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Urates of colubroid snakes are different from those of boids and pythonids

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Uricotelic species, such as squamate reptiles, birds and insects, effectively eliminate nitrogen as uric acid in a solid form commonly called urates. Observations made over a decade suggested that the voided urates produced by colubroids (modern snake species) exhibit remarkable differences from those of boids and pythons (ancient snake species). Here, we compare the urates generated by eight captive snake species fed the same diet. Although all fresh urates were wet at the time of excretion, those produced by modern snakes dried to a powdery solid, whereas those of ancient species dried to a rock-hard mass that was tightly adherent to surfaces. Powder X-ray diffraction and infrared spectroscopy analyses performed on voided urates produced by five modern and three ancient snakes confirmed their underlying chemical and structural differences. Urates excreted by ancient snakes were amorphous uric acid, whereas urates from modern snakes consisted primarily of ammonium acid urate, with some uric acid dihydrate. These compositional differences indicate that snakes have more than one mechanism to manage nitrogenous waste. Why different species use different nitrogen-handling pathways is not yet known, but the answer might be related to key differences in metabolism, physiology or, in the case of ancient snakes, the potential use of urates in social communication.

ADDITIONAL KEYWORDS: ammonium acid urate – behaviour – chemistry – ecology – powder X-ray diffraction – renal – Reptilia – snakes – uric acid.

INTRODUCTION

Every living organism needs a mechanism to manage excess nitrogen. Among vertebrates, nitrogen that cannot be utilized is excreted in the form of ammonia (NH_3), urea [$CO(NH_2)_2$] or uric acid ($C_5H_4N_4O_3$) (Walsh & Wright, 1995). Different animal species can access these interrelated nitrogenous waste products to various extents, such that species are typically classified as ammonotelic, ureotelic or uricotelic based on the dominant metabolic pathway. The production of uric acid as a final nitrogenous waste material is more energetically demanding compared with urea and ammonia, but its excretion as a solid requires significantly less water. The prevalence of uricotelism across reptiles, birds and insect species is largely thought to be an evolutionary adaptation to meet water conservation needs. In fact, some amphibian species, such as foam-nest tree frogs (*Chiromantis xerampelina*), have been shown to alter the activity of their waste nitrogen metabolism enzymes in response to the availability of water (Balinsky, 1972; Balinsky *et al.*, 1976).

The early study of metabolic wastes in squamate reptiles (lizards, amphisbaenians and snakes), particularly excess nitrogen, was focused primarily on understanding the structural and chemical properties of ureteral–cloacal urine and voided urine (Khalil, 1948a, b; Minnich, 1972; Minnich & Piehl, 1972). Subsequent work emphasized renal morphology and physiological (osmotic and ionic) regulation (King & Goldstein, 1985; Ditrich, 1996; Dantzler & Bradshaw, 2008; Urity *et al.*, 2012) and attempts to understand ecological and evolutionary trends (Schmidt-Nielsen, 1997). More recent work has concentrated on social aspects, such as territorial marking via scats (urates and faeces) in lizards (Duvall *et al.*, 1987; Bull *et al.*, 1999a, b; Shah *et al.*, 2006; Baeckens *et al.*, 2019).

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This study was prompted by a series of general observations made on a large number of snakes over the course of the past decade. It was noted that urate wastes (urates or urate pellets) produced and excreted by modern taxa (e.g. colubrids, elapids and viperids) and ancient snakes (e.g. boids and pythonids) differ qualitatively in several key aspects, including the timing of excretion, their physical appearance and their mechanical strength. Notably, modern snake species always excrete wet urates and faeces simultaneously, and the urates dry quickly (< 1 day) to a powdery, sand-like solid. In contrast, ancient species typically expel urates in two intervals: the first void alone and the second in tandem with faeces. Especially from large-bodied taxa, the freshly voided ancient snake urates initially have a thick, wet, toothpaste-like consistency but solidify to a very hard material over several days to weeks depending on mass (e.g. several grams in small-sized individuals to > 400 g in giant species). Furthermore, these solidified urates adhere tightly to hard surfaces (e.g. plastics, wood and stone) on which they are deposited, with the removal of such deposits typically requiring sharp tools.

