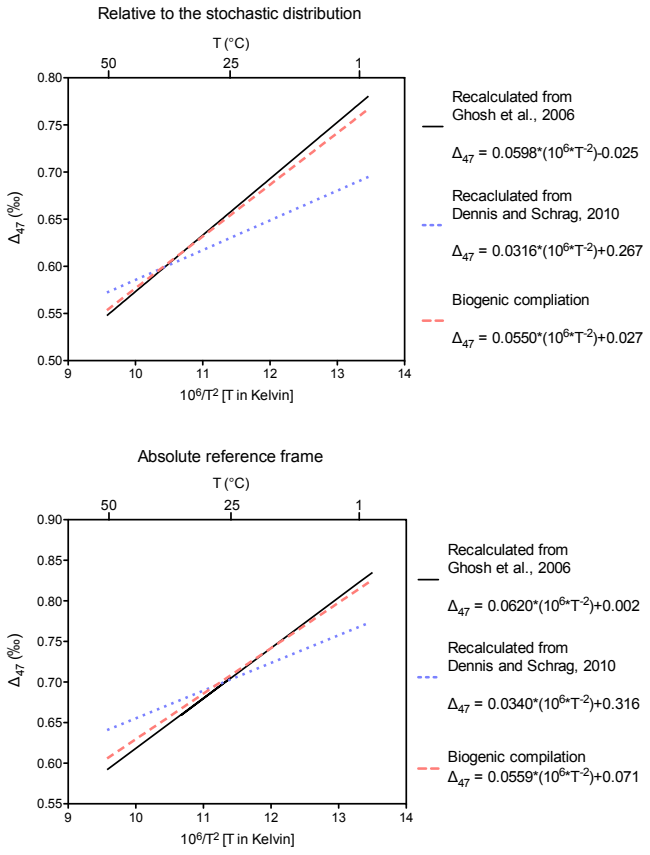

Fig. 1. Published calibrations of the carbonate clumped isotope thermometer. The top panel shows previously published inorganic calibration lines relative to the stochastic distribution, as described by Huntington et al. (2009) as well as a recalculation of the regression through the data compilation of Tripati et al. (2010) which drew on several original sources (Ghosh et al., 2006, 2007; Came et al., 2007; Eagle et al., 2010; Tripati et al., 2010); and now has the data from Thiagarajan et al., included (Thiagarajan et al., 2011). Data from Zaarur et al., was not included due to uncertainties over exactly what environmental conditions the materials analyzed should reflect (Zaarur et al., 2011), and the *Porites* coral analyzed by Ghosh et al., was also excluded due to apparent kinetic isotope effects on Δ_{47} values (Ghosh et al., 2006). Also shown is a regression through the same compilation of published materials now converted into the absolute reference frame (Table S1) via the secondary transfer function method (Dennis et al., 2011). Note that the $10^6/T^2$ scale with T in degrees Kelvin is the primary temperature scale used for data plots, with a secondary x-axis in degrees Celsius presented as a guide only. All regression lines were recalculated from original data (see methods for details).

is small and would not necessarily be measurable were it to be preserved in the solid phase (Guo et al., 2008), whereas more recent solution phase ab initio calculations predict a slightly larger effect which may potentially be measurable in carbonates precipitating from a large pH range but is still probably too small to be measured across the typical range of pH seen in the modern ocean (Hill et al., 2013).

The similarity in Δ_{47} between inorganic calcite and some biogenic carbonates (foraminifera, coccoliths and some corals) is consistent with pH effects on carbonate isotopic composition, though the effects are not necessarily required (Tripati et al., 2010; Thiagarajan et al., 2011), and suggest that any kinetic isotope effects must have negligible influence on Δ_{47} values. Conversely the discrepant Δ_{47} values of a *Porites* coral (Ghosh et al., 2006) are more consistent with a larger kinetic isotope effect and not a pH effect (Saenger et al., 2012). Here, we investigate the controls on ^{13}C–^{18}O bond abundance in the shells of bivalve mollusks, with the dual aim of providing an empirical proxy calibration for paleoclimate studies as well as giving some new perspectives on the fractionation of isotopes during carbonate biomineralization.

2 Methods

2.1 Mollusk culturing

We analyzed cultured bivalve specimens from several different laboratories. We briefly summarize the methods and materials of these culturing experiments here and refer to previous publications for more detailed descriptions of culturing conditions where appropriate.

Specimens of *Arctica islandica* were cultured at 10.3 and 15 °C at the Darling Marine Center in Walpole, Maine. Approximately 30 juvenile (~ 3 yr; shell height = ~ 40 mm) specimens of *A. islandica* were grown in muddy sediment in a temperature-controlled environment for 15 weeks. Ambient seawater (salinity = 30.4 to 30.7; Hydrolab® MiniSonde ± 0.2) from 10 m water depth was pumped into the flowing seawater labs, where the water flow was reduced (~ 6 L min^{-1}) and the water was heated or cooled to maintain the desired temperature in the 1500 L holding tank. Prior to the start of the growth experiment, individuals (ind.) were immersed and marked with a biomarker stain, calcein, according to methods outlined previously (Beirne et al., 2012). The clams were exposed to 10 °C seawater for five weeks (8 April to 12 May, 2011), then briefly removed from the growth experiment and re-marked with calcein stain. The animals were then reintroduced to the growth experiment and exposed to 15 °C seawater for 10 weeks (14 May to 21 July, 2011). The clams were only exposed to ambient food. On 21 July 2011, all animals were harvested. The soft tissues were removed and the intact valves were rinsed and air-dried. Samples were then shipped to Iowa State University. Prior to sampling the aragonitic shell material, the periostracum was physically removed with a Dremel® hand drill. Although growth marks were visible on the shell surface for each temperature treatment, sampling was further guided by the calcein stains (Beirne et al., 2012). Approximately 50 mg of $CaCO_3$ was removed from the outer shell layer of the left

valve of one shell with a Dremel® hand drill equipped with a diamond tipped bit on low speed.

5 °C cultures of *A. islandica* and *Mytilus edulis* were conducted at the Helmholtz Centre for Ocean Research Kiel (GEOMAR), Germany. Young *M. edulis* specimens were collected in Kiel Fjord (southwestern Kiel Bight) where salinity is on average 16.3 (±2.4 SD) and surface water temperatures range from 0.15 °C in winter to 23.4 °C (mean 10.48 ± 6.13 SD) in summer. *A. islandica* specimens were collected at 24 m depth at the station Süderfahrt (54°32.6′ N, 10°42.1′ E) in the central area of Kiel Bight where salinity is on average 21.8 (±2.4 SD) and temperatures vary between 0.6 and 17.5 °C (mean: 9.03 ± 4.23 SD; Bivalves were kept in temperature-insulated 4 L containers (with 10 ind. of *M. edulis*, and 7 ind. of *A. islandica* in each container) and were fed 0.5 mL ind.$^{-1}$ d^{-1} of a concentrated living-phytoplankton suspension 5 times a week (DT's Premium Blend; DT's Plankton Farm). Bivalve individuals were allowed to slowly acclimatize to the respective treatments. Temperature and salinity were kept constant for the experimental duration of 15 weeks. Salinity levels were set by admixing freshly collected Baltic Sea water with either ion-exchanged water or artificial marine salt (SEEQUASAL). The sample culturing setup is described in detail elsewhere (Hiebenthal et al., 2012). The shell material here used was grown at 5 °C and a salinity of 35. Shell sizes were measured at the beginning of the culturing phase and again prior to sampling using a caliper so that new growth could be identified. After 15 weeks of culturing, the whole soft tissue of the bivalves was removed from the shells and the shells were air-dried (7 d at 20 °C). Care was taken to remove with a Dremel® hand drill approximately 10 mg from the very outer shell layer, representing new shell growth.

