

# NEPHROLITHIASIS IN FREE-RANGING NORTH AMERICAN RIVER OTTER (*LONTRA CANADENSIS*) IN NORTH CAROLINA, USA

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**Abstract:** The North American river otter (*Lontra canadensis*) serves as an indicator species for environmental monitoring, is prized as a valuable furbearer, and is a popular display animal in zoologic collections. Nephrolithiasis has been reported as a frequent problem in other free-ranging and captive otter species but is rarely reported in North American river otters. In this study, we compared the prevalence of nephrolithiasis diagnosed using routine gross pathologic examination techniques with the use of computed tomography (CT) of excised kidneys. We also evaluated whether otter nephroliths could be accurately classified by their CT densities, and we examined the renal tissue uric acid concentrations in free-ranging otters in North Carolina, USA. Kidneys were collected from carcasses of legally trapped, free-ranging animals. Nephroliths were observed in 16.2% of the individuals ( $n = 229$ ). Associations were found between age and nephrolith status and between capture location and nephrolith status ( $P = 0.026$  and  $< 0.001$ , respectively). Computed tomography Hounsfield unit density measurements were not useful in determining nephrolith chemical composition in this study. Renal tissue uric acid concentrations were similar across genders, age groups, and stone status. The chemical composition of the nephroliths was determined by scanning electron microscopy–energy dispersive X-ray spectroscopy to be calcium phosphate in the carbonate form.

**Key words:** Calcium phosphate, computed tomography, energy-dispersive X-ray spectroscopy, *Lontra canadensis*, nephrolith, North American river otter, uric acid.

## INTRODUCTION

Nephrolithiasis is considered a common clinical problem of captive Asian small-clawed otters (*Aonyx cinerea*) and has been reported in captive and free-ranging Eurasian otters (*Lutra lutra*).<sup>4,5,26</sup> In a survey of captive Asian small-clawed otters in North America in 1988, 66.1% had renal calculi composed of calcium oxalate or urates.<sup>4</sup> More recently, the occurrence of urolithiasis has been documented in wild and captive Eurasian otters.<sup>5,26</sup> Uroliths composed of ammonium urate were found in 61.9% of all postmortem-examined captive Eurasian otters at a breeding center in France.<sup>5</sup> Renal calculi were also documented in 10.2% of wild Eurasian otters presented for postmortem examination in England, with 96%

of the stones composed of ammonium acid urate.<sup>26</sup>

A published report of nephrolithiasis in a North American river otter (*Lontra canadensis*) describes a severe case of bilateral nephrolithiasis and ureteral hypertrophy discovered during a study of 305 otters in the state of Washington (USA).<sup>16</sup> The otter was reported to be in normal body condition, but the kidneys were grossly and histologically abnormal, with expansion of the renal calyces and loss of medullary tissue, but without evidence of inflammation.<sup>16</sup> With the exception of that report, literature on the renal health parameters of North American river otters is relatively scant.<sup>1,18</sup> This raises the question of whether there is a real difference in species susceptibility among closely related mustelid species or whether nephrolithiasis is potentially being underdiagnosed in the North American river otter.

The objective of this study was to compare the prevalence of nephrolithiasis diagnosed using routine gross pathologic examination techniques with the use of computed tomography (CT) of excised kidneys in free-ranging otters in North Carolina, USA. The samples available for this work also provided us the opportunity to explore the range of renal tissue uric acid concentrations in this species. Having identified several stones in this study's cohort, the opportunity to assess the

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potential for nephrolith classification clinically by CT density in river otters was determined.

## MATERIALS AND METHODS

### Experimental animals

Carcasses of legally trapped, free-ranging North American river otters were collected from licensed trappers as part of a larger study of North Carolina otters from November through February 2009–2012. Otters were sampled from each of the North Carolina Wildlife Resources Commission furbearer management regions: mountain, piedmont, and coastal plain. Each individual was examined grossly, and morphometric and location data were recorded. During gross postmortem examination, both kidneys from each otter were removed, placed together in a plastic freezer bag, labeled, and frozen at  $-20^{\circ}\text{C}$ . Storage time was variable because of the large study size spanning multiple years of trapping. A lower canine tooth was collected for age estimation by cementum analysis (Matson's Laboratory LLC, Milltown, Montana 59851, USA).<sup>10,21</sup>

### Diagnostic imaging

Kidneys from a total of 229 individual otters were imaged. The kidneys were thawed overnight at  $4^{\circ}\text{C}$  and moved to room temperature for scanning. The kidneys were scanned using a Siemens Somatom Sensation 64-slice CT scanner (Siemens Medical Solutions USA Inc., Malvern, Pennsylvania 19355, USA) in an air-filled environment. The scanning technique was generally based on Deveci et al.<sup>9</sup> with some adjustments to better utilize the equipment available to us for the study. A 1-mm slice thickness at 120 kVp, 214 mAs, was scanned.

