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Carbon isotope fractionation between amorphous calcium carbonate and calcite in earthworm-produced calcium carbonate

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1 **Carbon isotope fractionation between amorphous calcium**
2 **carbonate and calcite in earthworm-produced calcium carbonate**

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16

17 **Abstract**

18 In this study we investigate carbon isotope fractionation during the
19 crystallization of biogenic calcium carbonate. Several species of
20 earthworm including *Lumbricus terrestris* secrete CaCO₃. Initially a
21 milky fluid comprising micro-spherules of amorphous CaCO₃ (ACC) is
22 secreted into pouches of the earthworm calciferous gland. The micro-

23 spherules coalesce and crystalize to form millimetre scale granules,
24 largely comprising calcite. These are secreted into the earthworm
25 intestine and from there into the soil. *L. terrestris* were cultured for 28
26 days in two different soils, moistened with three different mineral
27 waters at 10, 16 and 20 °C. The milky fluid in the calciferous glands,
28 granules in the pouches of the calciferous glands and granules excreted
29 into the soil were collected and analysed by FTIR spectroscopy to
30 determine the form of CaCO₃ present and by IRMS to determine δ¹³C
31 values. The milky fluid was ACC. Granules removed from the pouches
32 and soil were largely calcite; the granules removed from the pouches
33 contained more residual ACC than those recovered from the soil. The
34 δ¹³C values of milky fluid and pouch granules became significantly more
35 negative with increasing temperature ($p \leq 0.001$). For samples from
36 each temperature treatment, δ¹³C values became significantly ($p \leq$
37 0.001) more negative from the milky fluid to the pouch granules to the
38 soil granules (-13.77, -14.69 and -15.00 respectively at 10 °C; -14.37, -
39 15.07 and -15.18 respectively at 16 °C and -14.89, -15.41 and -15.65
40 respectively at 20 °C). Fractionation of C isotopes occurred as the ACC
41 recrystallized to form calcite with the fractionation factor $\epsilon_{\text{calcite-ACC}} = -$
42 1.20 ± 0.52 ‰. This is consistent with the crystallization involving
43 dissolution and reprecipitation rather than a solid state rearrangement.
44 Although C isotopic fractionation has previously been described

45 between different species of dissolved inorganic carbon and various
46 CaCO_3 polymorphs, this is the first documented evidence for C isotope
47 fractionation between ACC and the calcite it recrystallizes to. This
48 phenomenon may prove important for the interpretation of CaCO_3 -
49 based C isotope environmental proxies.

50

51 **Keywords:** Earthworms; calcium carbonate; calcite; carbon isotopes;
52 fractionation; crystallization

53

54 **Introduction**

55 Many earthworm species produce calcium carbonate (CaCO_3) granules
56 in specialised calciferous glands. In the earthworm *Lumbricus terrestris*
57 these occur in segments 11-12 as two pairs of swellings off the
58 oesophagus, and one pair of pouches anterior to the glands in segment
59 10 (Darwin, 1881; Canti and Pearce, 2003).

60 CaCO_3 production starts by secretion of an amorphous calcium
61 carbonate (ACC) suspension that we refer to as milky fluid. In the
62 pouches, small spherulites (1-5 μm) in the milky fluid accrete into
63 larger granules ($\leq 2.5 \text{ mm}$). These are released into the oesophagus and
64 excreted into the soil (Briones *et al.*, 2008; Gago-Duport *et al.*, 2008).

65 The granules retrieved from the pouches and the soil are

66 predominantly calcite, but can contain small amounts of ACC, vaterite

67 and aragonite (Gago-Duport *et al.*, 2008; Lee *et al.*, 2008; Fraser *et al.*,
68 2011; Brinza *et al.*, 2013; Brinza *et al.*, 2014a; Brinza *et al.*, 2014b;
69 Hodson *et al.*, 2015). The function of CaCO₃ production by the
70 earthworms remains unclear but is likely related to regulation of pH
71 and CO₂ concentrations in body fluids (Voigt, 1933; Aoki, 1934;
72 Kaestner, 1967; Kühle, 1980; Versteegh *et al.*, 2014).