Prompted by these general observations, we sought to establish whether the qualitative differences in urates produced by these different snake species were associated with differences in their chemical composition and/or structure. Several species of similar length from modern (Colubridae and Viperidae) and ancient (Boidae and Pythonidae) snake lineages were fed the same controlled diet and maintained in common conditions (e.g. temperature and water availability). Using X-ray diffraction and infrared spectroscopic methods, we show, for the first time, that the physicochemical properties of voided urates from modern and ancient snake taxa are chemically distinct. These results add to the growing literature illustrating that snakes (Greene, 1997; Burbrink *et al.*, 2020) are not a monolithic group insofar as behavioural, morphological and physiological traits are concerned (Secor & Diamond, 1998; Castoe *et al.*, 2013; Lillywhite, 2014; Booth & Schuett, 2016; Gamble *et al.*, 2017; Perry *et al.*, 2019) and that they might have an important role as unconventional models in addressing research questions related to efficient nitrogen management.

MATERIAL AND METHODS

Study species

Postprandial voided uric acid waste samples (urates) of adult snakes were obtained from eight different species (N = 15 animals) of ancient (lineages Boidae and Pythonidae) and modern (Colubroidea: lineages Colubridae and Viperidae) taxa (see Greene, 1997; Burbrink *et al.*, 2020) housed at the Chiricahua Desert Museum (Rodeo, NM, USA). The three species of ancient snakes studied included two species of African pythonids (Angolan python, *Python anchietae*, and ball python, *Python regius*) and one species of boid from Madagascar (Madagascan tree boa, *Sanzinia madagascariensis*). The five modern snakes, all New World taxa from North America, included three species of colubrids (Trans-Pecos rat snake, *Bogertophis subocularis*, desert kingsnake, *Lampropeltis splendida*, and the Mexican hog-nosed snake, *Heterodon kennerlyi*) and two species of viperids (western diamond-backed rattlesnake, *Crotalus atrox*, and Mojave rattlesnake, *Crotalus scutulatus*). All snakes were of similar size, ranging from ~80 to 100 cm in length.

Production and collection of samples

All snakes at the Chiricahua Desert Museum were offered one to four frozen (thawed) adult laboratory mice (a strain from a common local facility). Water (from a common source) was available from glass bowls *ad libitum*. Room temperature was 72–74 °F (~22–23 °C), and cage temperature permitted thermoregulation via a commercial heat strip at one end maintained at 90 °F (32 °C). The semi-arboreal boid (Madagascan tree boa) had an incandescent basking light (90–95 °F) and was housed in a commercial snake enclosure (61 cm × 61 cm × 61 cm). All other snakes studied were housed and maintained in identical enclosure conditions with the same dimensions (59 cm × 41 cm × 15 cm). Lighting and supplementary heat were maintained on a 12 h light–12 h dark cycle.

After feeding, the snakes were inspected every 12 h for potential wastes (urates and/or faeces). Owing to sample size, we analysed data from 20 trials on the timing of postprandial excretion in the ball pythons (N = 4) and the two species of rattlesnakes (N = 4). Urates naturally excreted by each individual were deposited on clean, fresh, commercial paper towelling. Only the urates (white material) that could be separated from the faeces (if present) cleanly and reliably were used in subsequent analyses. All urate samples were maintained in ambient temperature and humidity conditions and kept out of direct light until the time of analysis (Supporting Information, Fig. S1).

X-ray diffraction analysis

Powder X-ray diffraction (PXRD) data were collected on urate samples from all eight species of snakes ~2 months after excretion using a Bruker Apex DUO X-ray diffractometer (Cu Kα radiation, 50 kV, 30 mA current). Samples ground with a mortar and pestle were mounted in Kapton capillaries, with data

collection from 2θ = 5 to 50°. The PXRD patterns were compared against all known crystal forms of uric acid and its urate salts available in the Cambridge Structure Database (Groom *et al.*, 2016), including anhydrous uric acid [refcode: URICAC (Ringertz, 1966)], uric acid monohydrate [GEJQAO (Schubert *et al.*, 2005)], uric acid dihydrate [ZZZPPI02 (Parkin & Hope, 1998)], sodium urate monohydrate [NAURAT (Mandel & Mandel, 1976)], calcium urate hexahydrate [YODJAE (Presores *et al.*, 2013)], magnesium urate [BADTEX10 (Dubler *et al.*, 1986)], potassium quadriurate [PABRIW (Bazin *et al.*, 2016)] and ammonium acid urate [HOZSUL (Friedel *et al.*, 2006)].

