



# Hatchling turtles ingest natural and artificial incubation substrates at high frequency

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## Abstract

Geophagy is the consumption of hard objects with no caloric value (e.g. soil, sand, sediment) called gastroliths. This behaviour is widespread in animals, and among reptiles, geophagy has been reported in crocodilians and lizards and occasionally in chelonians. In this study, we calculated geophagy rates in snapping turtle (*Chelydra serpentina*) and painted turtle (*Chrysemys picta*) hatchlings under various incubation protocols, ranging from highly artificial to semi-natural conditions. Among multiple experiments where eggs were incubated atop the nesting substrate, 66% of painted turtle and 58–93% of snapping turtle hatchlings exhibited geophagy within 24 h of hatching. Snapping turtle hatchlings that experienced a simulated natural nest emergence had an 85–100% rate of gastrolith consumption. Hatchling snapping turtles from shallow simulated nests emerged earlier and had higher rates of geophagy compared with those from deeper nests. Given the high frequency, short time period (24–72 h) and variety of incubation protocols under which geophagy occurred, we suggest that this behaviour is intentional. We discuss multiple hypothesis for the adaptive and functional significance of geophagic behaviour with respect to chelonians, synthesize existing literature on chelonian geophagy and highlight the possible implications of hatchling turtle geophagy for ex situ captive breeding and head-starting programs. Given that hatchlings readily consume their incubation medium, caretakers should carefully consider the substrate their animals are exposed to. Future research should address how widespread geophagy is among hatchling turtles and the possible role of this behaviour for hatchling ecology and health, including effects on the gut microbiome.

## Significance statement

Animals regularly consume non-caloric foods, such as rocks and soils. This behaviour is exhibited by hatchling turtles, but why? Our literature review suggests that the ingestion of non-caloric foods by turtles is important for nutrition and, in particular, this behaviour may help establish gut health in hatchlings. Observational and experimental study demonstrated that nest characteristics in-part account for why hatchling ‘turtles eat dirt’. This has applications for hatchling ecology as well as captive rearing conservation programs, an increasingly common strategy for the conservation of these globally imperilled animals. This work complements several recent studies and review articles about geophagy in other major vertebrate groups (birds, mammals) and provides a comprehensive summary on the current state of knowledge of this behaviour for turtles.

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Mariel Terebiznik and Patrick D. Moldowan contributed equally to this work.

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## Introduction

Gastroliths (from Greek γαστήρ—‘stomach’ and λίθος—‘rock’; Panichev et al. 2013) are hard objects with no caloric value, such as soil, sand and sediments, that are retained in the digestive tract of an animal (Wings 2007). The act of ingesting gastroliths is known as geophagy, from Greek γη—‘earth’ and φάγειν—‘to eat’ (also see lithophagy, from Greek λίθος—‘rock’ and φάγειν—‘to eat’; Panichev et al. 2013). Geophagy has been identified in a large diversity of fossil taxa (Whittle and Everhart 2000), as well as extant invertebrates, fishes, birds (Downs et al. 2019) and mammals (Taylor 1993). Scattered observations of geophagy are reported in chelonians and other reptiles (Sokol 1971; Whittle and Everhart 2000; Wings 2007).

The study of geophagy is relevant for understanding nutrition (Beyer et al. 1994) and assessing environmental contaminant exposure (Hui 2004). Also, this facet of natural history has applications for animal husbandry and captive breeding programs such as head-starting, a common strategy used for turtle conservation (Burke 2015; Bennett et al. 2017). Many hypotheses have been developed to explain the adaptive significance of geophagy, including use of ingested substrate as a digestion aid, acquisition and assimilation of nutrients, parasite management, gut microbiome development and assistance in aquatic locomotion (Kreulen 1985; Taylor 1993; Krishnamani and Mahaney 2000; Wings 2007; Panichev et al. 2013; Downs et al. 2019). Geophagy and its explanatory hypotheses are better studied in birds (Downs et al. 2019) and mammals (Klaus and Schmid 1998; Krishnamani and Mahaney 2000; Slabach et al. 2015; Worker et al. 2015) in contrast to chelonians and other reptiles (Sokol 1971; Hui 2004). Among chelonians, most reports of geophagy are limited to terrestrial taxa, especially female tortoises (Testudinidae; e.g. Esque and Peters 1994; Walde et al. 2007; Moore and Dornburg 2014; Sullivan and Cahill 2019) and hatchlings of the painted turtle, *Chrysemys picta* (Packard et al. 2001; Costanzo et al. 2003; Packard and Packard 2003a, 2006). Despite reports of geophagy and its significance in animals, there exists no study comparing the intra- or interspecific frequency of this behaviour in chelonians, especially in early life stages. Additionally, most reports of chelonian geophagy come from scat observations and dissection (see Table S1). The limited number of direct geophagy observations, especially in hatchlings, makes it unclear whether gastrolith ingestion is an incidental rather than an intentional behaviour.

