Ontogenic increase of metabolic carbon in freshwater mussel shells (*Pyganodon cataracta*)

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[1] Incorporation of isotopically light metabolic carbon (C_M) can obscure carbon isotope records of dissolved inorganic carbon from biogenic carbonates. Ontogenic increases of C_M are common in marine bivalved mollusks, likely reflecting increasing absolute metabolism with increasing body size. Here we use shell aragonite from four specimens of *Pyganodon cataracta* to test if the same is true in freshwater bivalves. Annual $\delta^{18}O_{aragonite}$ values were not different between individuals suggesting that the aragonite samples represent the same time interval. $\delta^{13}C_{aragonite}$, however, showed an ontogenic decrease, with a strong linear relationship between shell height (H in mm) and $\%C_M$ ($R^2 = 0.96$; $\%C_M = 0.23*H + 13.33$), indicating that more C_M is incorporated into larger shells. We estimate that between 15 and 35% of shell carbon is derived from metabolic CO_2. Nevertheless, this vital effect does not exclude the use of $\delta^{13}C_{aragonite}$ records from freshwater shells as environmental proxies.

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1. Introduction

[2] The carbon isotopic signature of dissolved inorganic carbon (DIC) ($\delta^{13}C_{DIC}$) is a powerful tool for understanding biogeochemical cycling in aquatic systems. For example, $\delta^{13}C_{DIC}$ gives an indication of changes in stream respiration, primary production, stream nutrient loading, land use change (e.g., urbanization, deforestation), pH, bedrock geology, and atmospheric CO_2 levels [see *Mook*, 2000]. However, continuous records of $\delta^{13}C_{DIC}$ are usually not available and are costly to produce. An alternative to a time series of $\delta^{13}C_{DIC}$ measurements would be to find a proxy of $\delta^{13}C_{DIC}$, such as sequentially deposited carbonates precipitated in equilibrium with the water. Fortunately, many freshwater streams have abundant populations of freshwater mussels (e.g., Unionidae) which produce carbonate shells that could potentially be used as a proxy of $\delta^{13}C_{DIC}$.

[3] There have been several studies on the isotope geochemistry of shells of marine and estuarine mollusks, but comparatively fewer on freshwater mollusks. Freshwater mussels are not typically well preserved due to the acidic nature of freshwater streams [*Carroll and Romanek*, 2006]; however, they are usually well cataloged in museums and some species can live to be well over 100 years [e.g., *Mutvei and Westermark*, 2001]. Indeed, studies have obtained multicentury climate records from freshwater mussel shells [e.g., *Schöne et al.*, 2004].

[4] There are two carbon sources for shell construction: environmental dissolved inorganic carbon (DIC) and respired carbon, the latter which is ultimately derived from carbon in the diet [see *McConnaughey et al.*, 1997]. These two end-members can potentially have very different $\delta^{13}C$ values, with freshwater $\delta^{13}C_{DIC}$ typically around -10 to $-15‰$ [e.g., *Mook and Tan*, 1991; *Hellings et al.*, 1999], and respired carbon typically around $-28‰$ in streams draining areas with C3 vegetation dominating [see *Mook and Tan*, 1991; *Dettman et al.*, 1999]. However, these values are both highly variable. Ideally, in order to reconstruct the ambient carbon cycle, shells should record stream $\delta^{13}C_{DIC}$ with minimal respired (metabolic) carbon being incorporated (i.e., no "vital effects").

[5] Several authors have found that metabolic carbon (C_M) can significantly contribute to marine and freshwater bivalve shell carbonate (both [*Keith et al.*, 1964]; marine [*Tanaka et al.*, 1986; *Klein et al.*, 1996; *McConnaughey et al.*, 1997; *Lorrain et al.*, 2004; *Gillikin et al.*, 2006, 2007]; freshwater [*Veinott and Cornett*, 1998; *Dettman et al.*, 1999; *Aucour et al.*, 2003; *Kaandorp et al.*, 2003]). While in most marine bivalves the amount of C_M in the shell is typically less than 10% [*McConnaughey et al.*, 1997], more recently up to 37% C_M has been reported [*Gillikin et al.*, 2007]. *Gillikin et al.* [2007] also observed a strong ontogenic increase of C_M, with a 4‰ decrease in $\delta^{13}C$ in one shell. *Lorrain et al.* [2004] proposed that the ratio of the total amount of respired carbon produced by the bivalve to the total carbon demand for shell construction dictated the amount of C_M in the shell. Freshwater bivalve physiology is rather different than marine bivalve physiology considering that freshwater bivalves live in dilute media and must

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actively pump ions and regulate their acid-base balance [see *Raikow and Hamilton*, 2001]. Therefore, the ontogenic effects of metabolic carbon incorporation seen in marine bivalves are not necessarily expected in freshwater bivalve shells.