73 It is known that considerable $\delta^{13}\text{C}$ fractionation factors exist between
74 the different species of DIC and the various polymorphs of CaCO₃
75 (Fouke *et al.*, 2000; Romanek *et al.*, 1992; Szaran, 1997; Zhang *et al.*,
76 1995). In addition to thermodynamics, kinetics of precipitation plays an
77 important role in fractionation (Watson, 2004; DePaolo, 2011; Nielsen
78 *et al.*, 2012). Variable fractionation of carbon isotopes has been
79 observed in different calcium carbonate biominerals suggesting that
80 vital effects may also be relevant (e.g. Adkins *et al.*, 2003; Auclair *et al.*,
81 2003; Bernis *et al.* 2000; Lécuyer *et al.* 2012; McConnaughey, 1989;
82 Rollion-Bard *et al.*, 2016; Spooner *et al.*, 2016). Despite many calcium
83 carbonate minerals having an amorphous pre-cursor (Radha *et al.*,
84 2010; Rodriguez-Blanco *et al.*, 2011; Stephens *et al.*, 2011) and stable
85 ACC being increasingly observed in biominerals (Aizenberg *et al.*, 2003;
86 Jacob *et al.*, 2008; Wehrmeister *et al.*, 2011), carbon isotope
87 fractionation between ACC and calcite has not been previously reported
88 in the literature

89 Here we present results of stable carbon isotope analyses on milky
90 fluid collected from the calciferous pouches of earthworms, fresh
91 granules also collected from the pouches, and older granules collected
92 from the soil in which the earthworms were cultivated and address the
93 question: how does granule mineralogy influence $\delta^{13}\text{C}$ values?
94 Furthermore we make a first attempt at estimating the carbon isotopic
95 fractionation factor between calcite and ACC, produced by earthworms.

96

97 **Materials & Methods**

98 *Experimental setup*

99 Two soils were collected from agricultural fields in Berkshire, UK:
100 Hamble (SU 61968 70235) and Red Hill (SU 56060 80033); both
101 Typical Argillic Brown Earths (Avery, 1980; full soil characterisation in
102 Table 1, Versteegh et al., 2014). The soil was air-dried and sieved to 250
103 μm prior to use (Lambkin *et al.*, 2011). This ensures that no large
104 granules are present in the soil at the beginning of the experiment and
105 facilitates granule recovery at the end. Post-sieving soil pH and organic
106 matter content were 7.5 ± 0.3 and 3.8 ± 0.1 % for Hamble and 7.1 ± 0.1
107 and 7.4 ± 0.1 % for Red Hill. For each replicate, 300 g of soil were mixed
108 with one of three types of mineral water (initial $\delta^{18}\text{O}$ values -10.0, -7.3
109 and $-5.3 (\pm 0.2)$ ‰ VSMOW) to 65 % water holding capacity (BS ISO,
110 1998). The moistened soil was put in a zip-lock bag with 5 g air-dried

111 horse manure rehydrated with 10 ml demineralised water. One adult,
112 clitellate *L. terrestris* was added to each bag. Bags were closed and kept
113 at either 10, 16 or 20 °C. There were six replicates per treatment.
114 Earthworms were acclimatised for three weeks, and then transferred to
115 an identical treatment bag containing the same type and mass of soil
116 and manure at the same temperature. Experimental details are given in
117 Versteegh et al. (2013). After 28 days earthworms were removed from
118 the bags, killed by dipping them in near-boiling water, and the
119 calciferous glands were dissected out. Any CaCO₃ concretions present in
120 the pouches were also retrieved, rinsed in deionised water and air-
121 dried. Calciferous glands were put on a glass slide; MF was allowed to
122 leak from the glands, was left to air-dry overnight, and collected by
123 scraping it off the slide. The soil was wet-sieved to 500 µm to retrieve
124 granules which were air-dried.

125

126 *Stable-isotope analyses*

127 Milky fluid and individual granule CaCO₃ samples were analysed for
128 δ¹³C values using a Thermo Delta V Advantage IRMS with a GasBench II.
129 The Gasbench II sample preparation device uses 100% ortho-
130 phosphoric acid to transform CaCO₃ into CO₂ and hence only analyses
131 the mineral fraction of the samples (Paul and Skrzypek, 2007). The raw
132 δ¹³C values were converted to the VPDB scale after normalising against

133 NBS 18 and NBS 19 carbonate standards. The long-term standard
134 deviation of a routinely analysed in-house CaCO_3 standard was < 0.05
135 ‰. Statistical analysis of the ^{13}C data was carried out using SigmaPlot
136 12 for Windows 7.