A human baby lung window with a B60f# kernel provided the sharpest images for nephrolith evaluation. Hounsfield unit density (HUD) is a quantification of physical density made by comparing beam attenuation with water.<sup>24</sup> A single veterinarian evaluated all CT images. Each nephrolith observed was measured with the maximum diameter in the *x*- and *y*-plane calculated from the largest CT slice and the total number of slices for the maximum diameter found occurring in the *z*-plane. Three HUD measurements were obtained (OsiriX v.4.1.2 32-bit, Pixmeo, 1233 Bernex, Switzerland) on the largest nephrolith cross section visible and then averaged as the CT density. The average values were compared with the measured CT values of stones reported in Deveci et al.<sup>9</sup>

### Gross examination

Each kidney from the 229 individuals scanned was opened sagittally and grossly examined for nephroliths visually and by palpation. Any suspected nephroliths were collected and stored dry at room temperature until analysis.

### Determination of renal tissue uric acid concentration

A renal tissue sample was collected from a subsample of 125 otters included in the diagnostic imaging. Individuals were selected to minimize imbalances in the final age and gender distribution of the cohort. A relatively greater proportion of individuals from the older age groups were selected compared with the overall sample population to achieve a more evenly distributed sample size among groups. Each individual renal sample included cortex and medulla. Samples were frozen at  $-20^{\circ}\text{C}$  until processed for extraction.

The frozen kidney samples were homogenized in a mortar and pestle chilled with liquid nitrogen and extracted with cold 50 mM perchloric acid (1 M stock solution, Sigma-Aldrich, St. Louis, Missouri 63103, USA) at 200 mg of tissue/ml as described in Barja de Quiroga et al.<sup>2</sup> Individual sample size had a median of 195 mg (interquartile range [IQR] = 160–238 mg). The homogenized samples were vortexed and centrifuged at 3,000 *g* for 10 min, and the supernatant was collected and stored at  $4^{\circ}\text{C}$  until analysis.

A commercial uric acid assay kit (Amplex<sup>®</sup> Red Uric Acid/Uricase Assay Kit, Life Technologies, Grand Island, New York 14072, USA) was used to spectrophotometrically determine each sample's uric acid concentration. Percent dry weight for each extracted kidney sample was calculated using the percent dry weight of a representative kidney sample dried at  $40^{\circ}\text{C}$  until there was no further reduction in weight. Uric acid concentration (mg/100 g tissue) is reported on a dry matter basis.

### Nephrolith analysis

Suspected nephroliths, collected at gross examination, were submitted dry to the University of Minnesota Urolith Laboratory for analysis.<sup>29</sup> All nephroliths were returned to North Carolina State University College of Veterinary Medicine for additional processing and analysis.

All of the returned samples were placed in 95% ethanol under intermittent vacuum with constant stirring for 2 days. The samples were then transferred to 100% ethanol and maintained

under the same conditions for 2 days, with the ethanol being changed after 1 day. The samples were then placed in 50%, 80%, and 95% Technovit 7200 VLC embedding media (EXAKT Technologies Inc., Oklahoma City, Oklahoma 73116, USA) for 3 days at each concentration at the same stirring and vacuum conditions. Finally, the samples were placed in 100% Technovit 7200 for 14 days, with constant stirring and intermittent vacuum during the final 7 days. At the end of the infiltration process, the samples were placed in fresh 100% Technovit 7200 in the Exakt Light Polymerization Unit (EXAKT Technologies). Iced water was run through the unit to keep the samples cool. Three polymerization cycles of yellow and blue light were used to polymerize-cure the embedding media. The blocks of media containing the samples were cemented to acrylic glass slides with Exakt Technovit 4000 adhesive. The slides were rough ground using an Exakt Microgrinder and silica carbide grit grinding papers of 1,000–2,000 grit (EXAKT Technologies). The slides were then fine ground and polished with 1- $\mu$ m diamond paste (Buehler, Lake Bluff, Illinois 60044, USA).