[6] There are relatively few stable carbon isotope studies on freshwater bivalves. However, a few authors have suggested 5–80% C_M in freshwater bivalve shells [*Veinott and Cornett*, 1998; *Aucour et al.*, 2003; *Gajurel et al.*, 2006]. The higher values were reported by *Veinott and Cornett* [1998], but they did not analyze respired $\delta^{13}C$ (soft tissues) in their specimens and calculated C_M using the equation of *Tanaka et al.* [1986], which tends to overestimate C_M (see discussion). An ontogenetic increase of C_M incorporation has also been suggested [*Aucour et al.*, 2003; *Goewert et al.*, 2007], but has not been adequately tested. While shell $\delta^{13}C$ time series analyses can provide a good indication of ontogenic effects, they may also reflect real environmental changes through time which can then be mistaken as ontogenic. For example, pollutants or increased chloride concentrations may possibly affect how mollusks fractionate carbon isotopes [cf. *Gillikin et al.*, 2006].

[7] Here we present the first study to control for all environmental variations by collecting freshwater mussels of different ontogenic ages that grew at the same time and under the same environmental conditions; thereby allowing ontogenic effects to be easily separated from environmental effects. The aim of this study was to determine if the freshwater bivalve *Pyganodon cataracta* incorporates more metabolic carbon through ontogeny as marine bivalves do and to determine if metabolic carbon potentially obscures environmental $\delta^{13}C$ values.

2. Methods

[8] On 7 December 2006, four *Pyganodon cataracta* at various growth stages (shell sizes 88.30, 86.15, 43.35 and 20.75 mm) were collected from the Casperkill, on the campus of Vassar College in Poughkeepsie, NY, USA (41°44′N, 73°55′W). *P. cataracta* is one of the most common and widespread unionoids in central and eastern New York State [*Strayer and Jirka*, 1997]. The Casperkill has a catchment of 31 km^2 and drains into the Hudson River. Discharge in the Casperkill varies between 0.1 and 4 m^3 s^{-1}, with an average of 0.4 m^3 s^{-1} [*Minder*, 2004]. All shells were collected within the same 0.25 m^2 area of streambed. From each shell, foot, muscle and mantle tissues were collected and the remaining tissues were discarded. Shells were then air-dried. Each soft tissue sample was prepared for isotope analysis by drying at 60°C and grinding with an agate mortar and pestle. The periostracum was removed from the ventral margin of each *P. cataracta* shell using sandpaper and carbonate samples were removed from the shell surface using a dental drill and a raster pattern. Four samples were taken between the last growth line and the end of the shell so that the entire carbonate deposited between these two points was sampled (i.e., the entire last growth increment was sampled). Growth lines were assumed to be annual (but see *Kesler and Van Tol* [2000]). Shells were then embedded in epoxy and thin-sectioned to visualize internal growth lines. Water samples were collected from the same location on 1 December 2006,

26 December 2006, 26 March 2007, and 27 September 2007 for oxygen isotopes ($\delta^{18}O_w$) and $\delta^{13}C_{DIC}$ following the protocol outlined by *Gillikin and Bouillon* [2007]. Water temperature was measured monthly throughout the year prior to shell collection using a YSI handheld meter. Samples were collected near the bottom of the water column (at base flow water depth is ∼40 cm).