137

138 *Fourier transform infrared spectroscopy (FTIR)*

139 Three samples each of milky fluid, granules from pouches and granules
140 from soil were analysed by FTIR in the range $650 - 4000 \text{ cm}^{-1}$ using a
141 diamond internal reflection cell on a A2-Technology MicroLab Portable
142 mid-IR spectrometer of the Cohen Laboratories, University of Leeds.
143 Spectra were acquired by co-adding 512 scans with a 4 cm^{-1} resolution.
144 Crystalline carbonate phases have distinct bands at $\sim 714 \text{ cm}^{-1}$ (ν_4), \sim
145 866 cm^{-1} (ν_2), $\sim 1084 \text{ cm}^{-1}$ (ν_1) and $1420\text{-}1470 \text{ cm}^{-1}$ (ν_3) whilst ACC
146 lacks the distinct vibrational band at $\sim 714 \text{ cm}^{-1}$ (Chester and
147 Elderfield, 1967; Aizenberg *et al.*, 1996; Gago-Duport *et al.*, 2008;
148 Rodriguez-Blanco *et al.*, 2011). Areas for the ν_4 and ν_3 peaks covering
149 the wavenumber ranges between $651 - 725 \text{ cm}^{-1}$ and $1602\text{-}1243 \text{ cm}^{-1}$
150 respectively were determined using the Nicolet EZ OMNIC 5.1 Software.
151 Reference spectra for synthetic calcite and ACC were provided by Dr.
152 Juan-Diego Rodriguez-Blanco, University of Copenhagen, Department of
153 Chemistry.

154

155 Results*156 $\delta^{13}C$ values of $CaCO_3$*

157 For each individual earthworm, 10 granules were analysed from the soil
158 and one from each of the pouches (if available). For milky fluid, only one
159 analysis per earthworm could be undertaken, but sometimes this failed
160 because too little material was available. All analyses are reported in
161 the Supplementary material. Three-way Analysis of Variance (ANOVA)
162 with temperature, soil type and water type as factors indicated that
163 there were no significant differences in $\delta^{13}C$ values of granules
164 extracted from the soil between different treatments. In contrast, 3-way
165 ANOVA followed by pair-wise multiple comparison (Holm-Sidak
166 method) indicated that there were significant differences in $\delta^{13}C$ values
167 between different temperature treatments for the granules extracted
168 from the pouches and also for the milky fluid ($p \leq 0.01$); values became
169 increasingly negative from the 10 to 16 to 20 °C treatments. There were
170 no significant differences in $\delta^{13}C$ values for either the milky fluid or
171 granules from pouches between different soils or different mineral
172 water treatments. Consequently, the data for different soil-water
173 combinations but the same temperature were combined for analysis.
174 Kruskal-Wallis One-way Analysis of Variance (ANOVA) on ranks
175 followed by pair-wise comparison (Dunn's method) indicated that at
176 each temperature there were significant differences between the $\delta^{13}C$

177 values of the milky fluid, granules from pouches and granules from soil
178 with values becoming increasingly negative in that order (Fig. 1).
179 Ranges of $\delta^{13}\text{C}$ values were relatively narrow for milky fluid and
180 granules from pouches but wider for granules retrieved from the soil.