M. edulis and *Pecten maximus* cultures between 10 and 20 °C were carried out at the School of Ocean Sciences, Bangor University, UK. All animals were acclimated to the laboratory environment at a temperature of ∼ 13 °C for more than two months. Animals of similar size (< 1 yr) were then moved into separate aquaria and slowly acclimated to different but constant temperatures (maximum resolution of 1 °C), constant dimmed-light conditions and controlled food conditions; the aquaria were routinely cleaned of all detritus. Animals were fed a mixed algae solution from containers with a drip tap. For the duration of the experiments, animals were kept in individual plastic mesh cages within each aquarium. Natural seawater pumped from the Menai Strait was conditioned for a few days in settling tanks, and then pumped into holding tanks and introduced as a common supply into the laboratory aquaria. Due to variable growth rates, the duration of the experiments varied with species and aquarium temperature. Because of the limited number of aquaria available, separate temperature-controlled experiments were completed. Animals from the two species can be divided into three groups: one experiment with *M. edulis* at 12, 15 and 18 °C; a second experiment with *M. edulis* and *P. maximus* at 10, 15 and 20 °C; and a third with *P. maximus* and some *M. edulis* specimens at 18 °C. Seawater temperature was monitored every 15 min in each aquarium using submerged temperature loggers. Samples for pH measurements were obtained manually every other day by immersing 20 mL plastic syringes below the surface of the seawater in all the aquaria. The samples were subsequently allowed to warm up to room temperature (20 ± 2 °C) in the dark before measurement with a commercial glass electrode (Mettler Toledo Inlab 412). The electrode was calibrated using NBS pH 6.881 and 9.225 buffers (20 °C) and was then allowed to stand until a stable reading was obtained (∼ 1 min). Shell calcite from each specimen was sampled across each growth interval along the main axis of growth, as described previously (Freitas et al., 2008).

Bivalve specimens cultured at 25 °C and at different aragonite saturation states are described in Ries et al. (2009). Specimens of *Mytilus edulis*, *Mercenaria mercenaria*, *Argopecten irradians*, *Crassostrea virginica*, and *Mya arenaria* were collected from Nantucket Sound and then transferred into aquaria at the Woods Hole Oceanographic Institution. Briefly, seawater tanks were maintained at 25 ± 1 °C and were illuminated for 10 h per day with 213 W m^{-2} illuminance. Approximately every 24 days 75 % of the seawater was changed. Air-CO_2 mixtures of 409 and 2856 ppm pCO_2 were introduced into the aquaria with 6-inch micro-porous air stones. Salinity, temperature, and pH of aquarium seawater were measured weekly, and alkalinity biweekly using methods described previously (Ries et al., 2009). Aragonite saturation state, DIC, and pCO_2 were calculated from these parameters. Bivalve shells were sampled from their outermost growth line along their main axes of growth.

2.2 Field collected samples

Specimens were collected at the locations given in Table 2. The length of bivalve mollusk growing season will vary somewhat between taxa and this presents an additional source of uncertainty in the calibration. However, in the results section below we show that the slope of our calibration line is not significantly impacted by assumptions over the predominant season of field collected bivalve growth. In the figures and tables presented here we have assumed that there is a bias in the predominant season of shell growth to the three warmest months of the year. In order to obtain seawater temperatures at the sites where specimens were collected from we used the Levitus database (Levitus and Boyer, 1994), and in the case of the specimen from San Diego data from the Scripps Pier coastal water monitoring project (http://www.nodc.noaa.gov/dsdt/cwtg/spac.html).

2.3 Cleaning protocols

To evaluate the necessity of sample cleaning, 30–50 mg of each specimen were lightly crushed and treated for 60 min at

room temperature in a 3 % H_2O_2 solution. Samples were then washed three times in excess deionized water and dried in a 50 °C oven overnight. The majority of samples in this study were not cleaned as this cleaning was not found to impact Δ_{47} values, as described below.

2.4 Stable isotope measurements

Data were collected on two Thermo Finnigan MAT 253 gas source mass spectrometers. Carbonate samples and standards were reacted on the online common acid bath system with automated sample gas purification described previously (Passey et al., 2010). Acid digestion of carbonate minerals was carried out at 90 °C. For full details of analytical methods see previous publications (Huntington et al., 2009; Passey et al., 2010). In brief, 8–10 mg of calcium carbonate samples were crushed and reacted in phosphoric acid on an automated online acid reaction system (Passey et al., 2010) where evolving CO_2 gas is immediately frozen in a liquid nitrogen trap. Sample gases are passed through a Porapak Q 120/80 mesh GC (gas chromatograph) column held at −20 °C to remove potential organic contaminants. Gases are also passed through silver wool to remove sulfur compounds. Δ_{48} values were measured and were used as empirical indicators of potential organic contamination (not shown) as has been described previously (Huntington et al., 2009).

2.5 Data processing

Δ_{47} values are defined as

$$\Delta_{47}=[(R^{47}/R^{*47}-1)-(R^{46}/R^{*46}-1)-(R^{45}/R^{*45}-1)]-1, \quad (1)$$

where R^i represents mass i/mass 44 and R^* represents isotopologues in the random (stochastic) distribution (Affek and Eiler, 2006).

As measurements were made on CO_2 liberated from carbonates by digestion with phosphoric acid heated to 90 °C they are significantly offset from previous published data on carbonates reacted at 25 °C. Passey et al. (2010) empirically determined a value of 0.08 ‰ for this offset based on measurement of carbonate standards, and previous studies have assumed this offset to be constant (Passey et al., 2010; Eagle et al., 2010; Csank et al., 2011; Finnegan et al., 2011; Suarez et al., 2011; Eagle et al., 2011). Therefore, in order to compare mollusk data to previously published data reacted at 25 °C and reported relative to the stochastic distribution a correction of 0.08 ‰ was made.

We report data using both the stochastic reference frame for Δ_{47} values (as reported in previous studies such as Ghosh et al., 2006) and the "absolute reference frame" of Dennis et al. (2011), which assumes a certain value for the difference between heated gases and CO_2 gas standards equilibrated at other temperatures. As the majority of data here was collected before the proposition of the absolute reference frame, we convert Δ_{47} values to this reference frame using carbonate standards that were analyzed over the analytical time period. Accepted Δ_{47} values for Carrara marble and 102-GC-AZ01 on the absolute reference frame determined in our laboratory are 0.392 ‰ and 0.724 ‰ respectively (Dennis et al., 2011) and these were used to construct an empirical transfer function to generate Δ_{47} values on the absolute reference frame, as described previously (Dennis et al., 2011). For the conversion of the compiled published biogenic data (Tripati et al., 2010; Thiagarajan et al., 2011) and inorganic data to the absolute reference frame we also used the secondary transfer function approach, using standard values given in each publication, or where no standard data was given a Carrara marble or NBS-19 value of 0.392 ‰ was used (Dennis et al., 2011). All published data (Ghosh et al., 2006, 2007; Came et al., 2007; Eagle et al., 2010; Tripati et al., 2010; Thiagarajan et al., 2011) and new bivalve data converted to the absolute reference frame is given in Tables 3 and S1, which include the standard values and the slope and intercepts that were used in the transfer function used to convert from the "stochastic reference frame" to the absolute reference frame.

A carbonate standard was analyzed for every 5–6 samples of unknown isotopic composition. During the analytical period 44 analyses of Carrara marble yielded a $\delta^{13}C$ value of 2.3 ‰ (V-PDB, Vienna Pee Dee Belemnite), $\delta^{18}O$ of −2.0 ‰ (V-PDB), and Δ_{47} of 0.349 ± 0.006 (1 standard error, s.e., relative to the stochastic distribution). Twenty analyses of the standard Carmel chalk yielded a $\delta^{13}C$ value of −2.1 ‰, a $\delta^{18}O$ of −4.2 ‰, and Δ_{47} of 0.636 ± 0.005 ‰. Twelve analyses of the standard 102-GC-AZ01 yielded a $\delta^{13}C$ value of 0.5 ‰, a $\delta^{18}O$ of −13.1 ‰, and Δ_{47} of 0.656 ± 0.006 ‰. Fifteen analyses of the standard TV01 yielded a $\delta^{13}C$ value of 0.1 ‰, a $\delta^{18}O$ of −8.6 ‰, and Δ_{47} of 0.653 ± 0.009 ‰ .

For aragonite $\delta^{18}O$ calculations an acid digestion fractionation factor of 1.00854126 was used, calculated by extrapolation from a published calibration (Guo et al., 2009; Kim et al., 2007). For calcite a value of 1.00821000 was used (Swart et al., 1991).

3 Results

3.1 The effect of sample cleaning on stable isotope measurements from bivalve shell carbonate

Bivalves calcify onto a protein matrix (Addadi et al., 2006), which results in the interlocking of organic material and carbonate shell. Organic contamination has the potential to provide isobaric interferences with mass-47 CO_2 measurements, and so we investigated the effect of oxidative sample cleaning on measured Δ_{47} values using a treatment of 30 min in 3 % H_2O_2. We found that cleaning did not impact measured Δ_{47} in several samples analyzed (Table 1), and so we conclude that the automated sample reaction and cleaning apparatus described in Passey et al. (2010) is sufficient to remove the levels of volatile organic contaminants generally

produced from reaction of bivalve shell carbonate in phosphoric acid (Passey et al., 2010). It is also possible that the majority of the organic matter present in mollusk shell is refractory. This is a different result than seen in biogenic phosphate minerals where sample cleaning does seem to be necessary for accurate measurements (Eagle et al., 2010). This indicates either that phosphates tend to have higher levels of contaminants that provide isobars for Δ_{47} measurements or that the larger sample size reacted to produce CO_2 from phosphate minerals tends to lead to higher levels of contaminants or incomplete reactions of uncleaned samples. Therefore in the remaining analysis presented here we did not perform any sample cleaning.