The samples were examined with a Hitachi S-3200N (Hitachi High-Technologies America Inc., Dallas, Texas 75261, USA) scanning electron microscope (SEM) equipped with an energy-dispersive X-ray spectrometer (EDS) consisting of an X-ray spectrometer (Advanced Analysis Technologies, Dane, Wisconsin 53529, USA) and 4Pi pulse processor (4Pi Analysis Inc., Hillsborough, North Carolina 27278, USA). Digital micrographs and X-ray spectra were collected and analyzed with 4Pi Revolution software (v.1.6.0b214, 4pi Analysis). The samples were examined in variable pressure mode with helium as the backfill gas at a pressure of 150 Pa with a beam energy of 20 keV to avoid coating with a conductor. Scanning electron micrographs were collected with a Robinson back-scattered electron detector. X-ray spectra were collected from multiple regions within each sample where morphologic differences were observed. Standardless quantitative EDS analysis was performed on all of the X-ray spectra collected to yield the weight and atomic percentage of the various elements in the sample.

Statistical analyses

The otter cohort was examined by year class based on cementum analysis; otters  $\geq 4$  yr old were grouped into a single age class to better balance the group sample sizes. Nonparametric testing was used, including random sampling,

**Table 1.** Nephrolithiasis occurrence in North American river otters in North Carolina, USA, by age, as diagnosed by computed tomography (CT) and gross dissection.

Age group/ age (yr)	CT examination			Gross dissection		
	Positive	n	%	Positive	n	%
<1	2	50	4.0	0	50	0
1	10	61	16.4	2	61	3.3
2	10	47	21.3	1	47	2.1
3	8	27	29.6	1	27	3.7
$\geq 4^a$	6	44	13.6	0	44	0
4	2	16	12.5	0	16	0
5	0	13	0	0	13	0
6	1	7	14.3	0	7	0
7	1	3	33.3	0	3	0
8	1	1	100	0	1	0
9	1	1	100	0	1	0
10	0	2	0	0	2	0
11	0	0	0	0	0	0
12	0	1	0	0	1	0
Total	36 <sup>b</sup>	229	15.7	4	229	1.7

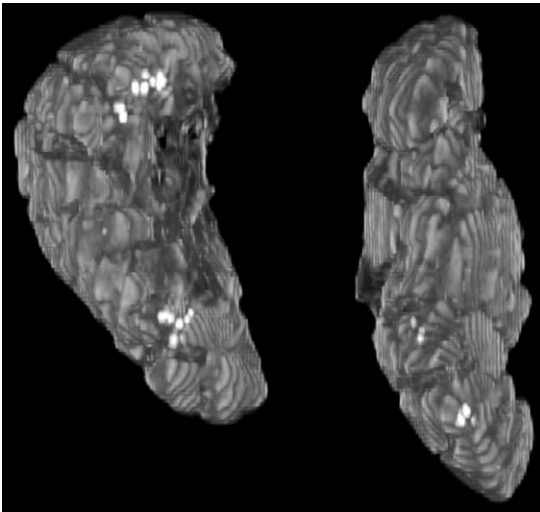
<sup>a</sup> Age group  $\geq 4$  yr old expanded in detail.  
<sup>b</sup> One individual was positive by gross dissection and negative by CT.

two-sample permutation tests, and permutation chi-square tests. A chi-square test was used when comparing gender and nephrolith status and sample location and stone status because all expected values were  $>5$ . All statistical analyses were performed with R 2.15.1.<sup>25</sup> Values of  $P < 0.05$  were considered statistically significant.

RESULTS

The cohort of otters imaged composed 59.5% (135/229) males, with overall median male and female weights being statistically different (random sampling two-sample permutation test, median difference,  $R = 1,000$ ,  $P < 0.001$ ) at 6.48 kg (IQR 5.61–7.57 kg) and 5.37 kg (IQR 4.44–6.22 kg), respectively. The age distribution was: first year of life, 21.8% (50/229); 1 yr, 26.6% (61/229); 2 yr, 20.5% (47/229); 3 yr, 11.8% (27/229);  $\geq 4$  yr, 19.2% (44/229).