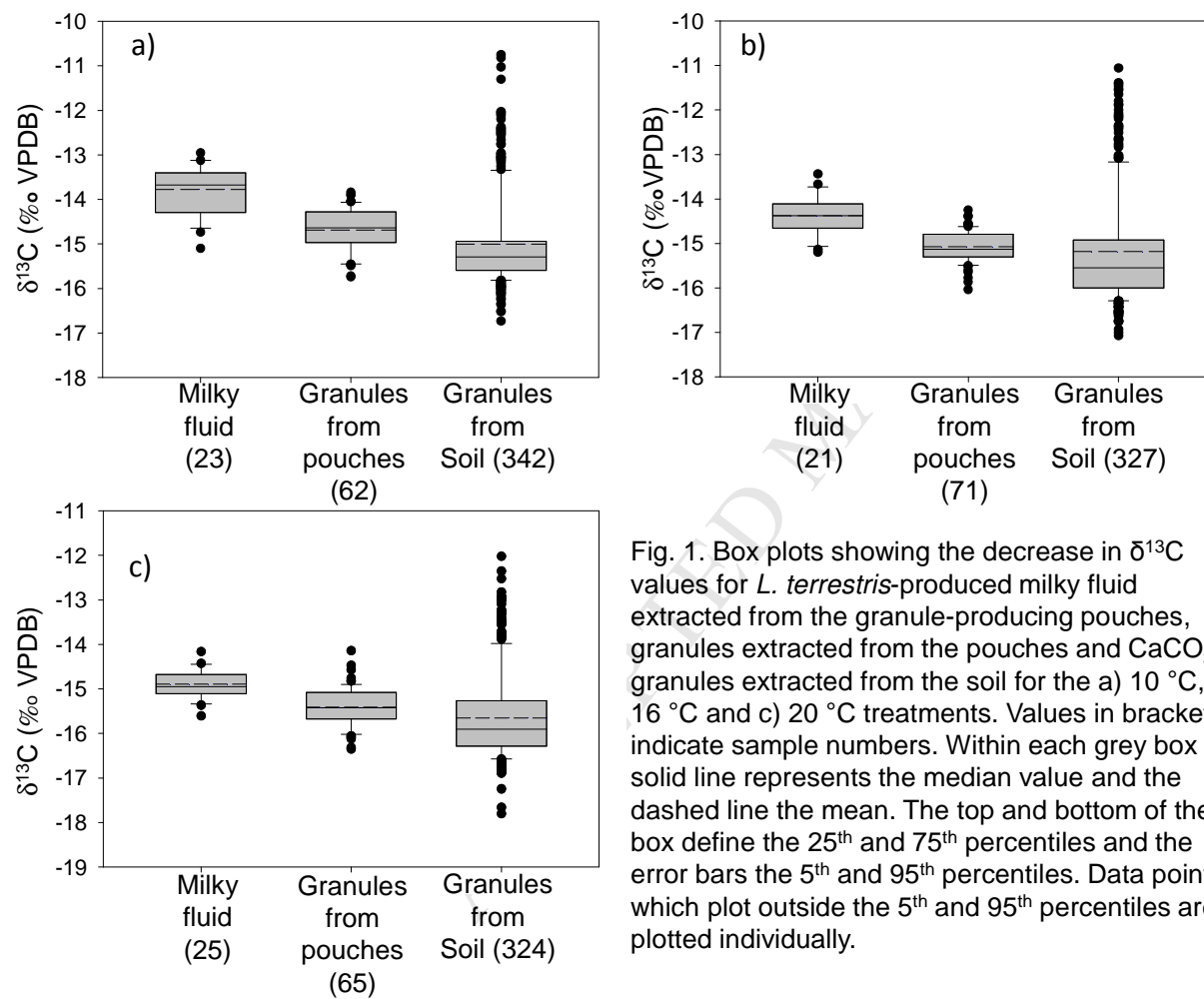


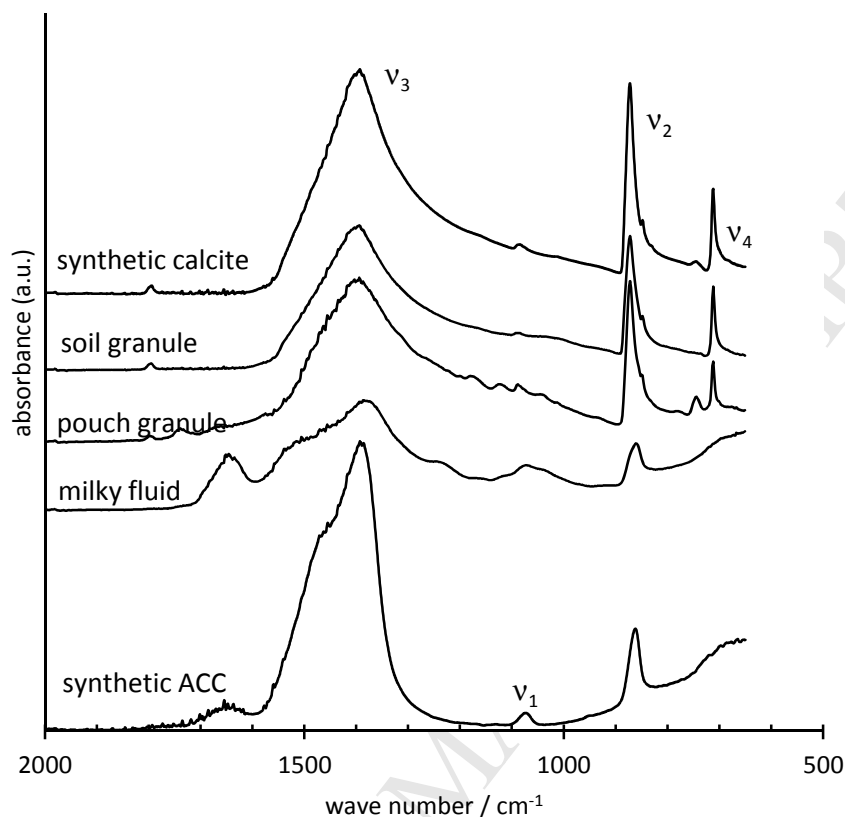
Fig. 1. Box plots showing the decrease in $\delta^{13}\text{C}$ values for *L. terrestris*-produced milky fluid extracted from the granule-producing pouches, granules extracted from the pouches and CaCO_3 granules extracted from the soil for the a) 10 °C, b) 16 °C and c) 20 °C treatments. Values in brackets indicate sample numbers. Within each grey box the solid line represents the median value and the dashed line the mean. The top and bottom of the box define the 25th and 75th percentiles and the error bars the 5th and 95th percentiles. Data points which plot outside the 5th and 95th percentiles are plotted individually.