3.2 The relationship between temperature and Δ_{47} values in bivalve mollusks

An initial study of the temperature effects on Δ_{47} values in modern bivalve mollusks examined three samples (Came et al., 2007). Here we greatly expand the number of specimens measured as well as the range of temperatures encompassed by the calibration.

We present data both relative to the stochastic reference frame (to aid comparison with previously published data) and in the recently proposed absolute reference frame (Tables 1–6 and S1). The most direct analysis of our data (i.e. involving a minimum of calculations) is the empirical correlation between known growth temperature and Δ_{47} value of bivalve carbonate relative to the stochastic reference frame, using a 90 °C phosphoric acid digestion reaction (Fig. 2; Table 3). This is the temperature that is now standardly used on our automated online sample reaction and gas purification systems (Passey et al., 2010). We then applied the empirically determined acid digestion correction of 0.08 ‰ to derive data relative to the stochastic distribution that could be compared to previously published data collected on CO_2 produced by digesting carbonates in phosphoric acid at 25 °C (Fig. 2). Linear regressions through each dataset are presented in Fig. 2, and are tabulated with calculated uncertainties and alongside previously published regressions in Table 4.

Individual bivalve samples generally conform reasonably well to the temperature relationship defined by the total population of bivalve data. However a small number of samples, for example the specimen of *Zygochlamys patagonica*, show a significant departure from this relationship (i.e. fall outside the 95 % confidence intervals of the linear regression; Fig. 2). This appears to represent a unique property of the sample (possibly a "vital effect") on Δ_{47} rather than an imprecise measurement as the result is confirmed by analysis of CO_2 extracted from this specimen 6 times (Table 2). The Levitus atlas of ocean temperatures also calls for a minor difference in mean annual temperature (~ 8 °C) versus warm summer month (~ 9 °C) temperature at the location and water depth on the Patagonian shelf where this sample was recovered from. Therefore if the database is correct, then

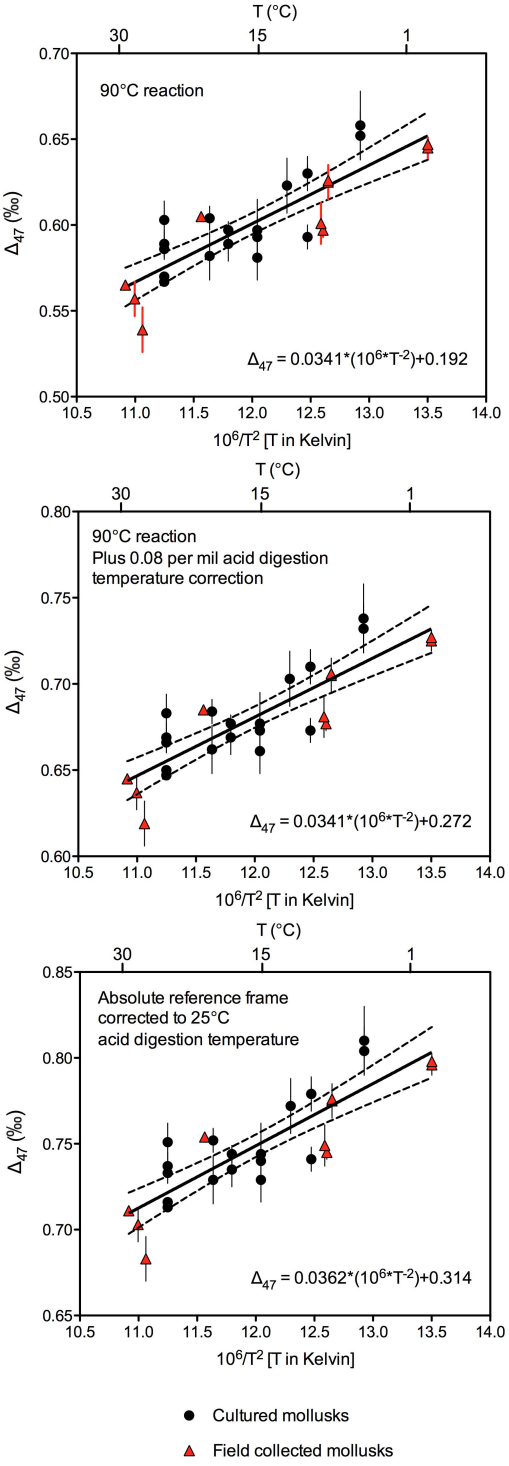

Fig. 2. Bivalve Δ_{47} calibration data. The top panel shows a linear regression with 95 % confidence intervals through Δ_{47} measurements made on both cultured (circles) and field collected (triangles) mollusks grown at different temperatures. Shells were reacted with phosphoric acid heated to 90 °C to produce analyte CO_2. These data are relative to the stochastic distribution as described previously (Huntington et al., 2009) and do not have the empirically derived acid digestion correction of 0.08 ‰ added (Passey et al., 2010), which is used to compare data to that derived from a 25 °C acid digestion reaction. The middle panel is the data with this correction. The bottom panel is bivalve calibration data with the acid digestion correction, then converted into the absolute reference frame (Dennis et al., 2011) using a secondary transfer function. Equations for the relationship between measured Δ_{47} and bivalve growth temperature are given in each case.

Table 1. Effect of oxidative sample cleaning on mollusk stable isotope data.

Taxa	Sample ID	Growth Temperature[1] (°C)	Sample Treatment	Mineralogy[2]	Total Number of Analyses[3]	$\delta^{13}C$ ‰ V-PDB	$\delta^{18}O$ ‰ V-PDB	Δ_{47} ‰ (SD)[4]	Δ_{47} ‰ (ARF)[5]
Crassostrea virginica	JR-126	25	None	C	6	−0.5	−1.7	0.650 ± 0.005	0.716 ± 0.005
Crassostrea virginica	JR-126	25	3 % H_2O_2	C	6	−0.4	−1.2	0.651 ± 0.012	0.716 ± 0.012
Mya arenaria	JR-131	25	None	A ≫ C	3	−1.0	−3.3	0.648 ± 0.005	0.714 ± 0.005
Mya arenaria	JR-131	25	3 % H_2O_2	A ≫ C	3	−1.0	−3.3	0.644 ± 0.002	0.709 ± 0.002

[1] Cultured specimen growth temperature is accurate to within 0.5 °C on average (see methods). For field collected specimens temperatures correspond to average temperatures for the three warmest months (assumed to be the predominant growing season), it is assumed that there is a 1 °C error in growth temperatures on average. Ocean temperatures determined from the Levitus database. All temperatures are rounded to the nearest integer.
[2] C, calcite; A, aragonite. ≫ refers to a mixed mineralogy with one mineral predominating. For the purpose of isotope calculations the dominant mineralogy is used.
[3] Represents the number of distinct extractions of CO_2 from a sample, which are then purified and analyzed.
[4] Relative to the stochastic distribution. Also referred to as data in the Caltech intralaboratory reference frame. Includes the acid digestion correction of 0.08. ±. Values are 1 s.e.
[5] Values given on the absolute reference frame.

incorrect attribution of the season of growth to the summer months in Fig. 2 does not seem a likely explanation (Levitus and Boyer, 1994). Additional work on specific taxa will be needed to confirm this observation. Amongst the most significant departures from previous calibration lines are those from both calcitic and aragonitic specimens forming in the coldest environments, near-freezing shallow marine waters of the Ross Sea off Antarctica that do not reach temperatures significantly above 0 °C all year.

The R^2 value of our bivalve mollusk calibration line is 0.7258 (Table 4) using data on the absolute reference frame, and the standard deviation of the residuals (SDR) is 0.017 ‰. This suggests that there is somewhat larger variability in bivalve Δ_{47} data compared to other biogenic calibration datasets. For example the linear regression through the foraminifera calibration of Tripati et al. (2010) has an R^2 value of 0.8998 and a SDR of 0.014 ‰, and for the study of corals by Thiagarajan et al. (2011) the R^2 value is 0.8703 with a SDR of 0.015 ‰ (Tripati et al., 2010; Thiagarajan et al., 2011). It is possible that this reflects very subtle biological or mineralogical effects on bivalve Δ_{47} data, although, as we describe below, we cannot resolve these effects in our dataset.