Two single crystals large enough for single-crystal X-ray diffraction were isolated from the Mojave rattlesnake sample. Single-crystal data were collected on a Bruker D8 Quest diffractometer (Mo Kα radiation = 0.71073) equipped with a Photon 100 CMOS detector (Bruker AXS) at 100 K. APEX 3 software and SHELX were used for structure solution and refinement. Both single crystals had a unit cell consistent with uric acid dihydrate (Parkin & Hope, 1998). Refinement of the higher-quality data set yielded a complete structure with a final *R*-factor of 4.67%. Whole-molecule disorder and twinning were observed. The CCDC deposition number is 2041488.

Infrared spectroscopy

Fourier-transformed infrared (FT-IR) spectra of ground (mortar and pestle) snake urate samples, ~20 months after excretion, were recorded on a Perkin Elmer Spectrum-Two FT-IR spectrophotometer equipped with a UATR-TWO diamond ATR attachment. Scans were collected on each sample over a 600–4000 cm^{-1} range, with each spectrum representing an average of ten scans.

Data availability

Optical micrographs and powder X-ray data are provided in the Supporting Information, Fig. S1. Any other data may be obtained from the corresponding authors on reasonable request.

RESULTS

Timing of postprandial urate excretion

After feeding, without exception, the adult ball pythons (*N* = 4 subjects) excreted urates at two different time points. In all 20 trials (20/20), the first excretion (urate-1) consisted only of urates (no faeces) and occurred within 3–7 days of feeding (*N* = 20 trials, z-test, z = 4.248; null = 0.5, two-tailed, $P < 0.01$). The second excretion of urates (urate-2) also involved a bowel movement (faeces) and occurred 7–15 days after urate-1. The second urate excretions were typically smaller in size (mass). Regardless of the time at which they were excreted (i.e. urate-1 or urate-2), all ball python urates exhibited the same initial toothpaste-like consistency and dried to a hard mass within several days (Fig. 1A). The pattern of urate excretion was similar in the Madagascan tree boa and Angolan python (i.e. urate-1 and urate-2) we examined.

In the conditions of this study, the two species of adult rattlesnakes (*N* = 4 subjects) always excreted urate and faecal wastes in tandem within 6–10 days after feeding (*N* = 20 trials, z-test, z = 4.248; null = 0.5, two-tailed, $P < 0.01$). Occasionally, but not reliably, a second void of urates and faeces occurred 5–8 days after the first one, especially when meals were large (e.g. two or three mice). Urates dried quickly over a period of ~1 day, yielding a sand-like consistency (Fig. 1B). Postprandial urate excretion in the other

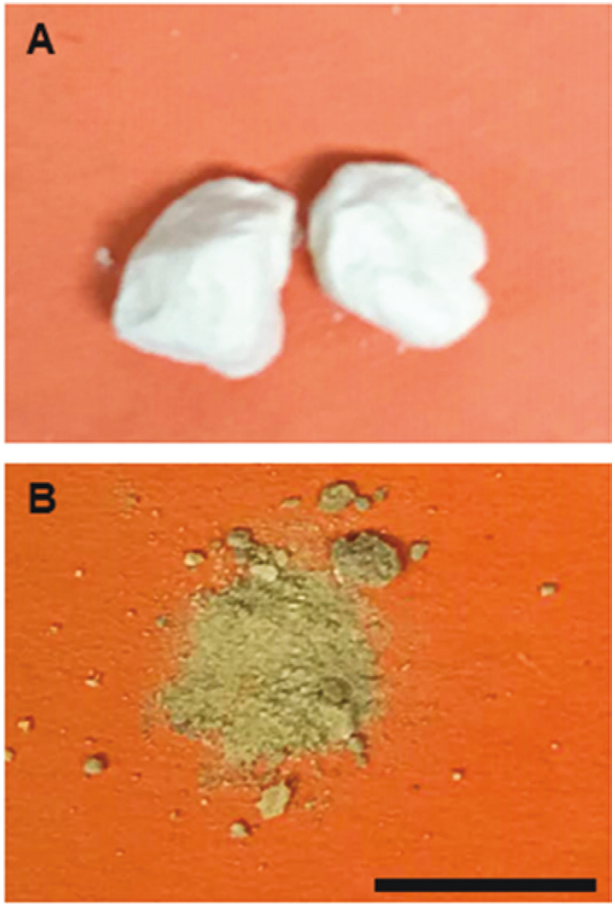

Figure 1. Optical micrographs of snake urate samples. A, air-dried urate sample (incomplete) from an adult ball python (*Python regius*), captive held. B, air-dried urate sample (complete) from an adult female Mojave rattlesnake (*Crotalus scutulatus*), captive held. Scale bar: 1 cm.

colubroids (Mexican hognose snake, desert kingsnake and Trans-Pecos rat snake) had patterns that were similar to the two species of rattlesnakes but occurred earlier (i.e. faeces and urates were excreted in tandem 4–8 days after feeding).