182

183 *FTIR data*

184 FTIR analyses revealed that in the milky fluid the ν_4 peak at 714 cm^{-1}
185 was absent (Fig. 2). In contrast, the granules recovered from the pouch
186 and from the soil both had a distinct peak at 714 cm^{-1} . The ratio of the
187 peak areas for the ν_3 and ν_4 vibrations was significantly greater (t-test,
188 $p \leq 0.05$) for the pouch granules (31.9 ± 2.2 , mean \pm standard deviation,
189 $n = 3$) than for the soil granules (21.3 ± 0.6).

190



191

192 FIG. 2. Typical FTIR spectra for milky fluid, a granule recovered from
 193 one of the pouches (pouch granule), and a granule recovered from the
 194 soil (soil granule); all spectra relate to samples from the same
 195 earthworm. Reference spectra for synthetic calcite and ACC are also
 196 shown. Spectra are vertically offset on the absorbance axis for clarity.
 197 The major calcium carbonate peaks (ν_1 to ν_4) are labelled. Calcite
 198 shows peaks at $\sim 714\text{ cm}^{-1}$ (ν_4), $\sim 866\text{ cm}^{-1}$ (ν_2), $\sim 1090\text{ cm}^{-1}$ (ν_1) and
 199 $1420\text{--}1470\text{ cm}^{-1}$ (ν_3); amorphous calcium carbonate lacks the $\sim 714\text{ cm}^{-1}$
 200 ν_4 peak.