The objectives of this study are several fold: (1) contribute to the body of geophagy literature by reporting on the

frequency of gastrolith ingestion in hatchling snapping turtles (*Chelydra serpentina*) and painted turtles (*Chrysemys picta*) under standard incubation procedure and/or simulated natural hatchling emergence conditions; (2) compare rates of geophagy between simulated natural nest emergence and standard incubation procedure to evaluate the possibility of incidental ingestion; (3) examine patterns and consider hypotheses explaining geophagy in hatchling chelonians; (4) discuss the adaptive significance of geophagy in chelonians; and (5) synthesize existing literature on chelonian geophagy.

## Methods

The geophagy observations reported in this paper were collated from several separate experiments performed over a 2-year period. There is no single turtle egg incubation protocol; however, it is commonplace for those incubating eggs for research and conservation purposes to place eggs at or near the surface (0 cm depth) of the incubation substrate for the duration of incubation, which allows for close monitoring of eggs without disturbance. This contrasts the natural state of incubation for turtle eggs, where eggs are buried in a subterranean cavity excavated by a nesting female. Thus, the phrase ‘standard incubation procedure’ refers to egg incubation on the substrate surface typical of *ex situ* methods.

The present study was conducted as four main experiments that took place in spring-summer 2018 and 2019 at the University of Toronto (Table 1). Snapping turtle (*Chelydra serpentina*) and midland painted turtle (*Chrysemys picta marignata*) eggs were collected from Algonquin Provincial Park, Ontario, Canada (45° 35' N, 78° 30' W), as part of a long-term study on temperature-dependent sex determination (Schwarzkopf and Brooks 1985; Bobyn and Brooks 1994; Massey et al. 2019). Dissection of hatchlings was required for sex identification associated with these independent experimental studies, therefore allowing data collection on gastrolith presence or absence in multiple experimental contexts. To minimize observer bias, blinded dissections occurred where the experiment was known, but hatchling treatment was unknown. All methods were approved by the Biological Sciences Local Animal Care Committee at the University of Toronto under Animal Use Protocol No. 20011948. Animals were approved for collection under a Scientific Collectors Permit from Ontario Parks No. 1093596.

Nesting of both species is concentrated in June in Algonquin Provincial Park (Rollinson and Brooks 2008; Edge et al. 2017; Francis et al. 2019). As outlined for each experiment (see below and Table 1), eggs were incubated at

**Table 1** Experimental methods and summary statistics of geophagy in hatchling snapping turtle (*Chelydra serpentina*, CS) and painted turtle (*Chrysemys picta*, CP)

Expt.	Protocol and experimental conditions	Year	Sp.	Nests	Hatchlings	% with gastroliths
1	Incubated <i>in situ</i> in wild for <i>ca.</i> 60 days, then incubated atop substrate (0 cm depth) at 27 °C	2018	CS	5	62	77
2		2019	CS	10	153	78
2A	Lab incubated, 0 cm depth at 24 °C				29	86
2B	Lab incubated, 0 cm depth at 28 °C				40	57.5
2C	Lab incubated, 0 cm depth, sinusoidal 24 ± 4 °C				29	93
2D	Lab incubated, 0 cm depth, sinusoidal 28 ± 4 °C				55	80
3	Incubated <i>in situ</i> in wild until late embryonic development (stage 25; Yntema 1968). Eggs collected, brought to lab, incubated in sand at room temperature (~ 22 °C) simulating natural nest environment. Eggs incubated in 2 × 2 factorial design: egg burial depth (shallow at 5 cm or deep at 15 cm) and simulated clutch size (solitary egg or a group of 10 eggs)	2019	CS	9	175	92
3A	Shallow and solitary; mean ( $\bar{x}$ ) ± standard deviation (SD) = 3.75 ± 2.46 days to emergence, range = 0.5–10 days to emergence				16	100
3B	Shallow and group; $\bar{x}$ ± SD = 3.63 ± 1.74 days to emergence, range = 1–12 days to emergence				75	96
3C	Deep and solitary; $\bar{x}$ ± SD = 6.27 ± 2.66 days to emergence, range = 3–14 days to emergence				15	93
3D	Deep and group; $\bar{x}$ ± SD = 4.99 ± 1.72 days to emergence, range = 2–12 days to emergence				69	85.5
4	Lab incubated at constant pivotal temperature of 27.5 °C	2019	CP	27	44	66

