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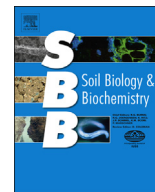
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Short communication

Environmental controls on the production of calcium carbonate by earthworms[☆]Emma A.A. Versteegh^{a,*}, Stuart Black^b, Mark E. Hodson^c^a University of Reading, Department of Geography and Environmental Science, School of Archaeology, Geography and Environmental Science, Whiteknights, P.O. Box 233, Reading RG6 6DW, UK^b University of Reading, Department of Archaeology, School of Archaeology, Geography and Environmental Science, Whiteknights, P.O. Box 227, Reading RG6 6AB, UK^c University of York, Environment Department, Heslington, York YO10 5DD, UK

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ABSTRACT

Lumbricus terrestris earthworms produce calcium carbonate (CaCO₃) granules with unknown physiological function. To investigate carbon sequestration potential, the influence of temperature and CO₂ concentration ([CO₂]) on CaCO₃ production was investigated using three soils, five temperatures (3–20 °C) and four atmospheric [CO₂] (439–3793 ppm). Granule production rates differed between soils, but could not be related to any soil characteristics measured. Production rates increased with temperature, probably because of higher metabolic rate, and with soil CO₂ concentration. Implications for carbon sequestration are discussed. CaCO₃ production in earthworms is probably related to pH regulation of blood and tissue fluid in the high CO₂ environment of the soil.

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Many earthworm species produce calcium carbonate (CaCO₃) granules in specialised glands (Darwin, 1881; Canti and Pearce, 2003). These granules are mainly calcite, with small amounts of amorphous calcium carbonate, vaterite and aragonite (Gago-Duport et al., 2008; Lee et al., 2008; Fraser et al., 2011; Brinza et al., 2013). Granule production is likely related to regulation of pH and CO₂ concentrations ([CO₂]) in body fluids, or regulation of Ca²⁺ and other potentially toxic cations (e.g. Dotterweich and Franke, 1936; Robertson, 1936; Crang et al., 1968; Pearce, 1972; Bal, 1977). *Lumbricus terrestris* is a major CaCO₃-producing species in temperate soils. Production rates range from 0.8 to 2.9 mg/earthworm/day (Canti, 2007; Lambkin et al., 2011a; Versteegh et al., 2013). With 1.9–61.8 individuals/m² (Berry and Karlen,

1993; Bernier and Ponge, 1998; Nuutinen et al., 2001; Briones et al., 2008) this equates to precipitating 2–261 kg C/ha/yr, a potentially significant contribution to carbon sequestration.

The aim of this study was to further elucidate the carbon sequestration potential of earthworms, by investigating the influences of temperature, and atmospheric and soil [CO₂] on CaCO₃ production rates in *L. terrestris*. Hypotheses were: 1) granule production increases with temperature due to increased metabolism, and, 2) granule production increases with soil [CO₂] due to increased demand for removal of CO₂ from blood and tissue fluids.

CaCO₃ production rates in *L. terrestris* were studied in laboratory experiments with a minimum of 6 replicates (individual earthworms) per treatment (Table 1). The experiments were designed to investigate the origins of the C in the calcium carbonate (results to be reported elsewhere) and so we selected soils based on the crops (C₃ or C₄) of the previous seasons. Three agricultural soils (all Typical Argillic Brown Earths; Avery, 1980) were sampled in Berkshire, UK: Hamble (SU 61968 70235, C₃), Red Hill (SU 56060 80033, C₄ > 10 years), and Winning Hand (SU 61213 69140, alternating C₃/C₄). Soils were air-dried and sieved to 250 μm (Lambkin et al., 2011b), ensuring soils were granule-free and facilitating granule recovery at the end of the experiments. For each replicate, 300 g of soil were mixed with demineralised water to 65% water holding capacity (BS ISO, 1998),

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Table 1
Experimental design and granule production rates.