[9] $\delta^{13}C$ of soft tissues were measured on a continuous-flow isotope-ratio mass spectrometer (IRMS; Finnigan Delta Plus^{XL}). Samples were wrapped in tin cups and were then combusted using an elemental analyzer (Costech) coupled to the IRMS. Standardization was based on NBS-22 and USGS-24 for $\delta^{13}C$ and precision was better than ±0.06‰ (1σ), based on repeated internal standards (reference values for standards taken from *Gonfiantini et al.* [1995]). All carbonate isotopic analyses were performed on a Finnigan MAT 252 IRMS equipped with a Kiel III automated sampling device. Samples were reacted with >100% orthophosphoric acid at 70°C. Results are reported relative to VPDB by calibration to the NBS-19 reference standard ($\delta^{13}C$ = +1.95‰ and $\delta^{18}O$ = −2.20‰ VPDB). $\delta^{18}O$ of water was measured on a Finnigan Delta S IRMS, samples were equilibrated with CO_2 gas at approximately 15°C in an automated equilibration device coupled to the IRMS. Standardization is based on international reference materials VSMOW and SLAP. Precision is 0.08‰ or better on the basis of repeated internal standards. The $\delta^{13}C$ of DIC was measured on the continuous-flow IRMS coupled with a Gasbench automated sampler (also manufactured by Finnigan). Samples were reacted for >1 h with phosphoric acid at room temperature in Exetainer (Labco) vials previously flushed with He gas. Standardization was based on NBS-19 and NBS-18 and precision was ±0.3‰ or better (1σ). All isotopic analyses were carried out by the Environmental Isotope Laboratory, Department of Geosciences, University of Arizona.

3. Results

[10] Soft tissue $\delta^{13}C$ values are presented in Table 1. All tissues were within 2.3‰ of each other. There was no significant difference between tissue types overall (one-way ANOVA, p = 0.34), but within individuals, mantle tissues were generally more negative than other tissues, followed by foot tissues, then muscle tissues. There was a significant strong positive correlation between shell height (in mm) and muscle tissues (R^2 = 0.98; p < 0.01; slope = 0.007), but not for foot versus height (p = 0.57) nor mantle versus height (p = 0.28).

[11] Aragonite oxygen isotope signatures ($\delta^{18}O_{ar}$) were similar between individuals with averages having an overall range of 1.1‰; with a similar variability within individual shells (Figure 1). There was no correlation between average $\delta^{18}O_{ar}$ and shell height (p = 0.13). Aragonite carbon isotopes ($\delta^{13}C_{ar}$) were different between individuals with a strong significant negative correlation between $\delta^{13}Car$ and shell height (R^2 = 0.95, p < 0.05; Figure 1). Shells were about 4 to 7‰ more negative than expected based on the equilibrium fractionation of *Romanek et al.* [1992] (Figure 1).

[12] Internal growth lines did not always correspond with external growth lines on all shells. Some prominent external growth lines toward the growing tip of the shell were

Table 1. Soft Tissue Carbon Isotope Data From Four *Pyganodon cataracta* of Various Sizes Collected on 7 December 2006 Within the Same 0.25 m^2 Area of the Casperkill in Eastern New York State

Shell Height (mm)	Foot δ^{13}C (‰)	Muscle δ^{13}C (‰)	Mantle δ^{13}C (‰)
20.8	−35.8	−35.2	−36.7
43.4	−35.0	−35.0	−35.5
83.2	−35.2	−34.8	−35.9
88.3	NA[a]	−34.7	−34.4

[a]NA, not analyzed.

represented internally (Figure 2a). However, we feel that the external growth lines (Figure 2b) are approximately annual and that internal growth lines in younger shell sections are not highly visible.
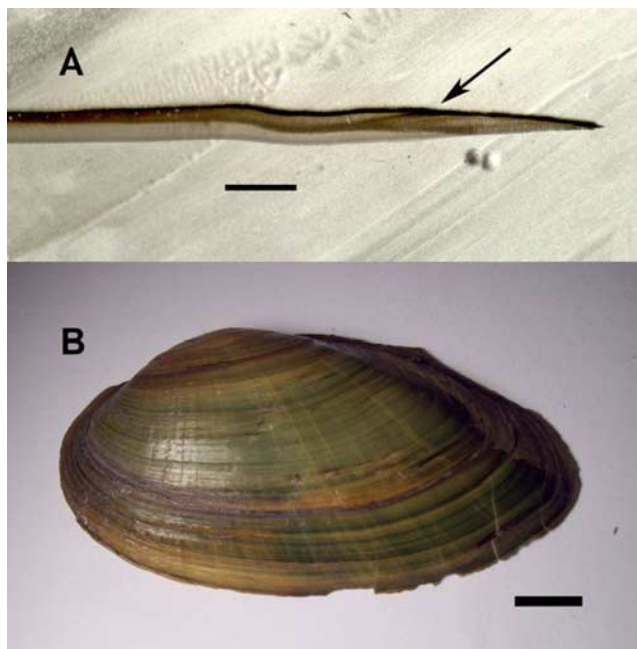
[13] The four water samples collected in fall, winter and spring had an average δ^{18}O$_w$ value of −8.2 ± 1.2‰ and an average δ^{13}C$_{DIC}$ of −9.1 ± 0.6‰. Water temperature ranged from 1.7°C (January) to 26.6°C (July) and was above 12°C from April to September. Continuously recorded pH (every 20 min between 7 June and 8 April (after shell collection) by a YSI model 6920-S sonde) ranged between 7.2 and 8.5 approximately 1 km downstream of the collection site (K. Menking, unpublished data, 2008), but averaged about 7.7 for most of the recorded interval except in the spring where it reached values as high as 8.5.