Sp. species

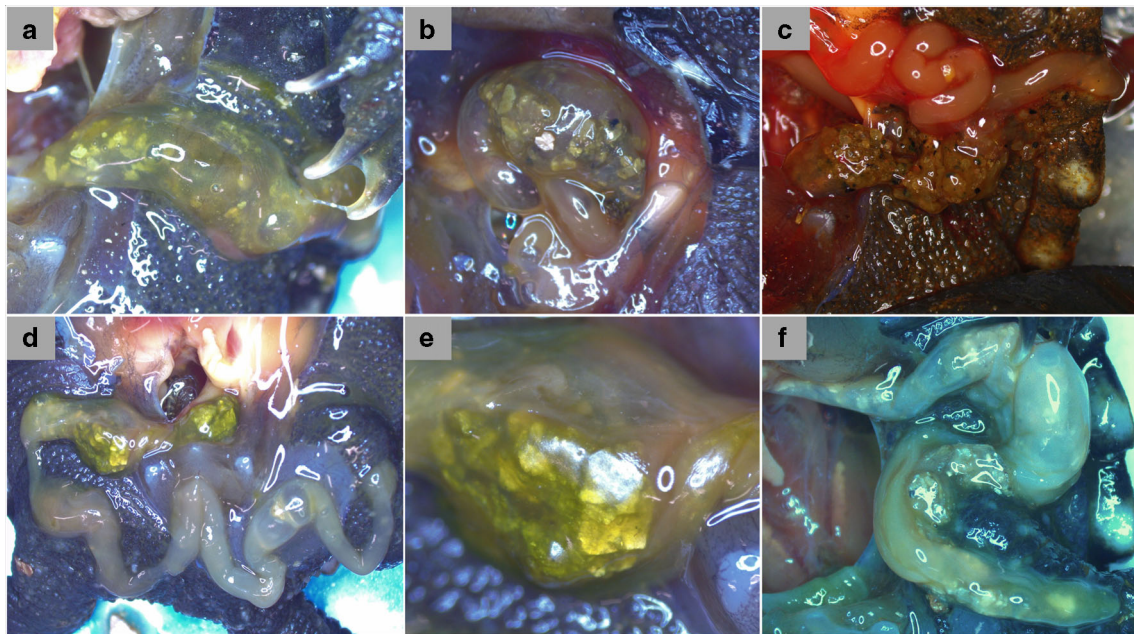
different depths relative to the substrate surface (0 cm, 5 cm or 15 cm depth), temperatures, group sizes (1 or 10 eggs) and substrates (vermiculite or coarse sand/fine gravel). The differences in temperature and group size among experiments are a consequence of other concurrent research projects. There is presently no clear biological reason to believe that geophagy in hatchlings is related directly to incubation temperature. Thus, we focus on the possible relationship between geophagy, nest depth and hatchling group size in this study.

**Experiment 1** In 2018, five snapping turtle nests were incubated *in situ* from time of laying until late August (i.e. for majority of the embryonic development period). In late August, eggs were removed from wild nests and transported to the University of Toronto, where they were incubated in individual containers at 27 °C on the surface (0 cm) of a layer of vermiculite substrate (1:1 water to substrate ratio, by weight) and monitored daily for hatching. The expanded (exfoliated) vermiculite incubation medium used in this study (Schultz®; Atlanta, GA) had a loosely layered accordion-like structure with soft granules of 2–4-mm diameter (Bush 2001; Bar-tal et al. 2019), typical of commercially available vermiculite used as a soil additive in horticulture and for reptile egg incubation. Within 24 h of hatching, the turtles were euthanized for macroscopic gonadal inspection and their gastrointestinal (GI) tract was examined for gastrolith presence or absence (i.e. whether an individual demonstrated geophagy) under a dissecting microscope ( $n = 62$  hatchlings, 5 nests; Fig. 1). A

hatchling was determined as having exhibited geophagy if fragments of vermiculite or sand were spotted anywhere in the GI tract from the oesophagus to the colon. Gastroliths were commonly observed through the translucent GI tract (e.g. Fig. 1a); however, subsequent dissection of the GI tract took place if the presence of gastroliths was ambiguous (e.g. Fig. 1c). Vermiculite presence was easily identified given its distinctive polygonal shape and semi-metallic shine (Fig. 1a, b, Fig. 1d–f) while sand/gravel particles were identified as multi-coloured coarse-grained sediment present in the GI tract (see Fig. 1c). If presence of geophagy was not able to be clearly ascertained (ex. yolk popped during removal spilling into the cavity and coating the GI tract), the hatchling was removed from the study ( $n = 44$  out of 501 hatchlings). Turtles had a maximum of 24 h contact time with the vermiculite incubation substrate. It was assumed that any substrate ingested after hatching was still present within the GI tract at the time of dissection.

**Experiment 2** In 2019, 10 snapping turtle nests ( $n = 153$  hatchlings) were collected at time of laying, brought to the University of Toronto, and incubated on vermiculite (1:1 water to substrate ratio, by weight) under four temperature regimes in the laboratory: constant temperature of 24 °C ( $n = 29$ ; experiment 2A); constant temperature of 28 °C ( $n = 40$ ; experiment 2B); 24-h sinusoidal wave cycle of 24 ± 4 °C ( $n = 29$ ; experiment 2C); and 24-h sinusoidal wave cycle of 28 ± 4 °C ( $n = 55$ ; experiment 2D) (Table 1). Upon hatching, turtles





**Fig. 1** Ingestion of substrate by hatchling snapping turtle, *Chelydra serpentina*. **a** Gut lightly packed with vermiculite incubation substrate. **b** Gut densely packed and distended with vermiculite. **c** Densely packed sand spilling from gastrointestinal tract ruptured during dissection. **d**

Boluses of vermiculite in the gut and intestinal tract. **e** Close-up of vermiculite bolus in intestinal tract from panel **d**. **f** Vermiculite spread throughout the gastrointestinal tract from oesophagus to intestine. Photos by M. Terebiznik

had up to 24 h of contact time with substrate. Turtles were processed as above.