Soil	WHC ^a (%)	pH ^b	Ca ^c (wt%)	Organic matter ^d (%)	T (°C)	[CO ₂] treatment	Atmospheric [CO ₂] ^e (ppm)	[CO ₂] in soil air ^f (ppm)	N ^g	Average CaCO ₃ production rate (mg/g earthworm/day)
<i>Experiment 1</i>										
Hamble	33%	7.5 ± 0.3	1.3 ± 0.02	3.8 ± 0.1	10	ND	ND	ND	18	0.27 ± 0.07a
					16				29	0.40 ± 0.08b
					20				17	0.49 ± 0.10c
Red Hill	56%	7.1 ± 0.1	0.6 ± 0.02	7.4 ± 0.1	10	ND	ND	ND	18	0.37 ± 0.06d
					16				18	0.47 ± 0.10e
					20				16	0.63 ± 0.11f
Winning hand	43%	7.4 ± 0.2	0.9 ± 0.02	5.6 ± 0.0	3	ND	ND	ND	6	0.21 ± 0.07g
					16				9	0.44 ± 0.06b,e
					18				6	0.56 ± 0.13b,e
<i>Experiment 2</i>										
Winning hand	43%	7.4 ± 0.2	0.9 ± 0.02	5.6 ± 0.0	16	Low	439 ± 79	1132 ± 395A	6	0.49 ± 0.07H
						Ambient	469 ± 43	891 ± 134A	5 ^h	0.40 ± 0.08H
						Medium	1590 ± 81	1924 ± 203B	6	0.47 ± 0.13H
						High	3793 ± 161	3122 ± 141C	5 ^h	0.57 ± 0.10H

ND = not determined; different letters (lower case for experiment 1, upper case for experiment 2) indicate significant differences between treatments at $p < 0.05$.

^a Water holding capacity.

^b Measured following Great Britain Agricultural Development and Advisory Service (1986).

^c Analysed with a handheld XRF.

^d Measured as loss on ignition.

^e Atmospheric [CO₂] in the glove boxes stabilised after 2–5 days, the “ambient” treatment showed a diurnal cycle.

^f [CO₂] in soil air at the end of the experiment, average ± 1σ of 3 replicates is shown (remaining analyses failed).

^g Number of replicates per treatment.

^h One earthworm died in each of these treatments leaving only 5 replicates per treatments; all errors are given as ± 1σ.

then put in a zip-lock bag with 5 g air-dried horse manure rehydrated with 10 ml demineralised water and one adult earthworm.

In Experiment 1 two soils were studied (Hamble and Red Hill) and each bag was placed in a constant temperature room at 10, 16, or 20 °C in darkness. During a later experiment, the Winning Hand soil was used at 16 °C as well as two additional temperatures, 3 and 18 °C, using the same methods.

In Experiment 2, earthworms were kept in open bags of soil with a mesh cover at 16 °C in glove boxes with a continuous 15 cm³/min through flow of air with different [CO₂]. [CO₂] of 210, 550 and 700 ppm were chosen to reflect the early Holocene, projected mid 21st century and end 21st century, respectively (IPCC, 2007). A fourth set of replicates was kept in ambient laboratory conditions. The mass of individual soil and earthworm-bearing containers was measured twice a week, and demineralised water added up to the original weight to compensate for evaporation. As it proved impossible to maintain the initially chosen [CO₂] in the glove boxes, beakers containing NaOH pellets or 46/48% NaOH solution were put in two of them to lower [CO₂], creating three different treatments, hereafter called “low”, “medium” and “high” (the fourth being ambient conditions in the laboratory). Atmospheric [CO₂] and temperature were measured every 10 min with an Extech SD800 datalogger. Soil air was sampled by placing a section of silicone tubing with a bung in each end in the bags of soil at the beginning of the experiment. Air was extracted from the tube using a syringe immediately at the end of the experiment (adapted from Clark et al., 2001).

For both experiments earthworms were acclimatised for three weeks, and then transferred to identical treatment bags containing the same type and mass of soil and manure at the same temperature and atmospheric [CO₂]. After 28 days earthworms were removed and the soil wet-sieved to 500 μm to retrieve granules, which were air-dried and weighed. In Experiment 2, soil gas was sampled and analysed for [CO₂] using a Thermo Fisher GC Box connected to a Delta Plus mass spectrometer.

Over both experiments, granule production ranged from 0.49 to 3.64 mg/individual/day, which equates to the sequestration of 1–329 kg C/y/ha.

Granule production rate differs significantly between the Red Hill soil and the other two (ANOVA: $F = 19.404$; $p < 0.001$; $n = 137$). This does not appear to be related to any of the soil characteristics measured (Table 1).

At higher temperatures earthworms produced more CaCO₃ (Fig. 1). The increase of CaCO₃ production rate with temperature can be explained by an increase in metabolism as expected for ectothermic animals.

In experiment 2, soil [CO₂] was measured on 3 replicates per treatment. The remaining [CO₂] analyses failed. Over these 12 replicates higher atmospheric [CO₂] resulted in higher soil [CO₂] ($[CO_2]_{soil} = 0.63 [CO_2]_{atmospheric} + 773$; $R^2 = 0.93$; $p < 0.001$; $n = 12$); soil pH showed no relationship to either atmospheric or soil [CO₂].