4. Discussion

[14] Our data clearly illustrate a trend of more negative δ^{13}C$_{ar}$ through ontogeny in *Pyganodon cataracta* shells (Figure 1). Considering that these shells were all collected at the same time and that the carbonate analyzed should represent the same time period (∼1 growth season), the ontogenic trends observed are not due to a change in environmental conditions. As δ^{13}C$_{DIC}$ changes over the

Figure 2. (a) Thin section of a *Pyganodon cataracta* shell illustrating an internal growth line (arrow) and (b) an image of one valve showing external growth lines. Both shells collected on 7 December 2006 from the Casperkill stream in eastern New York State. Scale bars are 1 mm in Figure 2a and 1 cm in Figure 2b.

year and shell δ^{13}C$_{ar}$ is also expected to change throughout the year [e.g., *Dettman et al.*, 1999; *Wurster and Patterson*, 2001; *Goewert et al.*, 2007] it is imperative that the average shell signal is representative of the whole year [see *Krantz et al.*, 1989]. The δ^{18}O$_{ar}$ of shell carbonate is dependent on both the δ^{18}O of water as well as water temperature. We expect δ^{18}O$_{ar}$ to change sinusoidally over the year, reflecting the variations in seasonal temperature and δ^{18}O$_{precipitation}$ in New York State (similar to *Wurster and Patterson* [2001]). Therefore, if some shells represent only part of the growth season, we would expect δ^{18}O$_{ar}$ values to differ between shells, which they do not. Moreover, shell δ^{18}O$_{ar}$ is close to what is expected using the paleo-temperature equation of *Böhm et al.* [2000] when averaging stream temperatures above 12°C (unionids have been shown to stop precipitating shell below 12°C [*Dettman et al.*, 1999]), suggesting both that *P. cataracta* precipitates its shell in oxygen isotope equilibrium with its environment similar to most mollusks and that we have captured the same time intervals in all shells.

[15] There are several hypotheses that can be put forth to explain why this ontogenic effect is present. Kinetic fractionation effects can probably be ruled out as these have never been recorded in bivalves (including freshwater bivalves), most likely due to the role of carbonic anhydrase in calcification physiology [see *McConnaughey and Gillikin*, 2008]. The remaining hypotheses involve either environmental or biological effects.

[16] Considering these mussels were collected within the same 0.25 m^2 area, microsite conditions are very unlikely and can be ruled out. The only potential environmental

Figure 1. Annual carbon and oxygen stable isotope data from four *Pyganodon cataracta* shells plotted against shell height (H). Four samples were milled from the last growth increment and were averaged (standard deviation of the four samples are shown as error bars). The gray bar represents approximate δ^{13}C$_{ar}$ equilibrium using the fractionation factor of +2.7‰ [*Romanek et al.*, 1992] and the measured average δ^{13}C$_{DIC}$ of −9.1 ± 0.6‰.

effect would be differences in burial depth with age. Because of high biological activity in sediments, pore water $\delta^{13}C_{DIC}$ can be expected to be more negative than overlying waters. If this ^{13}C depleted pore water seeped into the extrapallial fluid (EPF; the site of calcification in bivalves) it could potentially influence shell $\delta^{13}C$ [see *Krantz et al.*, 1987; *McConnaughey and Gillikin*, 2008]. However, fresh-water mussels in the Casperkill generally sit with much of the ventral margin of their shell above the sediment (personal observation) and smaller unionids have been shown to actually burrow deeper in the sediments than larger unionids [*Schwalb and Pusch*, 2007]. Therefore, if pore water was affecting these shells, we would expect an ontogenic increase of $\delta^{13}C_{ar}$, which is not the case, so we consider burial depth to be an unlikely factor.