In the case of field collected bivalves in the figures and regression analysis presented we assumed that preferential growth occurred in the three warmest summer months. However we accept that many taxa do also grow at other times of the year and so in order to assess the impact of our assumption on the resulting regression lines through Δ_{47} versus temperature data we also created a regression line using mean annual water temperatures (data not shown) for field collected specimens. The slope of a linear regression line through all bivalve data including field collected specimens assumed to reflect mean annual temperature (rather than warm month average temperatures as in figures and tables) is 0.0350 on the absolute reference frame. This compares to a slope of 0.0362 assuming the warm month average temperature is the predominant growing season for field collected bivalve shells (Table 4). These slopes are not significantly different in an analysis of covariance (ANCOVA) test ($p = 0.68$). Therefore we conclude that our assumptions over the predominant growing season for bivalve mollusks do not significantly impact the slope of the linear regression lines presented here.

3.3 Comparison of bivalve Δ_{47} calibration with other theoretical and empirical calibrations

A linear regression through the plot of 1/T versus Δ_{47} values for our measurements from bivalves produces a significantly shallower slope than a regression through previously published calibration materials analyzed in our laboratory (Fig. 3). Previous publications did not use the same software or approaches for calculating linear regressions (e.g., Ghosh et al., 2006; and Huntington et al., 2009). Therefore in order to compare regressions precisely, as in Figs. 3 and 4, we recalculate all linear regressions using GraphPad Prism software (Zar, 1984) and it is these values that are presented in Table 4. In practice however these different methods do not yield slopes and intercepts that are markedly different; for example the linear regression presented by Ghosh et al. (2006) yielded a slope of 0.592, whereas using the software utilized here we yield a slope of 0.598. Linear regressions presented here do not take into account errors in carbonate formation temperatures or isotope measurements; in this dataset these tend to be quite similar on average and do not significantly impact the slope of the regression (data not shown).

The slopes of the bivalve calibration regression and the Ghosh et al. (2006) inorganic calcite regression are significantly different (Table 5). Additionally, the bivalve mollusk calibration is shown to be significantly different than a compilation of published biogenic data from our laboratory (Table 5). The slopes of the bivalve calibration regression and the inorganic calibration regression of Dennis and Schrag (2010) are not significantly different (Table 5). However, the intercepts of the Dennis and Schrag regression and our bivalve data are significantly different ($p = 0.0012$). Thus, even though the slopes of these calibrations are statistically

Table 2. Average stable isotope data for all mollusk samples grown at seawater in equilibrium with present day pCO_2.

Taxa	Growth Temperature[1] (°C)	Location	Mineralogy[2]	Number Individuals Analysed	Total Number of Analyses[3]	Δ_{47} ‰ (SD)[4]	Δ_{47} ‰ (ARF)[5]
Cultured Specimens							
Arctica islandica	5	Kiel	A	4	4	0.738 ± 0.020	0.810 ± 0.020
Arctica islandica	10	Iowa State	A	1	2	0.673 ± 0.007	0.741 ± 0.007
Arctica islandica	15	Iowa State	A	1	3	0.661 ± 0.013	0.729 ± 0.013
Mytilus edulis	5	Kiel	C > A	3	3	0.732 ± 0.014	0.804 ± 0.014
Mytilus edulis	10	Bangor	C	3	3	0.710 ± 0.010	0.779 ± 0.010
Mytilus edulis	12	Bangor	C	4	4	0.703 ± 0.016	0.772 ± 0.016
Mytilus edulis	15	Bangor	C	4	4	0.677 ± 0.018	0.744 ± 0.018
Mytilus edulis	18	Bangor	C	3	4	0.677 ± 0.005	0.744 ± 0.005
Mytilus edulis	20	Bangor	C	4	4	0.662 ± 0.014	0.729 ± 0.014
Mytilus edulis	25	Woods Hole	C > A	2	2	0.683 ± 0.010	0.751 ± 0.011
Pecten maximus	10	Bangor	C	2	2	0.710 ± 0.003	0.779 ± 0.003
Pecten maximus	15	Bangor	C	4	5	0.673 ± 0.006	0.740 ± 0.006
Pecten maximus	18	Bangor	C	3	3	0.669 ± 0.006	0.735 ± 0.006
Pecten maximus	20	Bangor	C	3	3	0.684 ± 0.004	0.752 ± 0.004
Argopecten irradians	25	Woods Hole	C	2	8	0.670 ± 0.000	0.730 ± 0.000
Mercenaria mercenaria	25	Woods Hole	A ≫ C	2	10	0.664 ± 0.007	0.733 ± 0.006
Mya arenaria	25	Woods Hole	A ≫ C	2	7	0.649 ± 0.001	0.713 ± 0.002
Crassostrea virginica	25	Woods Hole	C	1	6	0.650 ± 0.000	0.716 ± 0.000
Field Collected Specimens							
Laternula elliptica	−1	Ross Sea, Antarctica	A	3	11	0.725 ± 0.006	0.796 ± 0.006
Adamussium colbecki	−1	Ross Sea, Antarctica	C	2	6	0.727 ± 0.001	0.798 ± 0.002
Mytilus sp.	8	Ushuaia, Argentina	C > A	2	9	0.705 ± 0.009	0.775 ± 0.010
Mytilus sp.	8	Seno Otway, Chile	C > A	2	7	0.706 ± 0.002	0.776 ± 0.002
Arctica islandica	9	Flatey, Iceland	A	2	10	0.677 ± 0.004	0.745 ± 0.004
Zygochlamys patagonica	9	Patagonian shelf	C	1	6	0.681 ± 0.012	0.749 ± 0.012
Mytilus californianus	21	Scripps Pier, USA	C > A	2	2	0.685 ± 0.002	0.754 ± 0.002
Tridacna gigas	28	Great Barrier Reef	A	1	3	0.619 ± 0.013	0.683 ± 0.013
Tridacna gigas	28	Cocos Islands	A	1	3	0.637 ± 0.010	0.703 ± 0.010
Tridacna gigas	29	Papua New Guinea	A	1	5	0.645 ± 0.002	0.711 ± 0.002

[1] Cultured specimen growth temperature is accurate to within 0.5 °C on average (see methods). For field collected specimens temperatures correspond to average temperatures for the three warmest months (assumed to be the predominant growing season), it is assumed that there is a 1 °C error in growth temperatures on average. Ocean temperatures determined from the Levitus database. All temperatures are rounded to the nearest integer.
[2] C, calcite; A, aragonite. ≫ refers to a mixed mineralogy with one mineral predominating. For the purpose of isotope calculations the dominant mineralogy is used.
[3] Represents the number of distinct extractions of CO_2 from all samples, which are then purified and analyzed.
[4] Relative to the stochastic distribution. Also referred to as data in the Caltech intralaboratory reference frame. Includes the acid digestion correction of 0.08. ±. Values are 1 s.e.
[5] Values given on the absolute reference frame.

indistinguishable, there could be an offset in the absolute values of the two. We also note that the apparently higher variability in the bivalve mollusk dataset compared to other biogenic calibration datasets is taken into account by the statistical analysis of slopes presented in Table 5 and so this variability itself cannot explain the statistically significant differences in slopes we observe.

In order to consider whether the slope of the bivalve linear regression could be significantly effected by a few anomalous datapoints we tested the effect of excluding the five specimens recovered from the coldest temperatures from Antarctica (*Laternula elliptica* and *Adamussium colbecki)* that are also amongst the most different from the calibration line of Ghosh et al. (2006), yielding Δ_{47} values of 0.72–0.74 ‰ relative to the stochastic distribution (Table 3) compared value of 0.80 ‰, which is predicted for carbonates growing at −1 °C if they conformed to the calibration of Ghosh et al. (2006). One possibility is that cold environments favor the expression of kinetic isotope effects on the ^{13}C–^{18}O bond abundance in carbonates, as, for example, the rate of reaction for the hydration of CO_2 in solution decreases significantly between 25 and 0 °C and is a potential source of disequilibrium isotope effects in the dissolved inorganic carbon pool from which carbonate forms (Johnson, 1982; Zeebe, 2009). In order to assess potential bias from these datapoints on regression lines we recalculated the linear regression through our dataset excluding taxa that grow in coldest environments and give potentially anomalous Δ_{47} values. Exclusion of the

Table 3. Stable isotope data for individual mollusk specimens grown at ambient carbonate saturation state and with no cleaning.

