

# Early decrease of ionized calcium and static symmetric dimethylarginine concentration in a model of renal tubular necrosis in corn snakes (*Pantherophis guttatus*)

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## OBJECTIVE

To determine if plasma concentrations of symmetric dimethylarginine (SDMA), *N*-acetyl-beta-D-glucosaminidase (NAG), GGT, ALT, AST, lactate, total calcium, and ionized calcium (iCa) and the calcium:phosphorus ratio are clinically relevant biomarkers to detect early stages of tubular lesions in snakes.

## ANIMALS

6 adult corn snakes (*Pantherophis guttatus*).

## METHODS

Corn snakes were administered 11 injections of gentamicin at 50 mg/kg, SC, q 24 h in an experimental model of induced tubular necrosis. Plasma biochemistry and blood gas analyses were performed at baseline and after the 3rd and 11th injections. Parameters were compared between time points using a paired Wilcoxon test. In 3 individuals, renal biopsies were collected at baseline before starting injections and at the 3rd and 11th injections, while renal tissue samples were procured after euthanasia in all individuals.

## RESULTS

Renal proximal and distal tubular necrosis and hepatic steatosis were present in all individuals at necropsy. Compared to baseline, decreased blood concentrations of lactate, ionized calcium, and total calcium and a decreased calcium:phosphorus ratio were noted. A significant decrease of lactate and ionized calcium was observed after 3 days. Conversely, no changes in SDMA, NAG, ALT, AST, GGT, and sodium were detected.

## CLINICAL RELEVANCE

Ionized calcium and lactate concentrations were the earliest parameters to decrease compared to baseline values in this experimental model. While SDMA is a sensitive indicator of renal disease in mammals, this biomarker did not increase in a model of induced acute tubular necrosis in corn snakes.

**Keywords:** kidney, NAG, *N*-acetyl-beta-D-glucosaminidase, SDMA, symmetric dimethylarginine

Renal lesions have been reported in 40% of captive chameleons<sup>1</sup> and 64% of Hermann tortoises (*Testudo hermanni*)<sup>2</sup> at necropsy. As renal disease is frequent in reptiles, there is a need to develop noninvasive diagnostic tests to detect early stages of disease. In snakes, collection of uncontaminated urine is challenging as they lack a urinary bladder, making

blood parameters particularly relevant as biomarkers of renal disease.

Other diagnostic modalities, such as glomerular filtration rate measurement and scintigraphy,<sup>3</sup> have been described in reptiles but remain marginally used in clinical practice due to their limited availability or patient hospitalization requirements. Glomerular filtration rate references have been published for lizards<sup>4,5</sup> and turtles,<sup>6</sup> but they vary with hydration status<sup>5</sup> and ambient temperature outside of the preferred optimal temperature zone,<sup>7</sup> complicating clinical interpretation of results. An ideal

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marker of renal disease in reptiles should be commercially available and provide a rapid single-point result with good sensitivity/specificity.

Symmetric dimethylarginine (SDMA) is a sensitive early marker of acute and chronic renal disease in dogs and cats.<sup>8-10</sup> Since 2019, SDMA has been included in the International Renal Interest Society staging criteria in dogs and cats. It has been detected in Hermann tortoises and proposed as a marker of renal injury in a retrospective study involving 28 individuals with hyperuricemia.<sup>11</sup> Reference intervals for SDMA have also been described in green sea turtles (*Chelonia mydas*).<sup>12</sup> However, the clinical relevance of this marker remains unclear in reptiles. A recent study<sup>13</sup> has shown that GGT and ALT activities were higher in the kidneys of healthy water snakes (*Nerodia* spp) than in other tissues evaluated. Therefore these biochemistry parameters could be of clinical interest if they are increased when compared to reference intervals in cases with renal lesions.

N-acetyl-beta-D-glucosaminidase (NAG) is a lysosomal enzyme, which has been described as a marker of acute tubular necrosis in birds.<sup>14-16</sup> It is detectable in reptile plasma.<sup>17</sup> Among tested organs, NAG is found preferentially in the renal tissue of pigeons,<sup>16</sup> but its tissue distribution is currently unknown in reptiles. Evaluating the organ distribution of NAG in snakes would be relevant to determine if this enzyme is predominantly present in renal tissue.

The objectives of this study were twofold: first, to compare the sensitivity of commercially available blood markers to detect early stages of renal disease in corn snakes (*Pantherophis guttatus*), and second, to evaluate the tissue distribution of NAG in this species. Our hypotheses were that plasma SDMA and NAG concentrations would be more sensitive than other biochemistry parameters to detect renal disease in snakes and that NAG would be mainly detected in renal tissue as opposed to other evaluated organs in a healthy corn snake.