[17] Since environmental factors are unlikely to cause the observed ontogenic effect, the most plausible hypotheses are biological effects. The similarity in $\delta^{13}C$ tissue values between individuals and organs (Table 1), suggests that the diet of these mussels is similar across different age classes A decrease in $\delta^{13}C_{ar}$ would also result if the percent C_M being sequestered to the shell remained constant, but diet switched to a source with more negative $\delta^{13}C$ values, however this is clearly not the case here. We actually see the opposite effect with muscle tissues becoming slightly more positive through ontogeny (see Table 1). Therefore, the hypothesis put forth by *Lorrain et al.* [2004] is the most parsimonious. They suggest that the amount of metabolic carbon incorporated into bivalve shells is dependent on the carbon demand during calcification. When the bivalve is young the total amount of respiring tissues between the valves is small and therefore less metabolic CO_2 is produced in total (absolute metabolism is low). In addition, shell growth is fast (shell carbon demand is high). Therefore, most of the shell carbon comes from the DIC and only little metabolic carbon is incorporated in the shell. When the bivalves age, shell growth slows (shell carbon demand is low), the amount of tissues increases, and thus total respired CO_2 increases (absolute metabolism is high), thereby increasing the amount of metabolic carbon incorporated in the shell. Shell carbon demand and absolute metabolism both increase through ontogeny, of course, but shell carbon demand does not increase as fast as absolute metabolism [*Lorrain et al.*, 2004]. We contend that this is the phenomenon responsible for the ontogenic decrease in $\delta^{13}C_{ar}$ seen in these shells.

[18] Although we have only limited environmental data, a rough estimate of the percentage of metabolic carbon in these shells can be calculated (a 1‰ change in $\delta^{13}C_{DIC}$ changes C_M by ~5%). For this, a simple mixing model is used where respired carbon and environmental DIC are the two end-members. Even though the $\delta^{13}C_{DIC}$ data were not collected while the mussels were growing, the data should still characterize the stream (large interannual changes are not expected). Soft tissue $\delta^{13}C$ can be used as an approximation of respired carbon $\delta^{13}C$ [*McConnaughey et al.*, 1997]. Muscle tissues in bivalves typically have the slowest turnover time [*Raikow and Hamilton*, 2001; *Lorrain et al.*, 2002], so we used muscle tissue $\delta^{13}C$ as a proxy for respired carbon for the shell's growth season. In freshwater systems, $\delta^{13}C_{DIC}$ can vary dramatically throughout the year, with intraannual changes up to 5‰ being common in temperate regions [*Mook and Tan*, 1991; *Dettman et al.*, 1999].

Therefore, our average of four samples certainly does not capture the full variability in this stream. Using the average of these four $\delta^{13}C_{DIC}$ samples taken throughout the year does however allow us to calculate a rough estimate of C_M. We use the mixing equation rewritten from *McConnaughey et al.* [1997]:

$$C_M = (\delta^{13}C_{ar} - \varepsilon_{ar-b} - \delta^{13}C_{DIC})/(\delta^{13}C_M - \delta^{13}C_{DIC}) \qquad (1)$$

where ε_{ar-b} is the fractionation factor between aragonite and HCO_3^- (+2.7‰ according to *Romanek et al.* [1992]) and $\delta^{13}C_M$ is the $\delta^{13}C$ of metabolic carbon. *Tanaka et al.* [1986] also proposed a model to calculate C_M which included the fractionation factor of aqueous CO_2 and $CaCO_3$ and typically results in a much higher C_M estimation. In addition to other problems with their model [see *McConnaughey and Gillikin*, 2008], at the pH of the Casperkill (~7.5 to 8) nearly all of the DIC is present as HCO_3^-, making the fractionation factor of aqueous CO_2 and carbonate in the *Tanaka et al.* [1986] model insignificant. Using equation (1), we calculate C_M to be between 21 and 34% with a strong relationship between shell height (H in mm) and C_M (Figure 3; $\%C_M = 0.23 (\pm 0.15) * H + 13.3 (\pm 9.5)$; $R^2 = 0.96$).