Radiographic opacities consistent with nephroliths were observed in 15.7% (36/229) of the otters (Table 1). The majority of otters with nephroliths had multiple opacities (73.0%; 27/37). A total of 134 nephroliths were detected. Nephroliths ranged in diameter from 1 to 7 mm on CT measurement. The median CT density was 413 HUD and highly variable (IQR 314–569). The median CT value falls within the range observed for uric acid stones.<sup>9</sup> However, a large amount of intra- and interindividual variation was observed,

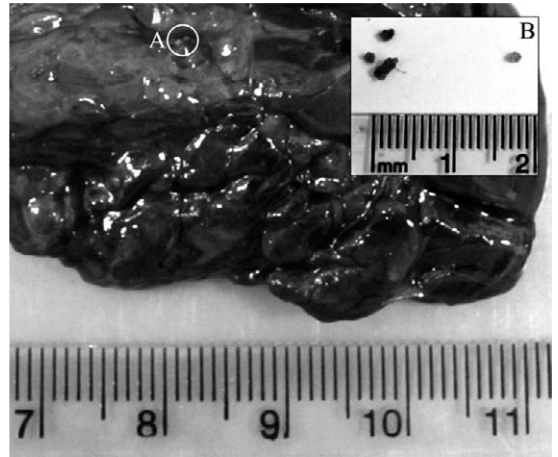


**Figure 1.** A three-dimensional computed tomography rendering of the kidneys of a male, 1-yr-old North American river otter depicting multiple radiographic opacities consistent with nephroliths.

and stones were observed in the ranges reported for uric acid, struvite, cystine, and calcium oxalate monohydrate.<sup>9</sup> A three-dimensional CT rendering (Fig. 1) depicted the typical appearance of the observed opacities. No difference was observed between body weights (random sampling two-sample permutation test, median difference,  $R = 1,000$ ,  $P = 0.61$ ) or gender (chi-square test,  $P = 0.23$ ) of otters with nephroliths versus those without. There was evidence of an association between nephrolith status and age (permutation chi-square test,  $R = 1,000$ ,  $P = 0.026$ ) and capture location (coastal plain vs. piedmont; chi-square test,  $P < 0.001$ ). Otters in their first year of life and otters  $\geq 4$  yr old had fewer individuals with stones than expected, and otters from the piedmont region had fewer individuals with stones than expected.

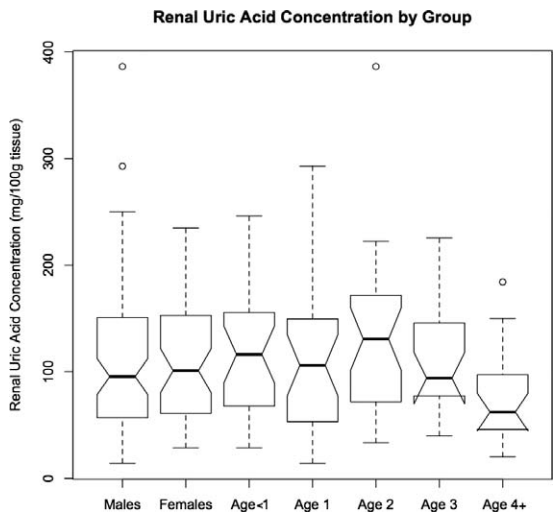
Nine grossly palpable nephroliths were found among four otters (1.75%; 4/229). The nephroliths were pale with a rough surface that was firmly adherent to the surrounding tissue (Fig. 2). The individual in Figure 2 had bilateral nephrolithiasis with a single stone in one kidney and three stones in the other. One of the four otters was found to have two stones unilaterally, both approximately 2 mm<sup>3</sup>, yet was negative on CT. The remaining two individuals both had unilateral nephrolithiasis with a single stone and two stones found.

The subset of otters selected for uric acid tissue concentration determination consisted of 60.8%



**Figure 2.** Photograph of a North American river otter kidney from Figure 1 with stones in situ (circle A) and removed (inset B).

(76/125) males and were distributed by age as follows: first year of life, 21.6% (27/125); 1 yr, 22.4% (28/125); 2 yr, 22.4% (29/125); 3 yr, 16% (20/125);  $\geq 4$  yr, 16.8% (21/125). Median renal tissue uric acid concentration was 97.18 mg/100 g tissue (IQR 58.53–151.71 mg/100 g tissue). No difference in tissue uric acid concentration was apparent between gender or age groups (Fig. 3). The uric acid concentration of otters with radiographic evidence of nephroliths ( $n = 23$ ) was not



**Figure 3.** Notched box plot of North American river otter renal uric acid concentration by gender and age group (years). Circles indicate possible outliers. The notches indicate a 95% confidence interval of the median; overlapping notches indicated strong evidence that the medians did not differ.



statistically significantly greater than those that did not have radiographic evidence of nephrolithiasis ( $n = 102$ ; random sampling two-sample permutation test,  $R = 1,000$ ,  $P = 0.849$ ).