Taxa	Sample ID	Growth Temperature[1] (°C)	Location	Mineralogy[2]	Total Number of Analyses[3]	$\delta^{13}C$ ‰ V-PDB	$\delta^{18}O$ ‰ V-PDB	Δ_{47} ‰ (SD)[4]	Δ_{47} ‰ (ARF)[5]
Cultured Specimens									
Arctica islandica	A 5 35/2	5	Kiel	A	1	−1.6	−0.4	0.767 ± 0.009	0.840 ± 0.009
Arctica islandica	A 5 35/1	5	Kiel	A	1	−1.6	−0.3	0.776 ± 0.005	0.849 ± 0.005
Arctica islandica	A 5 35/4	5	Kiel	A	1	−1.8	−0.5	0.690 ± 0.009	0.759 ± 0.009
Arctica islandica	A 5 35/3	5	Kiel	A	1	−2.6	−0.4	0.721 ± 0.013	0.792 ± 0.013
Arctica islandica	AI-10.3	10	Iowa State	A	2	2.2	−1.3	0.673 ± 0.007	0.741 ± 0.007
Arctica islandica	AI-15	15	Iowa State	A	3	2.3	−1.2	0.661 ± 0.013	0.729 ± 0.013
Mytilus edulis	M 5 35/1	5	Kiel	C > A	1	−2.8	−0.4	0.715 ± 0.011	0.786 ± 0.011
Mytilus edulis	M 5 35/3	5	Kiel	C > A	1	−3.2	−0.4	0.720 ± 0.017	0.792 ± 0.017
Mytilus edulis	M 5 35/2 + 35/3	5	Kiel	C > A	1	-3.5	−0.3	0.760 ± 0.014	0.834 ± 0.014
Mytilus edulis	E2 T10 A3	10	Bangor	C	1	−1.0	1.7	0.730 ± 0.009	0.800 ± 0.009
Mytilus edulis	E2 T10 B2	10	Bangor	C	1	−1.3	1.2	0.696 ± 0.011	0.765 ± 0.011
Mytilus edulis	E2 T10 F2	10	Bangor	C	1	−1.4	1.2	0.704 ± 0.014	0.773 ± 0.014
Mytilus edulis	E1 T12 C2	12	Bangor	C	1	−0.1	1.0	0.748 ± 0.015	0.819 ± 0.015
Mytilus edulis	E2 T12 A3	12	Bangor	C	1	−0.3	0.7	0.695 ± 0.007	0.763 ± 0.007
Mytilus edulis	E1 T12 A2	12	Bangor	C	1	−0.1	1.0	0.694 ± 0.011	0.762 ± 0.011
Mytilus edulis	E1 T12 F4	12	Bangor	C	1	−0.1	1.5	0.676 ± 0.009	0.743 ± 0.009
Mytilus edulis	E2 T15 B1	15	Bangor	C	1	−1.1	0.1	0.686 ± 0.008	0.754 ± 0.008
Mytilus edulis	E1 T15 F1	15	Bangor	C	1	−1.0	0.1	0.652 ± 0.010	0.718 ± 0.010
Mytilus edulis	E1 T15 A3	15	Bangor	C	1	−1.2	0.1	0.647 ± 0.013	0.712 ± 0.013
Mytilus edulis	E2 T15 E4	15	Bangor	C	1	−0.8	0.3	0.724 ± 0.009	0.794 ± 0.009
Mytilus edulis	E1 T18 E4	18	Bangor	C	1	−1.1	−0.2	0.689 ± 0.013	0.757 ± 0.013
Mytilus edulis	E1 T18 A1	18	Bangor	C	1	−0.9	−0.4	0.673 ± 0.008	0.740 ± 0.008
Mytilus edulis	E3 T18 A4	18	Bangor	C	2	−0.8	−0.2	0.669 ± 0.004	0.735 ± 0.004
Mytilus edulis	E2 T20 D3	20	Bangor	C	1	−0.8	−0.7	0.671 ± 0.009	0.738 ± 0.009
Mytilus edulis	E2 T20 C1	20	Bangor	C	1	−0.8	−0.8	0.674 ± 0.008	0.741 ± 0.008
Mytilus edulis	E2 T20 A4	20	Bangor	C	1	−0.1	−1.2	0.683 ± 0.012	0.751 ± 0.012
Mytilus edulis	E2 T20 A2	20	Bangor	C	1	−0.8	−0.5	0.621 ± 0.016	0.685 ± 0.016
Mytilus edulis	JR-107	25	Woods Hole	C > A	1	−0.5	−1.2	0.693 ± 0.006	0.761 ± 0.006
Mytilus edulis	JR-108	25	Woods Hole	C > A	1	−2.9	−2.4	0.673 ± 0.013	0.740 ± 0.013
Pecten maximus	E2 T10 P6	10	Bangor	C	1	0.9	1.8	0.713 ± 0.008	0.782 ± 0.008
Pecten maximus	E2 T10 P4	10	Bangor	C	1	0.9	1.4	0.706 ± 0.008	0.775 ± 0.008
Pecten maximus	E2 T15 P7	15	Bangor	C	1	0.5	0.3	0.681 ± 0.008	0.749 ± 0.008
Pecten maximus	E2 T15 P10	15	Bangor	C	1	0.6	0.3	0.683 ± 0.007	0.751 ± 0.007
Pecten maximus	E2 T15 P8	15	Bangor	C	1	0.5	0.4	0.657 ± 0.012	0.723 ± 0.012
Pecten maximus	E2 T15 P3	15	Bangor	C	2	0.5	0.4	0.670 ± 0.013	0.737 ± 0.013
Pecten maximus	E2 T18 P2	18	Bangor	C	1	0.4	−0.1	0.680 ± 0.008	0.747 ± 0.008
Pecten maximus	E2 T18 P7	18	Bangor	C	1	0.2	−0.2	0.666 ± 0.007	0.733 ± 0.007
Pecten maximus	E2 T18 P5	18	Bangor	C	1	0.3	−0.4	0.660 ± 0.009	0.726 ± 0.009
Pecten maximus	E2 T20 P2	20	Bangor	C	1	0.4	−0.7	0.679 ± 0.006	0.746 ± 0.006
Pecten maximus	E2 T20 P3	20	Bangor	C	1	0.3	−1.0	0.681 ± 0.009	0.749 ± 0.009
Pecten maximus	E2 T20 P9	20	Bangor	C	1	0.5	−0.4	0.692 ± 0.008	0.760 ± 0.008
Argopecten irradians	JR-113	25	Woods Hole	C	4	−2.6	−2.0	0.677 ± 0.011	0.728 ± 0.003
Argopecten irradians	JR-114	25	Woods Hole	C	4	−1.7	−1.6	0.661 ± 0.003	0.745 ± 0.011
Mercenaria mercenaria	JR-119	25	Woods Hole	A ≫ C	6	−1.3	−1.4	0.671 ± 0.009	0.739 ± 0.009
Mercenaria mercenaria	JR-120	25	Woods Hole	A ≫ C	4	0.0	−2.3	0.660 ± 0.006	0.727 ± 0.006
Mya arenaria	JR-131	25	Woods Hole	A ≫ C	3	−1.0	−2.9	0.648 ± 0.005	0.714 ± 0.005
Mya arenaria	JR-132	25	Woods Hole	A ≫ C	4	−0.8	−2.5	0.650 ± 0.008	0.716 ± 0.008
Crassostrea virginica	JR-126	25	Woods Hole	C	6	−0.5	−1.7	0.650 ± 0.005	0.715 ± 0.005
Field Collected Specimens									
Laternula elliptica	LE #1	−1	Ross Sea, Antarctica	A	4	1.3	4.4	0.721 ± 0.006	0.791 ± 0.006
Laternula elliptica	LE #2	−1	Ross Sea, Antarctica	A	3	1.4	4.4	0.718 ± 0.021	0.789 ± 0.021
Laternula elliptica	LE #3	−1	Ross Sea, Antarctica	A	4	1.3	4.5	0.736 ± 0.016	0.808 ± 0.016
Adamussium colbecki	AC #1	−1	Ross Sea, Antarctica	C	4	1.7	4.4	0.726 ± 0.004	0.796 ± 0.004
Adamussium colbecki	AC #2	−1	Ross Sea, Antarctica	C	2	1.9	4.1	0.727 ± 0.005	0.799 ± 0.005
Mytilus sp.	MTM #1	8	Ushuaia, Argentina	C > A	5	1.3	0.7	0.696 ± 0.004	0.765 ± 0.004
Mytilus sp.	MTM #2	8	Ushuaia, Argentina	C > A	4	−1.2	0.4	0.714 ± 0.003	0.785 ± 0.003
Mytilus sp.	MTM #3	8	Seno Otway, Chile	C > A	3	0.0	−0.3	0.708 ± 0.028	0.778 ± 0.028
Mytilus sp.	MTM #4	8	Seno Otway, Chile	C > A	4	1.6	0.3	0.704 ± 0.007	0.774 ± 0.007
Arctica islandica	AI-060967	9	Flatey, Iceland	A	3	1.4	3.5	0.681 ± 0.010	0.754 ± 0.002
Arctica islandica	AI-060971	9	Flatey, Iceland	A	7	1.9	3.1	0.674 ± 0.004	0.741 ± 0.004
Zygochlamys patagonica	Zygochlamys	9	Patagonian shelf	C	6	1.9	2.2	0.681 ± 0.012	0.749 ± 0.012
Mytilus californianus	KN-9	21	Scripps Pier, USA	C > A	1	0.6	−0.7	0.687 ± 0.016	0.756 ± 0.016
Mytilus californianus	KN-10	21	Scripps Pier, USA	C > A	1	0.5	−0.3	0.683 ± 0.017	0.752 ± 0.017
Tridacna gigas	TG GBR	28	Great Barrier Reef	A	3	2.4	−1.1	0.637 ± 0.010	0.683 ± 0.013
Tridacna gigas	TG Cocos	28	Cocos Islands	A	3	2.0	−1.4	0.619 ± 0.013	0.703 ± 0.010
Tridacna gigas	MT7	29	Papua New Guinea	A	5	2.0	−1.4	0.645 ± 0.002	0.711 ± 0.002