**Experiment 3** In 2019, eight snapping turtle clutches incubated *in situ* in wild nests for approximately 60 days until they reached late-stage embryonic development (Yntema stage 25; Yntema 1968;  $n = 175$  hatchlings). Upon excavation from wild nests, eggs were transported to a laboratory at the University of Toronto and were assigned to treatments that simulated natural nest emergence conditions. Eggs were divided among 48 wide-mouth plastic jars (3.8 L) filled with sieved substrate (sieve mesh size 5 mm) composed of medium-coarse consistency sand (0.25–2.0 mm diameter) and fine gravel ( $\geq 2.0$  to  $< 5$  mm) that was 25% saturated with water (McGehee 1990; Delmas et al. 2008) and incubated for the remainder of development at room temperature ( $\sim 22^\circ\text{C}$ ). These eight clutches were part of a  $2 \times 2$  factorial design, with two levels of egg burial depth (deep: 15 cm, shallow: 5 cm) and two levels of clutch size (solitary: 1 egg, grouped: 10 eggs) leading to four treatments: experiment 3A, shallow and solitary ( $n = 16$ ); experiment 3B, shallow and grouped ( $n = 75$ ); experiment 3C, deep and solitary ( $n = 15$ ); and experiment 3D, deep and grouped ( $n = 69$ ) (Table 1). Each egg was randomly assigned a treatment, but sibling eggs were always kept together in grouped treatments so that clutch ID could be later treated as a random factor. Jars were wrapped in black paper to eliminate potentially disruptive light cues for hatchlings during emergence. Turtles hatched from their eggs and evacuated the nest cavity by digging to the surface,

simulating a natural nest emergence. Jars were checked twice daily (morning and evening) for egg pipping and hatchling emergence, giving a maximum precision interval of 0.5 days when assigning the timing of these events. Once surfaced, time to emergence (date emerged subtract date pipped) was recorded, and turtles were processed as above. One time to emergence data point (a hatchling that was excavated from a simulated nest after 25 days) was identified as an outlier and removed from the experiment 3D dataset because the hatchling failed to emerge on its own accord and its time to emergence exceeded that of the next closest individual by 11 days.

**Experiment 4** In 2019, a sample of eggs from 27 painted turtle nests were collected soon after laying and transported to a laboratory at the University of Toronto. Eggs from the same nest were incubated together at a constant  $27.5^\circ\text{C}$  until hatching on the surface of a layer of vermiculite (1:1 water to substrate ratio, by weight). The eggs were separated into individual containers once pipping began and were monitored daily for hatching. Hatchlings used in this study ( $n = 41$  hatchlings) had up to 24 h of contact time with substrate. Turtles were processed as above.

**Statistical analyses** We calculated the proportion of individuals that demonstrated geophagy (i.e. presence of gastroliths in GI tract) across all experiments and treatments (Table 1). Using the data from experiment 3, we used three generalized linear mixed effects models to (model 1) evaluate the effect of the experiment variables egg burial depth, group size and

interactions thereof on the presence of gastroliths in the GI tract of hatchling snapping turtles; (model 2) evaluate the effect of time to emergence (i.e. contact time with substrate) on the presence of gastroliths of hatchling snapping turtles; and (model 3) determine if egg burial depth and group size explained time to emergence (Table S2). In all three models, maternal nest identity (accounting for the possibility that individuals of the same clutch may display systematic differences in behaviour) and experimental nest ID (accounting for possible systematic differences between experimental units) were included as random effects (Table S2). Significance was determined using  $p$  values provided by lmerTest (Kuznetsova et al. 2017) at  $\alpha = 0.05$ . In models 1 and 3, the interaction between egg burial depth and group size was non-significant and was removed from the analysis. Data analysis and modelling were conducted in R statistical software (R Core Team 2020), using the lme4 package, version 1.1-21 (Bates et al. 2015).

## Results

Across all experimental treatments, gastroliths were common inside the GI tract of hatchling snapping and painted turtles. Gastroliths were found in the oesophagus, stomach and large and small intestines of both species and ranged from a few small fragments to densely packed substrate (to the point of being distended) throughout areas of the GI tract (Fig. 1). When present, gut distension seems to have occurred due to the sizable volumes of ingested substrate but may also been a consequence of expansion by fluid-absorbing substrates, such as vermiculite. Although not specifically quantified, hatchlings that emerged from simulated nests (i.e. hatchlings that dug from below surface, experiment 3) appeared to have a more densely packed GI tract than did turtles that hatched from eggs positioned atop of nesting substrate (experiments 1, 2 and 4). Among hatchlings from surface-incubated eggs, a total of 77% (48 of 62; experiment 1) and 78% (119 of 153; experiment 2) of snapping turtles ingested vermiculite incubation substrate in 2018 and 2019, respectively (Table 1). In total, 92% (161 of 175) of snapping turtle hatchlings that experienced a simulated natural nest emergence (i.e. digging from depth to sand surface; experiment 3) had sand in their GI tract (Table 1). Sixty-six percent of surface-incubated painted turtles (29 of 44) ingested vermiculite (experiment 4; Table 1).