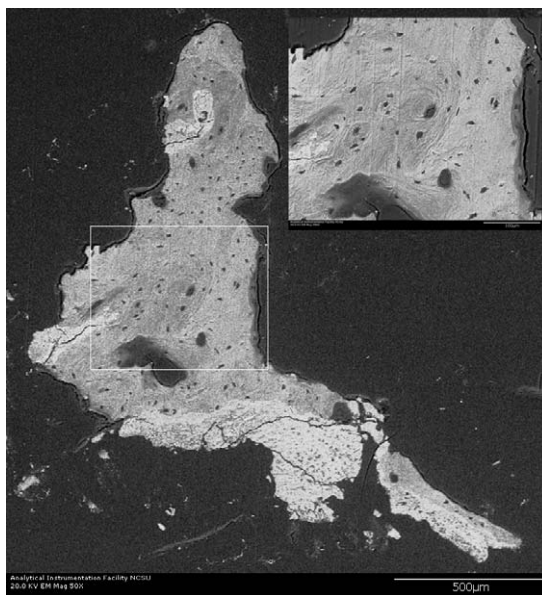
Only one of the grossly observed nephroliths submitted to the Minnesota Urolith Center was positively identified through routine analysis. It was identified as 100% calcium phosphate, carbonate form. The remaining stones were classified as osseous or miscellaneous material.

Analysis of EDS spectra supported chemical classification of all nine of the stones found on gross palpation as calcium phosphate, carbonate form  $[\text{Ca}_{10}(\text{PO}_4\text{CO}_3\text{OH})_6(\text{OH})_2]$ , also known as carbonate apatite. Many of the sites probed by EDS also contained trace amounts of sodium, magnesium, potassium, sulfur, or a combination of these elements. Observed by SEM, all of the examined stones had a lamellar appearance with frequent lacunae and areas that appeared heterogeneous on visual inspection (Fig. 4). Spectra were collected both from large areas of the sample and from areas that exhibited different observed morphologies. The spectra showed that the elemental makeup did not significantly change despite morphologic changes.

## DISCUSSION

It is very likely that nephrolithiasis is underreported in river otters examined by routine post-mortem examination techniques. The prevalence of nephrolithiasis (16.2%) in this cohort of otters is similar to that observed in wild Eurasian otters.<sup>26</sup> However, the small size of the nephroliths found in this study and the poor ability to detect stones by gross examination of dissected kidneys, compared with the use of CT imaging, suggest that the majority of nephroliths in North American river otter kidneys could go undetected without the additional benefit of diagnostic imaging. It is possible that prevalence of stones is underreported in other studies that limit analysis to gross dissection. The data collected on the otters that compose this current dataset did not include any health assessment information that would provide insight into whether the small stones detected would have clinical significance in otters.

Renal tissue uric acid concentrations were determined before performing EDS, based on the hypothesis that observed nephroliths would most likely be composed of uric acid, as is reported for European river otters. The lack of any difference in renal tissue uric acid concentration between North American river otters with



**Figure 4.** Scanning electron micrograph of a North American river otter nephrolith. The chemical composition was confirmed to be calcium phosphate, carbonate form, even in areas that visually appeared heterogeneous. Outline  $\times 50$ . Inset  $\times 250$ .

and without nephroliths is in accordance with this study's final analysis of actual composition of the larger stones. Renal tissue uric acid concentrations are not frequently reported, but this study's values for North American river otter renal tissue were similar to liver uric acid concentrations reported in Wistar albino rats (*Rattus norvegicus*) and kidney and liver uric acid concentrations found in a healthy Harris's hawk (*Parabuteo unicinctus*).<sup>17,19</sup>

The radiographic density measurements of the stones identified in this current study were spread across the expected HUD readings for a variety of stone compositions reported for humans.<sup>9</sup> In this study, only the composition of the few larger stones detected were analyzed, and the overall stone composition of the small stones detected by CT evaluation cannot be interpreted. The stones that were analyzed shared the same elemental composition across individual otters. Although this study's methods varied from those used in the human studies reported, the wide variability in HUD readings across renal stones of very similar composition suggests that the use of radiographic density identification schemes developed for stones in humans might not be reliable for determining

the chemical composition of an individual stone in river otters. This could be a function of the size of the stones examined. Edge effects affecting the HUD measurements of very small structures in CT scans can induce artificial variability.<sup>24</sup> When performing HUD measurements, it has been recommended to include a minimum of 1 mm of urolith exterior in the space being measured.<sup>24</sup> Given the small dimensions of observed nephroliths, the calculated HUD may have been variably falsely decreased by surrounding renal tissue. The CT beam passing through aligned nephroliths could also falsely decrease HUD measurement through beam hardening.<sup>24</sup>