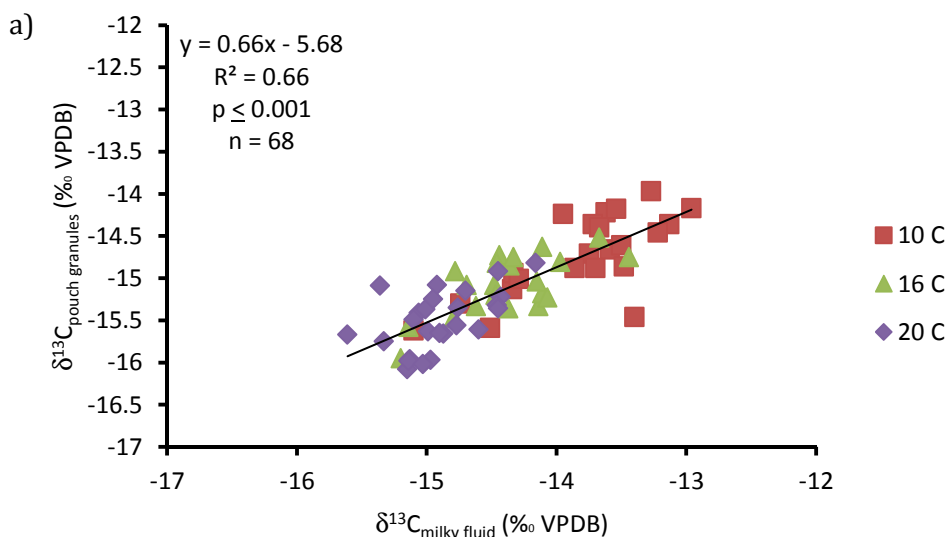
201

202 Discussion

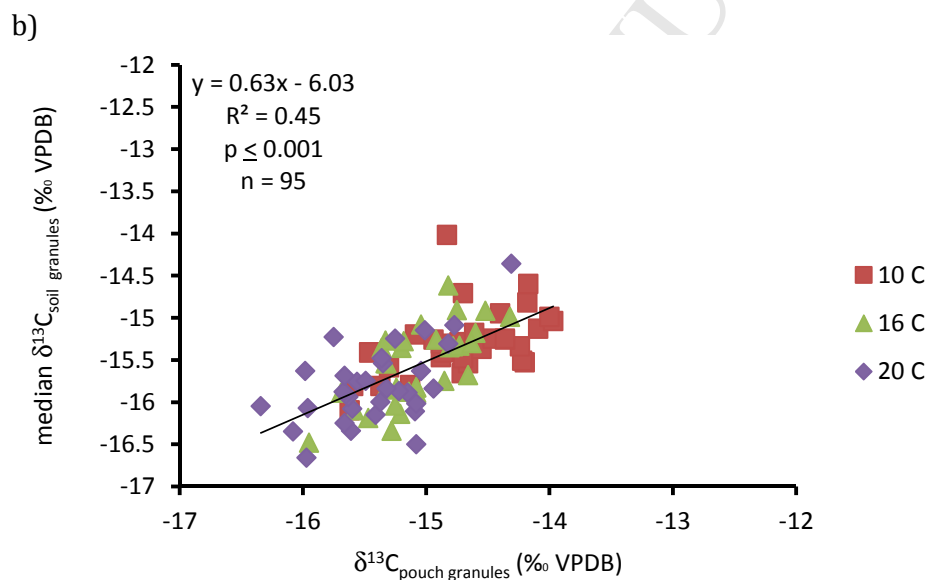
203 $\delta^{13}\text{C}$ values and polymorphs of CaCO_3

204 Ranges of $\delta^{13}\text{C}$ values are narrow for the milky fluid and granules from
 205 pouches, while $\delta^{13}\text{C}$ values for granules retrieved from the soil show a

206 wide range and are not normally distributed (Fig. 1). Therefore, for
207 each experimental replicate, the median $\delta^{13}\text{C}$ value for each set of 10
208 granules recovered from the soil per earthworm was used for
209 comparison with the $\delta^{13}\text{C}$ values of the milky fluid and pouch granules
210 recovered from the same earthworm that produced the soil granules.
211 Regression analyses for the entire dataset (combining the different
212 temperature treatments) revealed strong relationships between milky
213 fluid $\delta^{13}\text{C}$ values and $\delta^{13}\text{C}$ values of the granules retrieved from the
214 same earthworm's pouches (Fig. 3a), as well as between $\delta^{13}\text{C}$ values of
215 granules retrieved from the pouches and those secreted into the soil by
216 the same earthworm (Fig. 3b). Relationships were less strong when
217 individual temperature treatments were considered. Going from milky
218 fluid, to granules in the pouches, to granules in the soil, $\delta^{13}\text{C}$ values
219 show a gradual shift to lower values (Fig. 1, Fig. 3a-b).
220



221



222

223 FIG. 3. (a) $\delta^{13}\text{C}$ values of individual granules retrieved from the pouches
 224 versus $\delta^{13}\text{C}$ values of milky fluid recovered from the same pouch, note
 225 the depletion in ^{13}C in the former and the shift to lower values with
 226 increasing temperature; (b) Median $\delta^{13}\text{C}$ composition of granules
 227 recovered from the soil versus $\delta^{13}\text{C}$ values of the individual granules
 228 retrieved from the pouches of the earthworm cultivated in that soil;
 229 note increased scatter and lower R^2 . Data from all experiments is
 230 plotted.