Powder X-ray diffraction

Representative PXRD data of urates from all snakes tested are shown in Figure 2. All sample data shown were collected ~2 months after excretion. Comparison of the diffractograms obtained on urates from the three ancient snake species (one boid and two pythonids) showed that they were qualitatively similar. There were no obvious differences between urate-1 and urate-2 samples produced. Each sample exhibited only one broad diffraction line of reasonable intensity at $2\theta = 27.8°$. The peak position corresponds to an average d-spacing of ~3.2 Å, which is consistent with the expected separation distance between π-stacked heteroaromatic units. With only one broad diffraction line in the PXRD pattern, the sample is amorphous.

The PXRD patterns obtained on urates excreted by all five modern snake species appeared qualitatively similar to one another, but distinctly different from those excreted by ancient snakes. Given the irregularity of a second excretion, only the first voided urate in the modern species was tested. All modern snake urates exhibited diffraction peaks at $2\theta = 9.1$, 10.1, 15.6, 18.2, 19.1 and between 24.6 and 30.2° (±0.2°). The PXRD patterns were compared against several known uric acid and urate crystalline forms and found to match most closely the pattern for ammonium acid urate (Supporting Information, Fig. S2). During examination of the Mojave rattlesnake urate under polarized light microscopy, two individual optically transparent single crystals in the sample were identified and isolated from the larger sample (Supporting Information, Fig. S3). Structure determination with single-crystal X-ray diffraction confirmed that they were uric acid dihydrate.

Powder X-ray diffraction data were re-collected on the same ball python and Mojave rattlesnake samples ~12 months after their initial excretion. The ball python sample showed several additional diffraction lines, indicating that the sample had partly crystallized during this extended time period to a mixture of anhydrous uric acid and uric acid dihydrate. There were no apparent age-related changes in the PXRD pattern of the Mojave rattlesnake sample (Supporting Information, Fig. S4).

Infrared spectroscopy analysis

Comparison of infrared spectra of urates from modern snakes showed that they were qualitatively similar to each other, as in the PXRD analysis, but different from those excreted by the ancient snakes we examined (Fig. 3). Urates from ancient and modern snakes have broad absorption in the region