In experiment 3, egg burial depth tended to explain geophagy ( $z = 1.83$ ;  $p = 0.068$ ; Table S2; Fig. 2a). The probability of geophagy in the shallow egg burial treatments was 0.98 (asymptotic lower CL = 0.92; asymptotic upper CL = 1.00) compared with 0.93 in the deep treatments (0.79–0.98). Among experiment 3 treatments, time to emergence (i.e. substrate contact time) ranged from 0.5 to 14 days (Table 1). Time to emergence was a significant predictor of geophagy such that

gastroliths were more likely to be detected in individuals that emerged from simulated nests earlier (i.e. had reduced substrate contact time;  $p = 0.030$ ; Fig. 2b; Table S2). Egg burial depth was significantly related to time to emergence (i.e. substrate contact time;  $p = 0.002$ ; Table S2) with hatchling emergence from shallow nests (5 cm) on average 1.94 days earlier (SE = 0.56) compared with those from the deeper nests.

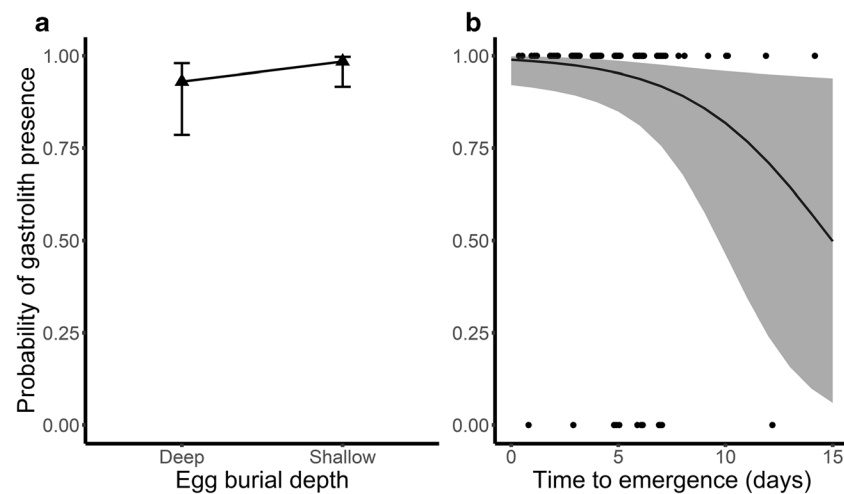
## Discussion

The overarching aim of the present study is to gain insight into the widespread but puzzling behaviour of geophagy in turtles. Here, we report both artificial (vermiculite) and natural (sand/gravel) substrates are consumed at high frequency in hatchling snapping and painted turtles. The probability of geophagy in hatchling snapping turtles was inversely related to time to emergence from underground nests, positively but weakly related to egg burial depth, and unrelated to hatchling group size. Our observations and analyses expand knowledge of geophagy in the early life of chelonians and promote discussion on the functional significance of geophagy.

### Geophagy by hatchling turtles

It is reasonable to speculate that propensity for geophagy in hatchling chelonians could be related to contact time with substrate, as greater contact time affords greater access to substrate. In turn, deeper nests result in prolonged contact time and access to substrate. Group size may therefore be negatively associated with geophagy, as more excavating hatchlings may accelerate time to emergence and reduce substrate contact time. Despite such speculation, our experimental approach and modelling showed that hatchling snapping turtles from shallow simulated nests emerged earlier and had higher rates of geophagy compared with those from deeper nests.

Ingestion of natural soils, vermiculite incubation medium and eggshells has been previously reported for hatchling snapping turtles (Packard et al. 2000) and painted turtles (Packard et al. 2001; Costanzo et al. 2003; Packard and Packard 2003a, 2006; Table S1). Research on geophagy in painted turtles has been investigated in a more detailed manner, driven by the possible significance of geophagy for overwintering success in this species (Costanzo et al. 2003; Packard and Packard 2003a, 2006). In our study, 66% of painted turtle hatchlings ingested vermiculite (Table S1), which is lower than previously reported ingestion rates (92%, Packard et al. 2001; 90–100%, Costanzo et al. 2003; 100%, Packard and Packard 2006; Table S1). Large differences in methodology exist between studies (e.g. how long hatchlings were permitted access to substrate, placement of eggs within or atop of substrate; Table S1), including the present study (Table 1), and probably led to different results. For example, it is



**Fig. 2** Geophagy in hatchling snapping turtle, *Chelydra serpentina*. **a** Egg burial depth (deep: 15 cm, shallow: 5 cm) tended to relate to the probability of gastrolith presence ( $z = 1.83$ ;  $p = 0.068$ ). Points show the probability of gastrolith presence from a model including egg burial depth and social treatment (together/single—i.e. model 1). Error bars indicated upper and lower 95% confidence intervals. **b** The effect of time to emergence on the probability of gastrolith presence (1 = gastrolith

presence; 0 = gastrolith absence). Geophagy was significantly related to time to emergence with individuals that emerged earlier (i.e. had reduced substrate contact time) more likely to ingest gastroliths ( $z = -2.18$ ;  $p = 0.030$ ). The line indicates the relationship between time and emergence and probability of gastrolith presence (i.e. model 2). The shaded area is the upper and lower 95% CIs