231

232 Previous studies show that the CaCO_3 in milky fluid mostly
233 comprises ACC (Briones *et al.*, 2008), while the granules mostly
234 comprise calcite but with trace amounts of vaterite (Gago-Duport *et al.*,
235 2008; Lee *et al.*, 2008; Hodson *et al.*, 2015). Our data are consistent with
236 this. Our FTIR analyses (Fig. 2) indicate that the milky fluid was
237 dominated by ACC (the ν_4 peak at 714 cm^{-1} was absent and the
238 spectrum resembled that of the reference synthetic ACC). In contrast,
239 the granules recovered from the pouch and from the soil both had a
240 distinct peak at 714 cm^{-1} , typical of crystalline forms of CaCO_3 and a
241 spectrum almost identical to reference calcite, though we note that the
242 FTIR spectrum of vaterite is almost identical to that of calcite (e.g.
243 Hodson *et al.*, 2015) and, in contrast to our previous studies, here we
244 did not carry out the X-ray diffraction data necessary to confirm that the
245 granules are calcite and not vaterite.

246 In previous studies relict ACC has been detected in granules (Gago-
247 Duport *et al.*, 2008; Lee *et al.* 2008; Fraser *et al.*, 2011; Brinza *et al.*,
248 2013, 2014a, b; Hodson *et al.*, 2015). The ratio of the peak areas for the
249 ν_3 and ν_4 vibrations was greater for the pouch granules than for the soil
250 granules. The ratio of ν_3 and ν_4 decreases as the amount of ACC
251 decreases (Hodson *et al.*, 2015) suggesting that the granules from the
252 pouches contain a larger amount of untransformed ACC than the
253 granules recovered from the soil.

254 It is known that considerable $\delta^{13}\text{C}$ fractionation factors exist between
255 the different species of DIC and the various polymorphs of CaCO_3
256 (Romanek *et al.*, 1992; Zhang *et al.*, 1995; Szaran, 1997). The observed
257 differences in $\delta^{13}\text{C}$ values between milky fluid and the two types of
258 granules could be due to analogous isotopic fractionation. Similarly
259 Guiffre *et al.* (2015) observed a change in both the Ca and Mg isotopic
260 composition of CaCO_3 as it transformed from ACC to calcite and
261 attributed this to a dissolution-reprecipitation mechanism for the
262 transformation. Thus, the observed C fractionation reported here
263 supports suggestions that the transformation of ACC into calcite occurs
264 through dissolution and re-precipitation (e.g. Pontoni *et al.*, 2003; Han
265 and Aizenberg, 2008; Bots *et al.*, 2012; Guiffre *et al.*, 2015) rather than
266 solid state dehydration and structural rearrangement (e.g. Beniash *et*
267 *al.*, 1999; Politi *et al.*, 2008; Weiner and Addabi, 2011; Gal *et al.*, 2013).
268 As we only analysed samples by FTIR we are unable to comment on
269 whether vaterite might form as an intermediate in this transformation.

270 Using the $\delta^{13}\text{C}$ values for the milky fluid, the pouch granules and
271 median values for the soil-recovered granules for individual
272 earthworms we estimated the isotopic enrichment factor (ϵ) between
273 calcite (soil granules and pouch granules) and ACC, defined by:

274

$$275 \epsilon_{\text{calcite-ACC}} = 1000 \cdot [(\delta^{13}\text{C}_{\text{calcite}} + 1000)/(\delta^{13}\text{C}_{\text{ACC}} + 1000) - 1] \quad (1)$$

276

277 The pouch granule - milky fluid ($-0.74 \pm 0.37 \text{ ‰}$), soil granule - milky
278 fluid ($-1.20 \pm 0.52 \text{ ‰}$) and soil granule - pouch granule (-0.46 ± 0.45
279 ‰) enrichment factors were significantly different from each other
280 (Kruskal-Wallis One Way Analysis of Variance on Ranks followed by a
281 post hoc Tukey test, $p \leq 0.01$) despite significant overlap between the
282 first two. The high level of overlap between these two enrichment
283 factors is undoubtedly due to the fact that the granules in the pouches
284 and the soil are predominantly calcite. This also helps explain the soil
285 granule - pouch granule enrichment factor that is almost equal to zero
286 within error; the majority of ACC will have converted to calcite in the
287 pouch granules and therefore little additional transformation occurs
288 following expulsion of the granules from the calciferous gland into the
289 earthworm intestine and from there into the soil. Both the pouch
290 granule - milky fluid and soil granule - milky fluid enrichment factors
291 indicate an increase in the incorporation of ^{12}C relative to ^{13}C as the ACC
292 crystallizes to calcite. This incorporation of the lighter isotope in the
293 final crystallization product is in agreement with existing kinetic
294 theories on the control of isotope fractionation (Watson, 2004; DePaolo,
295 2011; Nielsen et al., 2012) and has been observed in a variety of
296 biominerals (e.g. Auclair et al., 2003; Rollion-Bard et al, 2016; Spooner

297 et al, 2016) where the $\delta^{13}\text{C}$ of crystalline calcium carbonate is compared
298 to that of dissolved precursor ions.