[1] Cultured specimen growth temperature is accurate to within 0.5 °C on average (see methods). For field collected specimens temperatures correspond to average temperatures for the three warmest months (assumed to be the predominant growing season), it is assumed that there is a 1 °C error in growth temperatures on average. Ocean temperatures determined from the Levitus database. All temperatures are rounded to the nearest integer.

[2] C, calcite; A, aragonite. ≫ refers to a mixed mineralogy with one mineral predominating. For the purpose of isotope calculations the dominant mineralogy is used.

[3] Represents the number of distinct extractions of CO_2 from a sample, which are then purified and analyzed.

[4] Relative to the stochastic distribution. Also referred to as data in the Caltech intralaboratory reference frame. Includes the acid digestion correction of 0.08. ±. Values are 1 s.e.

[5] Values given on the absolute reference frame.

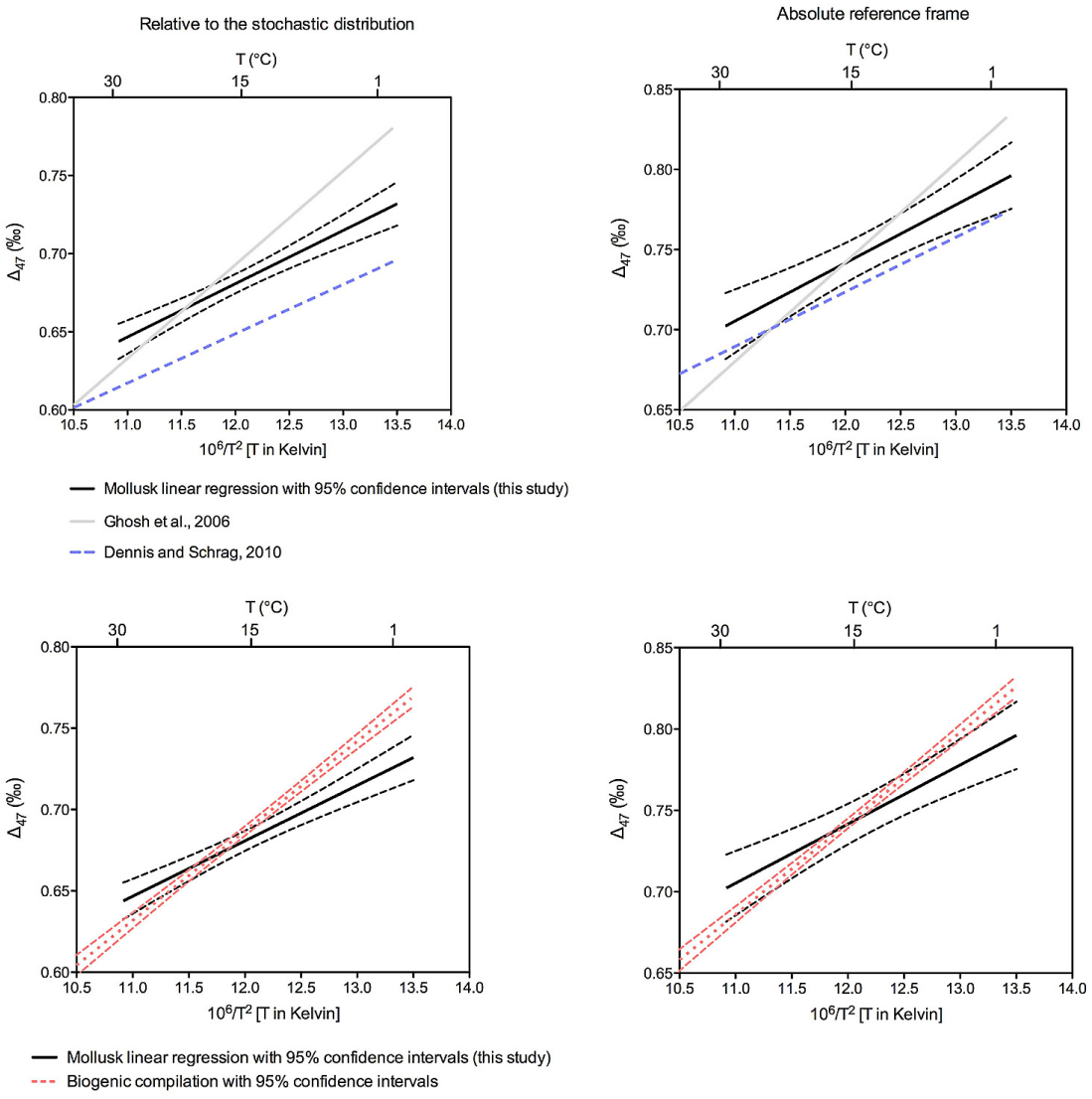

Fig. 3. Comparison of bivalve Δ_{47} measurements to previously published calibration data. Here we compare the linear regressions through our mollusk data shown in Fig. 2 to published calibration lines, relative to both the stochastic distribution (left panels) and the absolute reference frame (right panels). In all cases a correction of 0.08 ‰ was made to compare mollusk data to older data collected in our laboratory using 25 °C acid digestion reactions. Mollusk calibration lines have a clearly shallower slope than the inorganic calcite calibration line of Ghosh et al. (2006) and have a similar slope to the calibration of Dennis and Schrag, but with a slight offset to that calibration (Dennis and Schrag, 2010). The mollusk calibration line is also significantly shallower than the linear regression through the compilation of other published materials from our laboratory (bottom panels), with previously published data plotted in this graph given in Table S1.

specimens from Antarctica from the mollusk dataset does yield a steeper slope (Table 4) of 0.0402 ± 0.0050 (1 s.e.) on the absolute reference frame, however it does not change the results of our statistical analysis (Table 5) showing that the bivalve mollusk calibration dataset is has a significantly different slope to the previously published biogenic compilation produced in our laboratory and the inorganic calcite calibration of Ghosh et al. (2006).

Whilst it is useful to examine the effect of excluding these samples on the regression line, it is also important to note that at present we do not have a good reason to exclude these Antarctic specimens from the regression analysis in this way. There is some rationale for supposing that carbonates that form at low temperatures could be more prone to record kinetic isotope effects, as described above, however previously published studies on *L. elliptica* and *A. colbecki* from Antarctica report that their measured $\delta^{18}O$ are close to their expected equilibrium values (Barrera et al., 1994, 1990). Whilst we cannot rule out disequilibrium effects in Δ_{47} that do not manifest as significant disequilibrium effects on $\delta^{18}O$, this is perhaps unlikely. Therefore at present we

regard the regression line through all our mollusk data as the most robust calibration.

3.4 Calcite versus aragonite

Theoretical calculations predict that there would be an offset between Δ_{47} values derived from calcite compared to aragonite (Schauble et al., 2006; Guo et al., 2009). However measurements from foraminifera and corals have not resolved any mineralogical effect (Tripati et al., 2010; Thiagarajan et al., 2011). In our mollusk dataset there is a slight offset between the slopes of regression lines between calcitic and aragonitic mollusks (Fig. 4), however the offset is in the opposite direction to that predicted from theory (Schauble et al., 2006; Guo et al., 2009). The slopes of linear regressions through the temperature-Δ_{47} data for calcitic and aragonitic taxa (Fig. 4) were not significantly different ($p = 0.520$). If a difference between calcitic and aragonitic mollusks exists, then it is not easily resolvable. In some cases bivalves that precipitate shells with mixed mineralogy were selectively sampled to only acquire the calcite phase, such as the *M. edulis* specimens grown at Bangor University (Freitas et al., 2008). However, in other cases this distinction was not made and both mineralogies were sampled, as detailed in Table 4. For the calcite versus aragonite comparison samples with mixed mineralogy were excluded. When comparing the regression lines through the aragonite data to other calibrations (Table 5) it is worth noting that there does not appear to be enough data to statistically determine which of the two different inorganic calcite calibration lines (Ghosh et al., 2006; Dennis and Schrag, 2010) the aragonitic mollusk data fits best with. Therefore it remains possible that the lack of a mineralogical difference in our study could be further resolved in the future with larger datasets.