clear that substrate contact time and possibly egg burial depth (experiment 3) are important factors in predicting gastrolith presence. Finding higher geophagy rates among hatchlings that experienced shorter contact time with substrate seems initially counterintuitive; however, it is likely that geophagy is underestimated among individuals with longer contact time. It is clear that hatchlings consume substrate soon after emergence from the egg, but they also pass the ingested material quickly (< 12 days) especially when maintained under warm conditions (> 20 °C; Packard and Packard 2006). It is also possible that the propensity for geophagy may vary among populations. Previous geophagy studies have been limited to a cold-tolerant Nebraska population of painted turtle (Packard et al. 2001; Costanzo et al. 2003, 2008; Packard and Packard 2006), and our population of painted turtle is near the northern climatic range limit for the species (Edge et al. 2017; Janzen et al. 2018). Study of geophagy in less cold-exposed or cold-tolerant populations may be informative for discerning population-specific patterns of geophagy, if any. Differences in climate may be relevant for geophagy as hatchling painted turtles tolerate sub-zero temperatures while overwintering in shallow subterranean nests (Storey et al. 1988; Ultsch 2006). Under field conditions, the supercooling capacity of hatchlings is constrained by the complex interplay of ice, ice-nucleating agents in the environment (that gain access to hatchling tissues through oral and non-oral routes) and/or substrate ingestion (Costanzo et al. 2008). The ingestion of soil is seemingly problematic because soil contains ice-nucleating agents (e.g. fine particulates, microorganisms) that can freeze at sub-zero temperatures and permanently inhibit the supercooling capacity of hatchling, even after material has been purged from the GI tract (Packard et al. 2001; Costanzo et al. 2003; Packard and

Packard 2003a). Although substrates are a source of ice-nucleating agents, the action of ingestion may activate function of the gastrointestinal tract and may help purge the gut of high-risk endogenous sources of ice-nucleating agents (e.g. residual yolk, water; Packard et al. 2001; Costanzo et al. 2003; Packard and Packard 2003a). Experimental evidence suggests that gut contents must be purged prior to overwintering, a process taking 12–24 days depending on temperature (Packard and Packard 2006), in order to achieve a supercooling capacity that will support overwinter survival (Packard et al. 2001; Costanzo et al. 2003; Packard and Packard 2003a, b, 2006). Thus, it is perplexing that hatchling painted turtles would ingest substrate if such behaviour seemingly compromises their thermal tolerance and overwintering success.

Geophagy in species that are not capable of supercooling (e.g. snapping turtle; Obbard and Brooks 1981; Packard and Packard 1990; Packard et al. 1993; Costanzo et al. 1999; Ultsch 2006) combined with common observations of geophagy in terrestrial tortoise species (Table S1) suggests that there may be a broader adaptive significance to this behaviour.

### Geophagy across chelonian life stages

Among chelonians, geophagy has been reported from at least 16 species (11 genera, 5 families; Table S1), representing ~4.5% of extant chelonian species diversity (~11% generic, 43% family diversity; Turtle Taxonomy Working Group 2017). The bulk of geophagy observations are reported for females of terrestrial taxa and in the hatchlings of two particularly well-studied species,



*Chelydra serpentina* and *Chrysemys picta* (Table S1). This likely represents a sizable underestimate of geophagy, with respect to taxonomic and ecological diversity, in chelonians. The extent and frequency of geophagy in the wild are difficult to ascertain, especially owing to the cryptic behaviour of aquatic species. It is evident that the method used to assess geophagy strongly influences the detectability of this behaviour. For example, several thousand hours of field research over 11 years, comprising several hundred tortoises of both sexes, resulted in only six observations of female *Gopherus agassizii* exhibiting geophagy (Marlow and Tollestrup 1982). Geophagic behaviour was directly observed in <1% of wild *G. agassizii*, although radiographs revealed stones and soil in 67% of individuals (Esque and Peters 1994). Observations of geophagy for semi-aquatic and aquatic chelonians are nearly absent in the literature, excluding the terrestrial hatchling life stage (Table S1). Beyer et al. (1994) inferred that soil constitutes 5.9% of the semi-aquatic painted turtle diet based on faecal analysis, although it is unclear whether this represents incidental ingestion during feeding or intentional consumption of substrate. A paucity of information makes it unclear whether gastroliths occur naturally in marine turtles (Taylor 1993, but see Meylan 1988 and Table S1). A low frequency of gastroliths in aquatic turtles may be due to an absence of geophagic behaviour (e.g. because nutritional needs are generally met by their diet), caused by a general lack of suitable substrate in these environments, and/or difficulty of observation.

### Incidental and intentional geophagy

One explanation for geophagy is that the ingestion of substrate occurs passively or incidentally with other foodstuffs or while burrowing. Alternatively, geophagy is intentional and serves a beneficial purpose for the consumer (see below). Establishing incidental ingestion as opposed to intentional ingestion of gastroliths is challenging. For example, the small size (~1 mm diameter; <0.2 g) of gastroliths found in adult wild *Testudo hermanni* can be reasonably attributed to accidental ingestion (Gagno and Alotto 2010). On the contrary, a growing number of behavioural observations report juvenile and adult tortoises, namely females, actively forage for and consume gastroliths (e.g. Esque and Peters 1994; Stitt and Davis 2003; Sullivan and Cahill 2019; Table S1). There is no clear indication whether hatchling turtles incidentally and/or intentionally consume their nesting substrate. Naturally, geophagy would occur underground when hatchlings emerge from their nests and/or prepare to overwinter within the nest, out of sight of human observers. If geophagy in hatchling turtles is largely or strictly incidental, we expect that hatchlings in simulated nests would demonstrate a higher rate of geophagy compared with hatchlings that hatched on top of the substrate.