## Methods

This experimental study was approved by the Animal Care and Use Committee of the Université de Montréal (21-Rech-2134). Seven adult corn snakes (5 males, 2 females; body weight, 241 to 862 grams) were purchased from a breeder. Animals were considered healthy after a complete physical examination, a clinical follow-up over 3 weeks, and unremarkable fecal examinations (direct smear and flotation in zinc sulfate). One of the 7 snakes, a 4-year-old adult female, was kept as a control in the same environment as the other individuals involved in the experiment.

Snakes were kept individually in glass terraria with a surface of 90 X 45 cm (Exoterra; Hagen Inc). They were housed in a temperature and humidity-controlled room set at 29 °C and 38% to 53%, respectively. A warm area at 31 °C was provided in each terrarium by heat lamps. A large water bowl (Corner bowl; Zoomed) was provided where snakes could immerse themselves completely. The photoperiod

was set at a light:darkness ratio of 12:12. Paper was used as substrate, and a hiding box was provided in the colder area of each terrarium. Snakes were weighed twice weekly. They were fed thawed mice once a week. Prey items were offered again after 2 days in case of anorexia. Snakes were not handled for 24 hours after each successful prey ingestion.

The 6 corn snakes were randomly divided into 2 groups: 1 group (n = 3) underwent a renal biopsy per week, while the other group (3) only had a single renal biopsy before starting the experiment to confirm the absence of renal histological changes. The goal of having 2 groups was to evaluate the potential effects of renal biopsies on skeletal muscular enzymes (ie, creatine kinase, ALT, and AST<sup>13</sup>) independently of the effect of gentamicin. To induce renal tubular necrosis, SC injections of gentamicin (100 mg/mL Gentocin; Merck Animal Health) at 50 mg/kg<sup>18</sup> were administered in 6 corn snakes (5 males, 1 female) in the caudo-dorsal area of the body daily for 11 injections or until euthanasia. In case of ecdysis, injections were discontinued for 2 days to allow for complete shedding. Limit points used during the project were based on a grading system established before the study (**Supplementary Table S1**). Euthanasia was elected for individuals in which a score of 3 was reached for any 1 parameter in combination with a score  $\geq 2$  for another parameter or at 21 days as per study design. If gout was detected during coelomic ultrasounds, which were performed weekly (MyLab Sigma; Esoate Canada), euthanasia was also elected.

Before the beginning of the study (time point T0) and after a 3-week acclimation period, blood samples were collected in each of the 7 snakes under general anesthesia with isoflurane at 5% administered in 100% oxygen. Blood collection was performed via cardiocentesis to avoid lymph contamination and was stored in lithium heparin tubes (Lithium Heparin BD Microtainer Tubes; Becton, Dickinson and Company). The procedure was repeated in 6 snakes after 3 injections (time point T3) and 11 injections of gentamicin (time point T11). All blood samples were performed before surgery when applicable, and after at least a 48-hour fast. Plasma biochemistry panels were submitted immediately to the Clinical Pathology Service of the Centre de Diagnostic Vétérinaire de l'Université de Montréal for each snake and included uric acid, BUN, total calcium, phosphorus, total proteins, GGT, ALT, ALP, AST, total cholesterol, CK, glucose, LDH, NAG, and biliary acid concentrations using standard techniques (**Table 1**). The NAG concentration was measured using commercial reagents (Diazyme Laboratories), as described in birds.<sup>15</sup> Frozen plasma was shipped on ice to measure SDMA (Idexx Laboratories). No plasma biochemistry values published in peer-reviewed literature were available for corn snakes. Reference values obtained from a global database from various analyzers were used for interpretation (number of snakes = 65 to 140 depending on parameters).<sup>19</sup> Blood pH, ionized calcium (iCa), sodium, potassium, chloride, lactate, partial pressure in carbon dioxide, and other standard

**Table 1**—Analytic methods used to measure plasma concentration of metabolites in corn snakes (*Pantherophis guttatus*) using a laboratory analyzer (Beckman Coulter DxC 600)