The otters in this study were deemed generally healthy by field biologists. Gastrointestinal and renal parasitism were the only potential health issues found on gross postmortem examination, and these conditions were found both in otters with and without nephrolithiasis. This is comparable to findings in humans with urolithiasis, where no significant changes in glomerular filtration rate are associated with the presence of renal stones unless the affected individual is overweight.<sup>15</sup> However, it is possible that even a nonobstructing stone could cause pain,<sup>22</sup> which might result in stress and decreased fitness in a wild animal.

Given the lack of gross renal abnormalities in the majority of the kidneys, it is unsurprising that body weights of otters with and without nephroliths were similar. The apparent age association observed, where fewer stones than statistically expected were identified in <1-yr-old and ≥4-yr-old individuals, was similar to that established for humans, where nephrolithiasis is predominantly seen during the middle of life.<sup>20</sup> The understanding for this pattern is speculative. Perhaps a constellation of factors makes some otters prone to the condition where others are not. If the normal pathogenesis of nephrolithiasis involves accretion of minerals over a prolonged period, young animals may not have lived long enough to develop detectable stones. It can also be speculated that stones may contribute to mortality from a wide range of other causes, so that animals succumb in part from the effects of their stones without reaching old age.

Nephrolithiasis should not be considered an end diagnosis but suggestive of an underlying condition.<sup>22</sup> Calcium phosphate renal calculi, as found in the otters in this study, are the second most common stone type found in humans living in developed countries but are relatively more

common in female humans than calcium oxalate stones.<sup>1,20</sup> Calcium phosphate stones can be associated with distal renal tubular acidosis, primary hyperparathyroidism, magnesium depletion, idiopathic hypercalciuria, and hypocitruria.<sup>3,12,13,23</sup> Bacteria, both urease- and nonurease-forming, have been proposed as a main cause of calcium phosphate nephrolithiasis, but study results have not supported this hypothesis.<sup>12,14</sup>

The current data indicated a difference in prevalence of nephrolithiasis in otters between the coastal and piedmont management regions; the sample size from the mountain region was too small for statistical comparisons ( $n = 27$ ; one with nephrolithiasis). Human urolithiasis has been shown to have significant geographic variation within the United States, with the highest rates in the Southeast and lowest rates in the Northwest.<sup>28</sup> Within the United States, North Carolina has the highest prevalence of kidney stones in human males and the second highest prevalence for human females.<sup>28</sup> While there are no known edaphic factors in North Carolina that would explain this geographic difference in otters, it is possible that local variations in cations or minerals could predispose individuals to stone formation. Surrounding land use, environmental contaminants, presence of fossilized mollusks, water chemistry due to vicinity to saltwater, or other factors could influence these variations. The diet of otters from the coastal plain and piedmont is not expected to have significantly different components (e.g., proportion of fish vs. invertebrates, etc.), but the prey species composition may differ among the regions (Stoskopf, unpubl. data). These differences could result in varying protein content, which is known to affect stone formation in humans.<sup>11</sup> Human studies have noted high dietary calcium intake decreases the risk of kidney stones, whereas low potassium intake increases risk, and restricting dietary salt is recommended for prevention, although these associations may be specific to calcium oxalate urolithiasis.<sup>7,8,11</sup> It has also been hypothesized that the high prevalence of nephrolithiasis in humans in the Southeast may be related to increased exposure to sunlight and heat, leading to dehydration and changes in vitamin D metabolism.<sup>27,28</sup> Water hardness has also been implicated in stone formation, with a significantly increased prevalence in areas with soft water (i.e., <50 ppm), but many factors, such as diet and genetics, likely contribute to stone formation.<sup>6</sup>

Nephrolithiasis in North American river otters in North Carolina, USA, is much more prevalent than previously documented. If reniculate kidneys are more vulnerable to stone formation, it is interesting that the composition of nephroliths differs across otter species. While a definitive pathogenesis or population effect has yet to be determined, this study supports the hypothesis that mustelids are uniquely susceptible to this condition.

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