That is, digging and emergence from an underground nest provide more substrate contact time and opportunity for ingestion. In the present study, snapping turtle hatchlings emerging from buried eggs had greater rates of geophagy (91.5%, pooled from experiment 3) compared with those that hatched from eggs resting on top of the substrate (79%, pooled from experiments 1 and 2; but see low value in experiment 2b; Table 1). However, geophagy rates were slightly higher among hatchlings emerging from shallow nests (96–100%, experiment 3A–B) compared with those emerging from deeper nests (85–93%, experiment 3C–D), contradicting the expectation of incidental ingestion. This contradiction makes it uncertain whether hatchling geophagy in our study was incidental and/or intentional. However, in our other experiments (experiments 1, 2, 4), turtles typically had access to substrate for 24 h or less, and high rates of geophagy were observed in these hatchlings. Given that the eggs in experiments 1, 2 and 4 were not buried, these observations suggest that turtles actively ingested substrate and did so almost immediately after coming into contact with the incubation medium, making incidental ingestion an unlikely explanation for geophagy. This idea is supported by other studies in which hatchlings have exhibited high rates of geophagy under various incubation protocols and substrate contact times (Table S1). Aquatic freshwater turtles, such as snapping and painted turtles, are considered to have a limited capacity for aerial ingestion (instead using water to establish a suction feeding mechanism; Bramble 1973; Bramble and Wake 1985; Stayton 2011; Moldowan et al. 2015). On the contrary, it is evident from high rates of geophagy involving relatively large volumes of ingested substrate in short periods of time (Packard et al. 2001; Costanzo et al. 2003; Packard and Packard 2006; this study) that hatchling chelonians are very much capable of aerial ingestion. This all begs the question: why consume gastroliths?

### Adaptive hypotheses explaining geophagy

Several works have speculated on the adaptive significance of geophagy. Gastroliths often serve as mineral supplementation for animals (Krishnamani and Mahaney 2000; Wings 2007). Female *Gopherus* tortoises mine, consume and repeatedly visit calcium-rich mineral deposits during egg production to replenish their calcium levels (*G. agassizii*, Marlow and Tollestrup 1982; Esque and Peters 1994; *Gopherus polyphemus*, MacDonald and Mushinsky 1988; Moore and Dornburg 2014; *G. morafkai*, Stitt and Davis 2003; Sullivan and Cahill 2019). For example, female *G. morafkai* were observed to consume calcium-rich caliche rock only during the hot/dry spring period, but not in early spring or during the monsoon/fall season (Sullivan and Cahill 2019). Soil calcium level at sites mined by tortoises is significantly higher than that of surrounding areas, demonstrating targeted consumption of calcium-rich substrate (Marlow and Tollestrup 1982; Sullivan and Cahill 2019). Detailed descriptions from direct

observation and accompanying photos of tortoises feeding at mine sites and on caliche clearly demonstrate that the consumption of gastroliths can be intentional (Marlow and Tollestrup 1982; Sullivan and Cahill 2019). Presumably consumption of substrate materials meets essential minerals, namely calcium and phosphorus, for growth and reproduction. Similarly, the opportunistic scavenging of bones (osteophagy) is reported among box turtles (Legler 1960) and tortoises (Bally 1946; Milton 1992; Esque and Peters 1994; Walde et al. 2007; Moore and Dornburg 2014; Table S1). Turtles have the heaviest bony skeleton of any extant vertebrate (Iverson 1982, 1984), and owing to the high costs of reproduction, females will draw on skeletal calcium reserves to meet the demands of egg production (Edgren 1960; Stone 2009). Thus, where observed, the importance of geophagy and osteophagy for acquiring essential minerals seems obvious. The consumption of eggshells and select substrates by hatchlings may assist with calcium supplementation and short-term post-hatching skeletal growth (Packard et al. 2000). The hypothesis that ingested eggshell fragments provide mineral supplementation has been initially discounted based on their proportionately small contribution to hatchling gut contents and their intact condition in the gut (Costanzo et al. 2003), although explicit testing is still required.

Gastroliths have also been proposed to function as hydrostatic ballasts in crocodilians and plesiosaurs, helping to control aquatic movements (Taylor 1993; Henderson 2003, 2006; Uriona et al. 2019). However, the hydrodynamic ballast hypothesis has been questioned for turtles on the premise that the weight of the shell would act as a sufficient ballast and that the feeding of (marine) chelonians on sessile or slow-moving food would negate the need for a ballast for hydrodynamic foraging (Taylor 1993). Given that most hatchling painted turtles in our focal population overwinter in the nest (56–92%, Riley et al. 2014; 100%, Storey et al. 1988), and would presumably pass the gastroliths before overwintering, it seems unlikely that gastroliths are related to buoyancy in hatchling turtles.