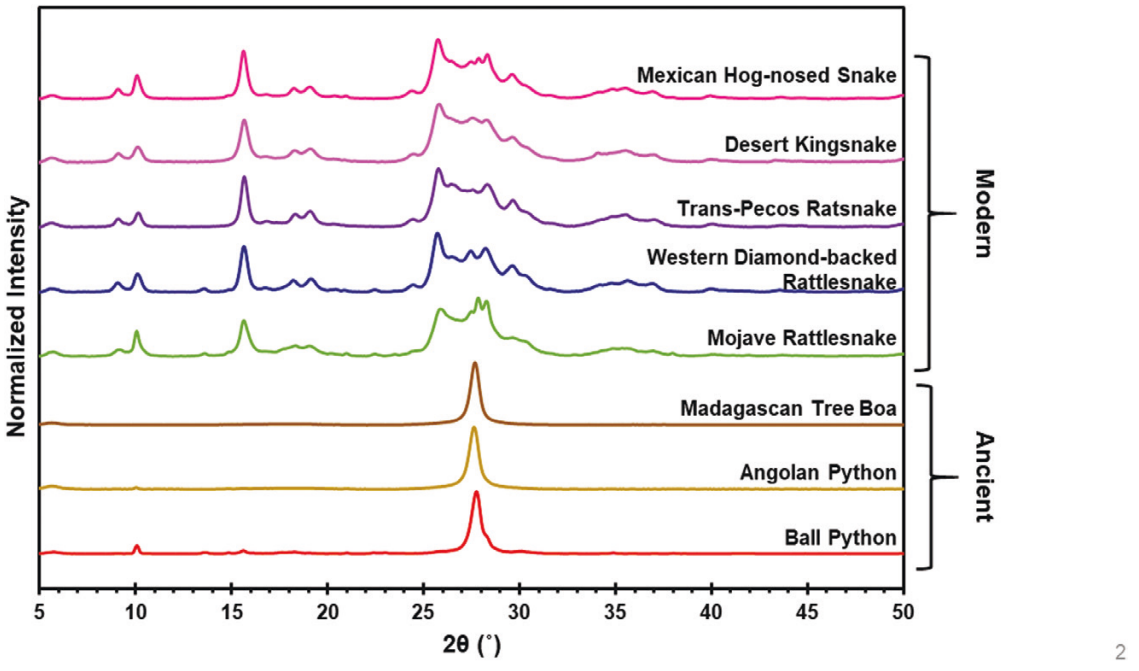

Figure 2. Representative powder X-ray diffractograms obtained on snake urate samples excreted from eight different ancient (boid and pythonid) and modern (colubroid) species. All individuals were fed the same controlled rodent diet and maintained in common enclosure conditions.

between 3500 and 2500 cm^{-1}, and distinct vibrations in the lower fingerprint region point toward different compositions. Reference infrared spectra for many crystalline constituents identified in renal deposits have been reported previously (Modlin & Davies, 1981), including those of the most common forms of uric acid/urate: ammonium acid urate, sodium urate monohydrate, anhydrous uric acid and uric acid dihydrate. Ammonium acid urate has a two sharp peaks at 740 and 780 cm^{-1}, whereas both anhydrous and dihydrate forms of uric acid have three strong absorption bands between 700 and 500 cm^{-1}. The infrared spectra of urates from all the colubroid taxa (modern snakes) tested were entirely consistent with ammonium acid urate, especially when taken in combination with the PXRD data. In contrast, each of the three urates from the boid and pythonids (ancient snakes) had three absorptions in the region between 700 and 500 cm^{-1}, which is consistent with uric acid in its protonated form. Data are summarized in Table 1.

DISCUSSION

In physiological solutions, uric acid (a weak acid, with a pK_a ~5.5; Finlayson, 1974) exists in equilibrium with urate, its deprotonated anion. Studies of this compound have shown that it can precipitate in many different solid forms depending on the pH and ionic strength of the fluid in which it is found. In mammals, uric acid concentrations that exceed the solubility limit can result in kidney stones and gout deposits. In humans, the solid form identified in uric acid kidney stones is most often the dihydrate or anhydrate (Herring, 1962). In controlled laboratory conditions, the former has been shown to be less stable, such that it can convert to the latter over time (Zelellow *et al.*, 2010; Presores & Swift, 2014). The most common salt forms identified in mammalian species include sodium urate monohydrate, which is widely associated with gout in humans (Mandel, 1976), and ammonium urate, which is frequently identified in the kidney stones of Dalmatian dogs (Bartges *et al.*, 1994) and managed bottlenose dolphins (*Tursiops truncatus*) (Venn-Watson *et al.*, 2010). The crystal structures of some of these forms are shown in Figure 4.

Unlike mammals, uricotelic species within the lineages Amphibia and Reptilia (avian and non-avian reptiles) are thought not to suffer from such maladies, owing to anatomical differences whereby excretory products are released directly into the cloaca. Nevertheless, to our knowledge at the outset of this