[19] These values are higher than the typical 10% C_M recorded for most aquatic mollusks [see *McConnaughey and Gillikin*, 2008], but are within the values reported for at least one marine species (*Mercenaria* sp.; see *Gillikin et al.* [2007] and Figure 3). Interestingly, the slopes between $\%C_M$ and shell height are not statistically different between the marine *Mercenaria* shells and the freshwater *P. cataracta* shells (p > 0.05 for both; Figure 3). The similarity in the slopes suggests that similar mechanisms may be responsible for the ontogenic effect seen here, despite the vast difference between the water chemistries in which these animals make their shells.

[20] It can be argued that equation (1) does not necessarily account for all fractionation processes. Respired carbon is produced in the form of CO_2, which then must convert to HCO_3^-. This conversion results in a ~−10‰ fractionation at 15°C [*Mook*, 2000], unless all CO_2 converts to HCO_3^-. Considering freshwater mussels typically have a hemolymph (an internal body fluid) pH of around 7.5 to 8 [e.g., *Byrne and Dietz*, 1997], nearly all (~97%) respired CO_2 should convert to HCO_3^- resulting in little to no fractionation. Therefore, the fractionation between aragonite and HCO_3^- should be used (see equation (1)). However, CO_2 passes biological membranes much more easily than HCO_3^- [*Gutknecht et al.*, 1977], so perhaps some of the small amount of CO_2 that is present in the water makes it to the EPF disproportionately and contributes to the negative $\delta^{13}C_{ar}$ disequilibrium recorded in bivalve shells (see also *McConnaughey and Gillikin* [2008] for more detailed discussion on this). This could in part explain why *Gillikin et al.* [2007] found that hemolymph in marine clams contained only about 10%C_M, but shells contained much more (up to 37%C_M).

5. Conclusion

[21] This study demonstrates that C_M increases through ontogeny in this freshwater mussel species and that the
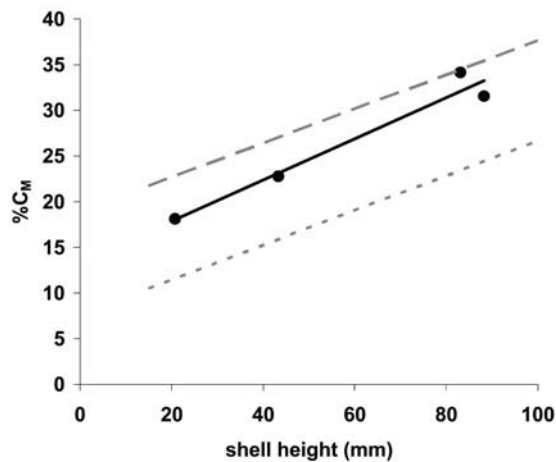
Figure 3. Estimated percent metabolic carbon (C_M) for the four shells calculated using the equation of *McConnaughey et al.* [1997] showing a strong relationship between shell height (H in mm) and C_M ($\%C_M = 0.23\ (\pm 0.15)\ *\ H + 13.3\ (\pm 9.5)$; $R^2 = 0.96$) (black symbols and soil black line). Regressions from *Mercenaria* sp. shells are also plotted as dashed gray lines (data from *Gillikin et al.* [2007]). The slopes of the gray lines are 0.190 (± 0.035) (n = 129, $R^2 = 0.48$) for the lower line and 0.187 (± 0.092) (n = 13, $R^2 = 0.64$) for the upper line.

amount of C_M can be considerable (up to 34%). While our data set is small, $\%C_M$ increases from approximately 15–35% over shell heights of 20–90 mm. This ontogenic effect, however, does not exclude $\delta^{13}C_{ar}$ in freshwater mussel shells as useful environmental proxies. For example, although *Goewert et al.* [2007] reported an ontogenic decrease in $\delta^{13}C_{ar}$, shells had clearly different $\delta^{13}C_{ar}$ values between a corn (C4) dominated and soybean (C3) dominated watershed. Therefore, as long as the ontogenic effect is accounted for, these shells can still provide useful information. Even if $\delta^{13}C_{ar}$ data cannot provide absolute $\delta^{13}C_{DIC}$ records, they can still be used as indicators of environmental processes that occurred during shell growth.

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