3.5 The influence of seawater carbonate saturation state on bivalve stable isotopes

In a number of biogenic carbonates it has been suggested that changes in solution pH can influence carbonate $\delta^{18}O$ (Spero et al., 1997; Rollion-Bard et al., 2003; Adkins et al., 2003). The effect of changing solution pH and carbonate chemistry on ^{13}C–^{18}O bond abundance in carbonate minerals has not been explicitly investigated. Here we analyzed specimens of *Mya arenaria*, and *Argopecten irradians* that were cultured at 25 °C and with CO_2 bubbled into the aquarium at either 409 ppm or 2856 ppm producing seawater that was either supersaturated or undersaturated with respect to aragonite (Ries et al., 2009). *M. arenaria* predominantly precipitates aragonite, whilst *A. irradians* precipitates low-Mg calcite. Both species showed a reduction in calcification in undersaturated seawater, but care was taken to only sample new growth in each case (Ries et al., 2009). In both cases no significant effects on $\delta^{18}O$ and Δ_{47} values were observed in carbonate that was formed by specimens cultured in seawater undersaturated with respect to aragonite (Table 6).

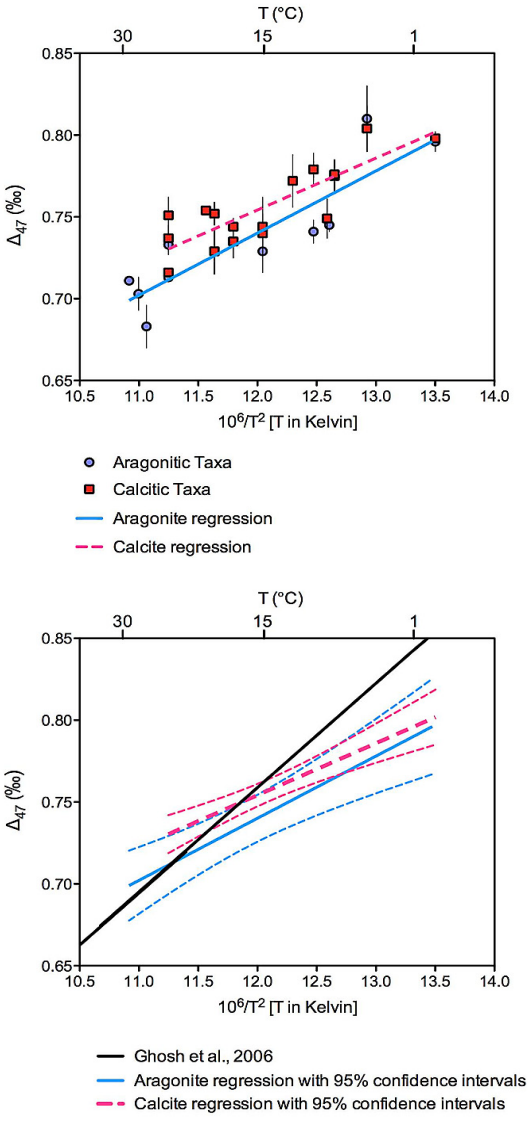

Fig. 4. Comparison of bivalve Δ_{47} data derived from calcitic and aragonitic taxa. The top panel shows data split between calcitic (squares) and aragonitic (circles) mollusks, with a linear regression through each. Here cultured and field collected samples are not distinguished in the figure. The bottom panel shows linear regressions with 95 % confidence intervals. There is an offset between the regressions between calcite and aragonite, but it is not statistically significant.

4 Discussion

The data presented here reaffirms the potential of Δ_{47} measurements to provide independent constraints on mineral formation temperatures and provides an empirical calibration that can be applied to paleoclimate studies using bivalve

Table 4. Slopes and intercepts of linear regressions through Δ_{47} and temperature data for samples with known growth temperatures.

	Relative to the stochastic distribution					Absolute reference frame				
Dataset	Slope[e]	1 s.e.	Intercept	1 s.e.	R^2	Slope[e]	1 s.e.	Intercept	1 s.e.	R^2
Inorganic calcite[a] Ghosh et al. (2006)	0.0598	0.0094	−0.0248	0.1046	0.8896	0.0620	0.0099	0.0021	0.1095	0.8877
Inorganic calcite[a] Dennis and Schrag (2010)	0.0316	0.0036	0.2697	0.0382	0.8587	0.0340	0.0038	0.3155	0.0408	0.8600
Published biogenic data compilation[b]	0.0550	0.0019	0.0267	0.0223	0.9140	0.0559	0.0019	0.0708	0.0232	0.9105
All bivalve mollusks This study	0.0341	0.0041	0.2719	0.0496	0.7246	0.0362	0.0044	0.3140	0.0527	0.7258
Bivalve mollusks minus Antarctic specimens[c] This study	0.0378	0.0050	0.1488	0.0601	0.7094	0.0402	0.0054	0.2686	0.0638	0.7098
Calcitic bivalve mollusks This study[d]	0.0342	0.0054	0.2725	0.0658	0.7685	0.0364	0.0058	0.3140	0.0706	0.7656
Aragonitic bivalve mollusks This study[d]	0.0383	0.0074	0.2094	0.0893	0.8180	0.0407	0.0078	0.2483	0.0095	0.8179

[a] See Table S1 for the data used for these regression line calculations.
[b] Includes coral data from Ghosh et al., 2006 (but excludes Red Sea *Porites*), and data from Ghosh et al. (2007), Came et al. (2007), Tripati et al. (2010), Eagle et al. (2010), and Thiagarajan et al. (2011). See Table S1 for values for these data.
[c] Excluding data from the five individuals of *Laternula elliptica* and *Adamussium colbecki* (which are Antarctic specimens from the coldest environments sampled in this study) as a means for determining whether the calibration slope could be significantly influenced by these samples alone.
[d] Excluding specimens with mixed mineralogy.
[e] Linear regressions through previously published data are all recalculated here using GraphPad Prism software (Zar, 1984) so that they are directly comparable to the new mollusk data presented here, and as a result may have slight differences from the slopes and intercepts given in original publications at the third or fourth decimal place. All regressions are on data that include an acid digestion temperature correction where appropriate (Passey et al., 2010). Errors are given as 1 s.e.

Table 5. ANCOVA p values derived by comparing linear regressions through the dataset generated in this study to previously published data.

Dataset[a]	Inorganic calcite Ghosh et al. (2006)	Inorganic calcite Dennis and Schrag (2010)	Published biogenic data compilation[d]
All bivalve mollusks This study	$p = 0.0035$ (Y)	$p = 0.7020$ (N)	$p < 0.0001$ (Y)
Bivalve mollusks minus Antarctic species[b] This study	$p = 0.0139$ (Y)	$p = 0.5453$ (N)	$p = 0.0006$ (Y)
Calcitic bivalve mollusks This study[c]	$p = 0.0196$ (Y)	$p = 0.9354$ (N)	$p = 0.0013$ (Y)
Aragonitic bivalve mollusks This study[c]	$p = 0.1274$ (N)	$p = 0.4664$ (N)	$p = 0.0126$ (Y)

[a] Linear regression lines through different subsets of our mollusk Δ_{47} calibration dataset in the first column are statistically compared to using ANCOVA tests (Zar, 1984) to linear regressions through other previously published calibration studies datasets. Calculations are done with values on the absolute reference frame (ARF). The table displays the ANCOVA p value and whether the two slopes being compared are statistically different; (Y) = Yes, (N) = No. In this case we consider a p value < 0.05 as indicating statistically significant differences between the two slopes.
[b] Excluding the five specimens of *Laternula elliptica* and *Adamussium colbecki* (which are specimens from the coldest Antarctic environments) as a means for determining whether the calibration slope could be significantly influenced by these samples alone.
[c] Excluding specimens with mixed mineralogy.
[d] Includes coral data from Ghosh et al. (2006) (but excludes Red Sea *Porites*), and data from Ghosh et al. (2007), Came et al. (2007), Tripati et al. (2010), Eagle et al. (2010), and Thiagarajan et al. (2011). See Table S1 for values for these data.

mollusks. We also show that changing solution pH and carbonate chemistry should not be a confounding factor in the interpretation of bivalve based Δ_{47} or $\delta^{18}O$ measurements, at least in the taxa studied, and that there is no significant mineralogical difference between calcite and aragonite. The errors in slope and intercepts for linear regression lines given in Table 4 highlight that successful calibration of the carbonate "clumped isotope" thermometer is dependent on having large datasets. For example, a linear regression through the initial inorganic calcite calibration dataset (Ghosh et al., 2006) has much larger uncertainties than a calibration line based on all the published biogenic calibration data from our

Table 6. Stable isotope data for individual cultured mollusk specimens grown at ambient carbonate saturation state and undersaturated conditions.