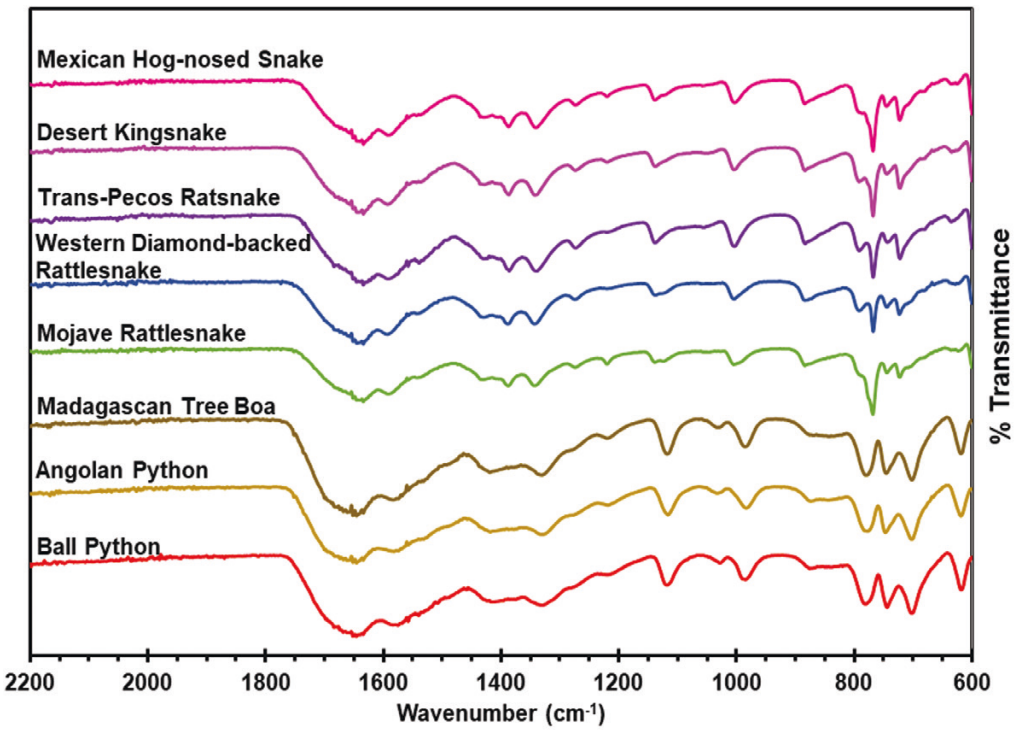

Figure 3. Experimental Fourier-transformed infrared spectra obtained on snake urate samples excreted by eight different ancient and modern species snakes used in this study. All individuals were fed the same controlled rodent diet and maintained in common conditions.

Table 1. Species of modern (colubrid and viperid) and ancient (boid and pythonid) snakes investigated in this study, with a descriptive summary of general observations and powder X-ray diffraction data

Species	Clade	Ecology	Consistency*	Powder X-ray diffraction	Major component
Mexican hog-nosed snake	Colubridae	Semi-fossorial	Powdery	Multiple peaks	NH_4 urate
Desert kingsnake	Colubridae	Terrestrial	Powdery	Multiple peaks	NH_4 urate
Trans-Pecos rat snake	Colubridae	Terrestrial	Powdery	Multiple peaks	NH_4 urate
Western diamond-backed rattlesnake	Viperidae	Terrestrial	Powdery	Multiple peaks	NH_4 urate
Mojave rattlesnake	Viperidae	Terrestrial	Powdery	Multiple peaks	NH_4 urate
Madagascan tree boa	Boidae	Semi-arboreal	Hard chunks	Amorphous	Uric acid
Angolan python	Pythonidae	Terrestrial	Hard chunks	Amorphous	Uric acid
Ball python	Pythonidae	Terrestrial	Hard chunks	Amorphous	Uric acid

*Refers to consistency of urates at the time of powder X-ray diffraction, which were dry when tested.