A comparison of average CaCO₃ production rates reveals no significant differences between the different CO₂ levels in Experiment 2. A regression analysis however, shows a weak relationship

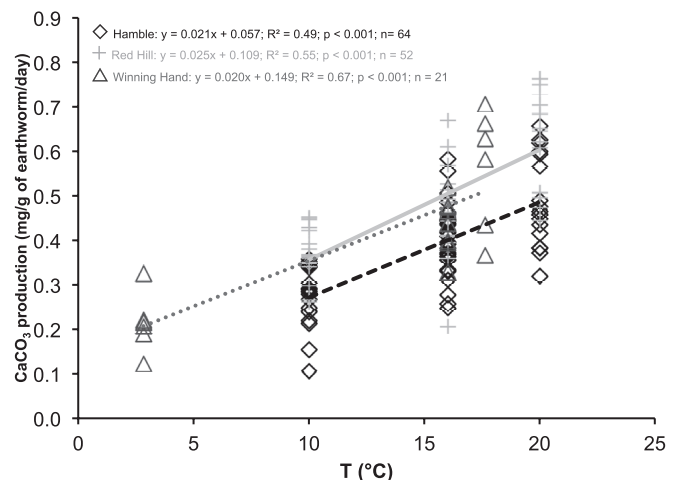


Fig. 1. The relationship between temperature and CaCO₃ production by *L. terrestris* for three different soils.

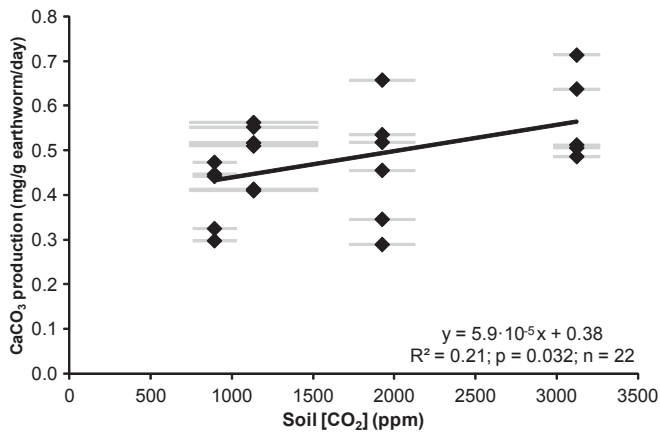


Fig. 2. Granule production rates per replicate, plotted against average soil [CO₂]. CaCO₃ production by *L. terrestris* increases with soil [CO₂] following the relationship $[\text{CO}_2]_{\text{soil}} = 5.9 \cdot 10^{-5} \text{ CaCO}_3 \text{ (mg/g earthworm/day)} + 0.38$ ($R^2 = 0.21$; $p = 0.032$; $n = 22$) consistent with increased [CO₂] causing more HCO₃⁻ being removed from earthworm tissues by granule production.

with higher soil [CO₂] resulting in higher CaCO₃ production rates (Fig. 2; $p = 0.032$; $R^2 = 0.21$).

There is on-going debate as to whether earthworms increase soil greenhouse-gas emissions or carbon sequestration and the timescale and nature of experiments required to determine this (Lubbers et al., 2013; Zhang et al., 2013). Our study shows that it is likely that at higher temperatures and atmospheric [CO₂], earthworm CaCO₃ production will increase. As granules can survive in soils for >300,000 years (own data), the potential sequestration of C in the form of CaCO₃ is on a longer-timescale than e.g. roots and soil organic matter. More studies are needed to elucidate the C sequestration potential of earthworms under field conditions.

Our results of increased granule production under elevated CO₂ support the interpretation that granule production buffers earthworm tissue HCO₃⁻, which would otherwise increase due to higher [CO₂]. This is consistent with the findings of Kühle (1980) who observed increased incorporation of ¹⁴C-labelled CO₂ in calciferous gland tissue at 5.0% CO₂ compared to 0.2% CO₂, although granule production was not recorded. Voigt (1933) carried out experiments at far higher [CO₂] > 14% and recorded reduced granule production. At this extreme level of CO₂, HCO₃⁻ may have been retained in tissues/fluids to buffer potential pH changes. The work of Kaestner (1967) and the negative relationship between pH and granule production observed by Lambkin et al. (2011b) support this interpretation. The restricted pH range of the soils used in this study may have prevented this relationship from being apparent here. Thus it appears that granule production can increase or decrease depending on whether HCO₃⁻ is potentially in excess or is required for pH buffering of tissues. However, more research is needed to establish how CaCO₃ production rates vary under a wider range of soil [CO₂], including measurements of earthworm tissue fluid [CO₂] concentrations and pH under different soil [CO₂] regimes.

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