Geophagy can also act to neutralize dietary toxins and enhance the bioactivities of foodstuffs (Kreulen 1985; Gilardi et al. 1999; Klein et al. 2008). However, this function is not applicable to non-feeding hatchling turtles that are reliant on yolk reserves. This explanation is likely of little relevance for hatchling aquatic turtles but may be relevant for the fibrous diet of many adult tortoises. For instance, the role of gastroliths in triturating food is established in birds (Wings 2007; Downs et al. 2019), and additional evidence from birds (Robinson et al. 2008) and mammals (Knezevich 1998) suggests that the ingestion of substrates can alleviate or counteract the symptoms caused by endoparasite infection. While there is no evidence linking this latter function to turtles, geophagy may also allow hatchling turtles to inoculate their gut with beneficial microbial flora (Costanzo et al. 2003, 2008). For

example, the eggshells of red-eared sliders (*Trachemys scripta elegans*) can host bacteria (e.g. *Salmonella*) during incubation (Holgersson et al. 2016) that may later be ingested, inoculating the otherwise uncolonized gut microbiome. Female turtles commonly urinate prior to and during nesting, an action that serves to loosen soil and help retain the shape of the nest chamber while digging (Legler 1954; Patterson 1971; Ehrenfeld 1979). While there are seemingly no bacteriostatic properties associated with turtle urine (Patterson 1971), there could be some mineral and/or microbial component(s), like urea, that is sought for consumption by hatchlings. Urea is an important cryoprotectant in hatchling painted turtles, among many other species (Costanzo et al. 2006). Ureolytic bacteria in the gut of wood frogs (*Lithobates sylvaticus*), for example, is key to reclaiming nitrogen that is then used to manufacture urea as an overwintering cryoprotectant (Weibler et al. 2018). Geophagy may inoculate the gut of hatchling turtles with ureolytic microorganisms (urease enzyme), which could provide similar cryoprotectant properties. Ureolytic microorganisms commonly occur in natural substrates and their populations can dramatically respond to the urea level in their immediate environment (Lloyd and Sheaffe 1973; Hasan 2000; Singh et al. 2009). It is conceivable that mother turtles increase local abundance of ureolytic microorganisms by urinating over the nest during oviposition (i.e. urea fertilization) thereby provisioning young with accessible urease. Juvenile tortoises engage in coprophagy, which functions as a way to establish microbial flora in the gut (Lance and Morafka 2001; Moore and Dornburg 2014). The consumption of gastroliths soon after hatching, the relatively rapid evacuation of these materials from the gut and a possible lack of subsequent reingestion (i.e. absence of gastroliths from guts of later-stage hatchlings; Packard and Packard 2006; this study) suggest that the benefit of gastroliths at the early life stage may be realized after consuming substrate once, consistent with the microbiome inoculation hypothesis.

Lastly, geophagy and associated gastrointestinal impaction have been considered a pathological disorder of some captive animals that may be caused by under stimulating environments and/or poor diet (Rhodin 1974; Warwick et al. 2013). This seems an unlikely explanation for geophagy in the present study. The simulated nest chamber of experiment 3 provided an environment similar to that found in nature and the short post-hatching period of time (in most cases 24–72 h; see “Methods” and Table 1) during which turtles exhibited geophagy gives little reason to think that individuals had developed behavioural aberrations.

Many of the aforementioned explanations for the functional significance of geophagy are not mutually exclusive and could shift across life stages. Preliminary data across chelonian species suggests that there may be female-biased patterns in geophagy, particularly for reproductive females (Table S1). For



example, in an intensively monitored population of *G. morafkai*, geophagy was recorded among 40% of adult females, never in adult males, and is suspected in juveniles (Sullivan and Cahill 2019). Gravidity is also associated with elevated rates of geophagy in *G. polyphemus* (Moore and Dornburg 2014). In contrast, no sex bias in geophagy, measured as total gastrolith mass, was found for *Testudo hermanni* when controlled for body size (Gagno and Alotto 2010). Geophagic behaviour may also be seasonally restricted.

## Conclusion

Knowledge of geophagy in a species can contribute to estimation of nutrient budgets and the study of environmental contaminant exposure (Beyer et al. 1994; Hui 2004). For turtles, geophagy is relevant for *ex situ* egg incubation and head-starting programs. Chelonians are globally imperilled (Turtle Conservation Coalition 2018) and a common conservation intervention involves captive breeding, *ex situ* egg incubation on a range of substrate types (e.g. soil, sand, moss, vermiculite, perlite) and offspring head-starting (Burke 2015; Bennett et al. 2017). As preliminary evidence suggests that geophagy is common in hatchling turtles (Table S1), caretakers should carefully consider the incubation substrate that animals are exposed to. Future research may wish to address whether access to natural substrates for ingestion may be of benefit to hatchling chelonians, and studies should explore whether there is a correlation between (artificial) substrate ingestion and traits such as hatchling gut microbiome, digestive efficiency and survival. Future research should also address how widespread geophagy is among chelonians and the possible functional significance of his behaviour for hatchling ecology and overall health.

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MDM contributed project conceptualization, writing, and editing.

CL contributed to data collection and editing.

JC contributed to data collection and editing.

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**Data availability** Data will be made available upon reasonable request to the authors.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethics approval** All methods were approved by the Biological Sciences Local Animal Care Committee at the University of Toronto under Animal Use Protocol No. 20011948. Animals were approved for collection under a Scientific Collectors Permit from Ontario Parks No. 1093596.

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