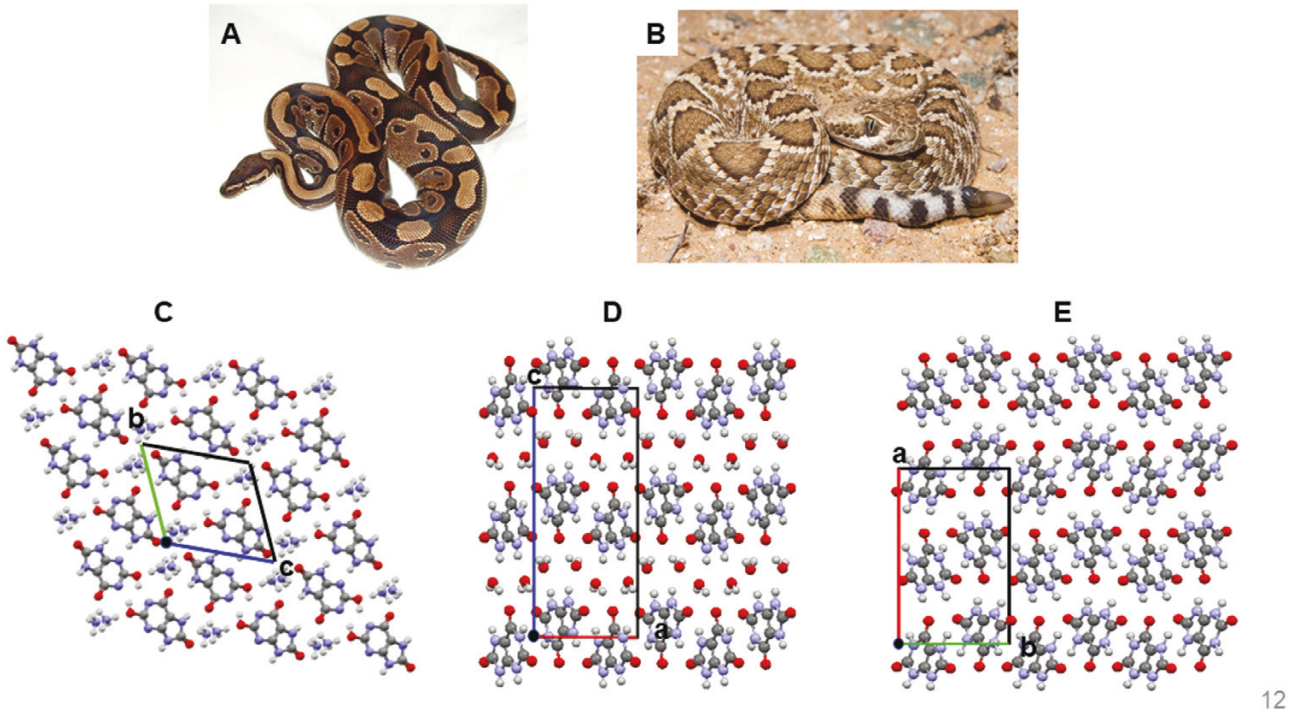

Figure 4. (A) Ball python (*Python regius*). (B) Mojave rattlesnake (*Crotalus scutulatus*). Urates from the Mojave rattlesnake consist primarily of (C) ammonium acid urate with small amounts of (D) uric acid dihydrate. Urates produced by the Ball python, after extended ageing contain both (D) and (E) anhydrous uric acid. Crystal structure diagrams (C–E) were generated in MERCURY from the corresponding cif files.

study, there were no reports detailing the structural or chemical composition of any postprandial snake urates. Unlike previous work, which provided incomplete descriptions of laboratory care (e.g. diet) of test animals (e.g. Minnich, 1972), we intentionally minimized several variables in our study by controlling multiple environmental factors (i.e. enclosure type and size, type and source of rodents, source of water and temperature of the enclosure). Yet even in the controlled conditions of this study, the postprandial urates excreted by the boid and pythonids were consistently different in several qualitative aspects (e.g. production time after feeding, mechanical strength and adhesion) compared with the urates produced by

the colubroids. Accordingly, it was not unexpected that finer examination of the structural properties of these urates would reveal important differences.

The PXRD analyses of the urates from the five colubroid species we tested had multiple diffraction lines, which, although broad, had a pattern consistent with what is expected for ammonium urate. This particular assignment is also in agreement with infrared spectral bands in the fingerprint region for this phase. However, neither piece of evidence precludes the existence of minor amounts of other forms, as demonstrated by the presence of a few small, isolatable uric acid dihydrate crystals from the Mojave rattlesnake urate sample. In contrast, the urates of the boid and pythonid species were amorphous and had infrared spectra consistent with uric acid in its protonated state. The time between sample excretion and testing also appears to be a crucial variable, because at least in the boid and pythonid species, changes in crystallinity in the samples were found to occur over extended time periods.

To the best of our knowledge, the qualitative differences in 'adhesiveness' and mechanical strength in the urates produced by ancient and modern snakes have not been discussed in the scientific literature. The closest to commenting on this aspect are Minnich & Piehl (1972), who indicate that the urates of geckos and other lizards dry to a harder material than those of most snakes, which are much less compact. Their reasoning for the difference in this property was the presence of more ammonium urate and sodium chloride that minimize aggregation of snake urates. The mechanical properties of some, but not all, forms of solid uric acid have been reported previously (Liu *et al.*, 2018), although rigorous mechanical measurements on pure laboratory samples and biologically derived materials are likely to be different owing to vast differences in purity. Additional testing would be required to gain a better understanding of this aspect.