299 Although significant differences exist between the enrichment
300 factors calculated for different temperatures for the pouch granule -
301 milky fluid (ANOVA, $p \leq 0.01$) and soil granule - milky fluid (Kruskal-
302 Wallis One Way Analysis of Variance on Ranks, $p \leq 0.01$) linear
303 regression indicates only a small dependence of this variation on
304 temperature ($R^2 < 0.2$), consistent with previous abiotic calcite-
305 bicarbonate enrichment factors (Romanek et al., 1992), but not with
306 theories considering kinetic controls on isotopic fractionation. The lack
307 of an apparent temperature dependence may be due to either or both
308 the metabolism of the earthworms maintaining a more constant body
309 temperature in the calciferous gland than in the surrounding soil
310 (though note that oxygen fractionation is temperature sensitive to the
311 temperature of the surrounding soil, see Versteegh et al., 2013) or the
312 scatter in the $\delta^{13}\text{C}$ values.

313 Guiffre *et al.* (2015) found that the Ca and Mg isotopic composition of
314 calcite formed from ACC was sensitive to the amounts of ACC present. In
315 a similar fashion, the relatively high standard deviation values for the
316 enrichment factors may be due to a lack of end member ACC and calcite
317 used in our calculation. Although the FTIR spectra for the milky fluid
318 indicate that the only form of CaCO_3 present is ACC (Fig. 2), studies have

319 found trace amounts of calcite in the milky fluid (Gago-Duport *et al.*,
320 2008) and it is possible that that is the case here with the calcite below
321 detection levels. Trace amounts of calcite may have been produced if
322 some ACC transformed whilst the milky fluid was drying out. Our
323 results suggest that this would result in more negative $\delta^{13}\text{C}$ values.
324 Similarly the granules, although predominantly calcite, may contain
325 varying, but small, amounts of ACC (e.g. Lee *et al.*, 2008; Hodson *et al.*,
326 2015)). Small amounts of ACC appear to be unusually stable in the
327 granules and may be preserved indefinitely in the granules. Further,
328 granules recovered from the soil were secreted over a 28 day period
329 and therefore potentially show different degrees of transformation
330 from ACC to calcite. Varying levels of transformation from ACC to calcite
331 from the milky fluid to the granules recovered from the soil are also
332 consistent with the wider range of $\delta^{13}\text{C}$ values and enrichment factors
333 observed for these granules compared to those present in the pouches
334 which will have a more similar age and, therefore potentially have
335 experienced the same amount of ACC transformation.

336 Methods have recently been developed to synthesise ACC that can
337 remain stable for several days (Rodriguez-Blanco *et al.*, 2008). This
338 opens up the possibility of direct and accurate determination of $\epsilon_{\text{calcite-ACC}}$
339 for abiotic systems in the near future. More detailed carbon isotope
340 and mineralogical studies of the calciferous gland and calcite granules

341 together with other biominerals, e.g. echinoderm spines, in which both
342 ACC and calcite are present are required to better understand the role
343 of vital processes in this fractionation. As many biominerals are
344 precipitated from an ACC precursor phase, and can contain stable ACC
345 in their mature state, this will be an important step in understanding
346 the mechanisms of biomineralisation and implications for the
347 environmental interpretation of biomineral proxies.

348

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362

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Earthworms produce granules of calcium carbonate that form from an amorphous calcium carbonate suspension

The microspherulites of amorphous calcium carbonate coalesce and recrystallize

Fractionation of C isotopes occurs as the ACC recrystallizes with $\epsilon_{\text{calcite-ACC}} = -1.20 \pm 0.52\%$

This is consistent with a dissolution-reprecipitation pathway rather than solid state rearrangement

This may be important for the interpretation of CaCO_3 -based C isotope environmental proxies.