Taxa	Sample ID	pCO_2 (ppm)	Alkalinity	pH	$\Omega_{aragonite}$	Total Number of Analyses[1]	$\delta^{13}C$ ‰ V-PDB	$\delta^{18}O$ ‰ V-PDB	Δ_{47} ‰ (SD)[2]	Δ_{47} ‰ (ARF)[3]
Mya arenaria	JR-131	409	1833	8.02	2.11	3	−1.0	−3.3	0.648 ± 0.005	0.714 ± 0.005
Mya arenaria	JR-132	409	1833	8.02	2.11	4	−1.0	−3.3	0.644 ± 0.002	0.716 ± 0.008
Mya arenaria	JR-135	2856	2063	7.45	0.71	3	−0.8	−2.8	0.650 ± 0.008	0.723 ± 0.018
Mya arenaria	JR-136	2856	2063	7.45	0.71	3	−1.0	−3.0	0.657 ± 0.018	0.721 ± 0.016
Argopecten irradians	JR-113	409	1833	8.02	2.11	4	−1.7	−1.6	0.661 ± 0.003	0.728 ± 0.003
Argopecten irradians	JR-114	409	1833	8.02	2.11	4	−2.6	−2.0	0.677 ± 0.011	0.745 ± 0.012
Argopecten irradians	JR-117	2856	2063	7.45	0.71	2	−1.3	−2.1	0.664 ± 0.004	0.730 ± 0.004
Argopecten irradians	JR-118	2856	2063	7.45	0.71	3	−5.2	−2.0	0.663 ± 0.010	0.730 ± 0.010

Culture conditions and seawater chemistry measurements are from Ries et al. (2009).
[1] Represents the number of distinct extractions of CO_2 from all samples, which are then purified and analyzed.
[2] Relative to the stochastic distribution only. Also referred to as data in the Caltech intralaboratory reference frame. Includes the acid digestion correction of 0.08. ±. Values are 1 s.e.
[3] Values given on the absolute reference frame.
$\Omega_{aragonite} = [Ca^{2+}][CO_3^{2-}] / K_{sp}$, where K_{sp} is the stoichiometric solubility product of aragonite. $\Omega_{aragonite}$ was calculated as described in Ries et al. (2009).

laboratory due to having fewer datapoints. However we have shown statistically that the uncertainties in these calibration lines cannot alone explain the difference between our bivalve mollusk calibration line and other data produced in our laboratory, which (i) highlights that empirical calibrations of the carbonate clumped isotope paleothermometer are vital for each type of material and experimental setup, and (ii) suggests that initial papers showing close similarity of some biogenic materials to the inorganic calcite calibration of Ghosh et al. (2006; Eagle et al., 2010; Tripati et al., 2010; Thiagarajan et al., 2011) should not be assumed to hold in all cases. We also note that after this manuscript was published as a discussion paper, another study of brachiopods and mollusks in a different laboratory also reported a similarly shallow slope (Henkes et al., 2013), although as these measurements were conducted using a very similar methodology to that used in our study the similarity between our calibration slopes does not entirely resolve the possible methodological differences between calibration studies described below.

There are two possible explanations that are immediately apparent for the differences between calibration lines generated from different materials in our laboratory. First, the bivalve mollusk data presented here was obtained using the automated online sample reaction system described in Passey et al., 2010, whereas the in-depth calibration studies of corals, foraminifera and coccoliths were conducted using offline reactions with cryogenic and gas chromatography cleanup steps performed manually (Passey et al., 2010; Tripati et al., 2010; Thiagarajan et al., 2011). The calibration study on bioapatite (Eagle et al., 2010) was conducted on the automated system, but it did not examine specimens grown at temperatures lower than $\sim 24\,^{\circ}C$ and so would not necessarily have resolved a difference in slope that would be most apparent at low temperatures. Therefore we must consider the possibility that an experimental effect, such as fractionation of gases in either offline or online systems, or an effect due to the differences in acid digestion temperature between the two systems (25 °C for the offline reactions, 90 °C for the automated systems, which is presently addressed using a correction of 0.08 ‰ on the Caltech intralab reference frame) is not being correctly accounted for. Evidence against an experimental artifact from these two sources comes from the broadly comparable results that have been generated in different labs that use different systems for purifying CO_2 gas and different acid digestion temperatures as part of an interlaboratory comparison, which included measurements on a cold-water coral standard in four laboratories that consistently yielded a Δ_{47} value in the range of 0.78–0.80 ‰ on the absolute reference frame (Dennis et al., 2011). Additionally a number of applied studies using the automated sample preparation system have found that the calibration of Ghosh et al. (2006) generally yields plausible results including on modern specimens where we have good controls over growth temperature (e.g., Passey et al., 2010; Eagle et al., 2010, 2011, 2013; Finnegan et al., 2011; Csank et al., 2011; and Suarez et al., 2011). Nevertheless most applied studies have focused on samples formed at temperatures of 20 °C or more, and so there is a possibility that experimental differences such as small amounts of gas fractionation or equilibration during sample gas purification could preferentially affect samples with heavier Δ_{47} values (> 0.75 ‰). This is an area that should be explored in the future. Another possibility is that there are variations in acid digestion fractionation factors for samples of different isotopic composition or of different mineralogy, and whilst the aragonitic cold-water coral did not show this effect (Dennis et al., 2011) it would be useful to check if this is the case in other materials.

A second possible explanation for the differences in calibration lines revolves around fundamental differences in shell calcification in bivalve mollusks compared to other

biogenic carbonates that could result in "vital effects" on Δ_{47}. In this scenario the closer match of deep sea corals to the calibration of Ghosh et al. (2006) at cold temperatures actually reflects the expression of a small kinetic isotope effect in all of these materials, one that is not found in mollusks. The data from foraminifera at cold temperatures is relatively sparse, with some samples from the Arctic Ocean showing deviations from the Caltech inorganic calcite calibration and so are analogous to the mollusk data presented here, but other datapoints from specimens from slightly warmer environments fall closer to the calibration of Ghosh et al. (2006), and Tripati et al. (2010). This highlights the relative paucity of data from carbonates forming at low temperatures and this is an obvious area to focus on in future calibration studies.

Bivalve mollusks frequently precipitate their shells close to equilibrium with respect to oxygen isotopes, with maximum deviations typically in the range of 0.5 ‰ (e.g., Horibe and Oba, 1972; Romanek and Grossman, 1989; Grossman and Ku, 1986; Barrera et al., 1994; and Wanamaker et al., 2006). This is in contrast to deep-sea corals, which exhibit nonequilibrium $\delta^{18}O$ values of up to 4–5 ‰ in some cases (e.g., Adkins et al. 2003). Therefore we might expect that bivalve mollusk derived Δ_{47} values may also record close to equilibrium values, unless there is a source of biological fractionation of Δ_{47} in bivalves that has not yet been identified but hypothetically could be linked to mollusk specific mechanisms of biomineralization such as the use of organic templates for carbonate precipitation (Weiner and Dove, 2003; Addadi et al., 2006). If it was the case that mollusks are recording close to equilibrium values, the calibration of Ghosh et al. (2006) would have to include a kinetic isotope effect that fortuitously matches "vital effects" in previously published biogenic data from a temperature range of 0–10 °C that falls close to the inorganic calcite values. Finally, we note that even though a mineralogical difference between calcite and aragonite could not be resolved in our dataset it is still possible that very subtle mineralogical effects do exist and these effects contribute to the variability in measured Δ_{47} values. Larger datasets may be required to constrain this possibility with more certainty.

In conclusion, if the experimental effects described above can be either ruled out or better constrained, we will be able to say more about whether there may be small biological fractionations in Δ_{47} that differ between corals, foraminifera, and bivalves, and why these fractionations are most apparent at cold temperatures.

Supplementary material related to this article is available online at: http://www.biogeosciences.net/10/4591/2013/bg-10-4591-2013-supplement.pdf.

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