Although the number of snake taxa we studied here is a tiny fraction of the 3800 or so extant species (THE REPTILE DATABASE, www.reptile-database.org), the snakes included in this study allowed for the same dietary input. In these controlled feeding conditions, the analyses performed confirm that fundamental differences exist in the urates across lineages. Additional testing of urate samples from other species in the clades we studied and species from other clades, both ancient (e.g. scolecophidians, cylindrophiids, uropeltids, loxocemids and xenopeltids) and modern (e.g. acrochordids, elapids, lamprophiids and natricids), would help to confirm whether the trends we report here are substantiated across the broader phylogeny of snakes. Also, we do not know to what extent varying the dietary input would influence the production and physicochemical attributes of the snake urates, although diet might be significant (Greene & Cundall, 2000). This question might be addressed easily in future studies in snake species where their diet is broad (e.g. earthworms, insects and rodents) and can be manipulated easily.

Of course, why snakes fed the same diets produce urates with different chemical compositions in the first place remains an open question. It is certainly possible that the results derive from fundamental physiological or metabolic differences. For example, there might be differences in the levels of various enzymes that regulate nitrogen metabolism across snake species. At a minimum, it must be the case that the colubroids examined here have higher ammonium levels in their urate wastes than do boas and pythons. However, it is not clear whether this is because colubroids have a decreased ability to convert ammonia to glutamine and other higher nucleotides or if they are simply better able to handle the ecological consequences of direct ammonia excretion. Addressing these hypotheses would require rigorous enzyme assays. Fundamental differences in the microbiome in different species might also be relevant. For example, if nitrogen-fixing bacteria that convert ammonia to nitrogen gas are abundant in the guts of boas and pythons, efficient ammonia conversion to N_2 might simply reduce ammonium concentrations to levels too low to result in the crystallization of ammonium urate.

At this time we are not able to provide experimental evidence for the ecological or evolutionary significance of the different types of urates produced by these snakes. Unquestionably, additional research is required. Owing to a paucity of comparative data on this topic, especially for snakes and other reptiles (Danzler 1996, 2005; H. Lillywhite, pers. com), new research on the anatomy and physiology of snake kidneys (and associated renal structures) and metabolic processes would be needed to explore potential differences in snakes from ancient and modern lineages. Other research needs to be directed at the potential functional role(s) of deposited urates in nature. In many vertebrates, including squamate reptiles such as lizards, urine (urates) and faeces serve communicative and social functions (Müller-Schwarze, 2006; Fenner & Bull, 2010; Apps *et al.*, 2015; Marneweck *et al.*, 2017; Baeckens, 2019). Some of the properties of urates from boids and pythonids, for example, make them good candidates for behavioural and physiological assays in the laboratory and the field. Accordingly, important next steps based on the new observations made in the present study might include analysis of chemical profiles, such as hormones, pheromones and signature mixtures (Halpern & Martínez-Marcos, 2003; Mason & Parker, 2010; Wyatt,

2010), coupled with behavioural experiments (Hebets & Papaj, 2005; Hebets *et al.*, 2016).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Samples of the snake urates used in this study. A, ball python (*Python regius*). B, Angolan python (*Python anchietae*). C, Madagascan tree boa (*Sanzinia madagascariensis*). D, Mojave rattlesnake (*Crotalus scutulatus*). E, western diamond-backed rattlesnake (*Crotalus atrox*). F, Trans-Pecos rat snake (*Bogertophis subocularis*). G, desert kingsnake (*Lampropeltis splendida*). H, Mexican hog-nosed snake (*Heterodon kennerlyi*). Scale bar: 1 cm.

Figure S2. Experimental powder X-ray diffraction (PXRD) diffractograms obtained on urate samples excreted from five different species of ancient snakes compared with simulated PXRD patterns of known crystalline forms of uric acid (anhydrous uric acid, uric acid monohydrate and uric acid dihydrate) and urate salts (sodium urate monohydrate, calcium urate hexahydrate, magnesium urate, potassium quadriurate and ammonium acid urate). Simulated PXRD diffractograms were generated in MERCURY from cif files obtained from the Cambridge Structure Database.

Figure S3. Sharp diffraction lines at 2θ = 10.1, 27.8 and 28.3° in the Mojave rattlesnake urate correspond to intense peaks expected for uric acid dihydrate. Peaks at the higher 2θ values appear shifted owing to effects of temperature. The inset is an optical micrograph, which shows individual crystals (red arrows) present in the sample. Scale bar: 100 μm. Single-crystal X-ray diffraction confirmed them to be uric acid dihydrate.

Figure S4. Experimental powder X-ray diffraction (PXRD) diffractograms obtained on the same ball python and Mojave rattlesnake urate samples collected ~2 and 12 months after excretion.