Parameter	Analytical method
Glucose	Hexokinase, 340 nm
Urea	Enzymatic, 340 nm
ALT	Henry, 340 nm
AST	Henry, 340 nm
ALP	Kinetic rate, 410 nm
CK	Rosalki, 340 nm
GGT	Szasz, 410 nm
LDH	Lactate to pyruvate, 340 nm
Total calcium	Endpoint, indirect ion selective electrode, calcium ionophore membrane
Phosphorus	Phospho-molybdate, 340 nm
Total cholesterol	Enzymatic, 520 nm
Total proteins	Biuret, 560 nm
Bile acids	Enzymatic, 405 nm
NAG	Colorimetric, 505 nm

CK = Creatinine kinase. NAG = *N*-acetyl-beta-D-glucosaminidase.

analyte concentrations were obtained on a handheld blood gas analyzer at 31 °C (Heska Element POC; Epocal Inc). For pH and iCa, temperature-adjusted values were calculated as previously described in snakes.<sup>20</sup> Blood concentrations of BUN were measured on both the biochemistry panel and blood gas analysis. Since the detection limit for BUN was 3 mg/dL on the blood gas analysis and 0.3 mg/dL on the laboratory analyzer, plasma BUN concentrations measured by the laboratory analyzer were used for statistical analyses. Reference values using the same blood gas analyzer for free-ranging snakes of the genus *Pantherophis* from a previous study (number of snakes = 19)<sup>21</sup> and reference values for iCa published in ball pythons (*Python regius*)<sup>22</sup> exposed to artificial ultraviolet light were used for interpretation.

Surgical renal biopsies were performed under general anesthesia before the beginning of the study in all snakes and after 3 and 11 gentamicin injections in 3 of the 6 snakes. Premedication included 0.5 mg/kg hydromorphone (2 mg/mL HYDRomorphone; Sandoz Canada Inc) SC administered 1 hour before induction and 5 mg/kg alfaxalone, SC (10 mg/ml Alfaxan Multidose; Jurox Pty Ltée) both in the cranial third of the body, although no significant difference in anesthesia duration or physiological variables has been reported in corn snakes among injection sites for alfaxalone.<sup>23</sup> Snakes were induced by mask with 5% isoflurane (USP; Fresenius Kabi Canada Ltd) in 100% oxygen and intubated with uncuffed endotracheal tubes constructed from a 16-G catheter (Terumo Medical Canada Inc). Snakes were manually ventilated twice per minute. Monitoring included a Doppler flow detector (Ultrasonic Doppler Flow Detector; model 811-B; Parks Medical Electronics) placed over the heart and a capnometer (Covidien N-85; Nellcor). All surgeries were performed in a temperature-controlled room set at 29 °C. The caudal part of the snake was placed in right lateral

recumbency. The left kidney was located at a distance of 90% of the head to cloacal length and visualized by ultrasound. Then, 4 mg/kg lidocaine (20 mg/ml Lurocaine; Vetoquinol) was injected SC at the incision site. The left kidney was approached in a standard fashion. Two mosquito hemostatic forceps were placed laterally on the kidney for 2 minutes before obtaining a 3-mm wedge biopsy. Hemostasis was verified, and the biopsy was placed in a cassette in buffered 10% formalin. If hemostasis was partial, a 5-mm section of procoagulant absorbable gelatin sponge (Surgifoam; Ethicon Inc) was placed on the biopsy site. The coelomic muscles were closed with a continuous pattern using polydioxanone suture (PDS 4.0; Ethicon; Johnson and Johnson Medtech). The skin was closed with interrupted sutures in an everting pattern using PDS 4.0. An injection of 2 mg/kg ketoprofen (100 mg/ml Anafen; Merial Canada) was administered in the cranial epaxial muscles.

Euthanasia was performed under isoflurane anesthesia with an intracardiac injection of 100 mg/kg pentobarbital sodium (340 mg/ml Euthanyl; Bimeda-MTC Animal Health) and 2 mEq/kg potassium chloride (2 mEq/ml; Pfizer Canada) followed by brain pithing. A complete necropsy was then performed, and tissue samples were taken and fixed in buffered 10% formalin, including the kidneys, heart, lung, liver, spleen, stomach, and reproductive tract. Four sections along the length of the right kidney were obtained and examined. Tissues were stained with hematoxylin, phloxine, and saffron using a routine staining protocol. Histopathologic scoring of kidney sections (biopsies and necropsy specimens) was performed, without prior knowledge of individuals and time points by a single veterinary pathology resident (SF) under the supervision of a trained and experienced veterinary pathologist (SL). A total of 5 randomly selected, renal fields at a magnification of X200 were evaluated from each biopsy and/or necropsy submission. Each field was scored using a standardized semiquantitative scale (0, within normal limits/absent; 1, mild changes [ $< 10\%$  of the cells or field affected]; 2, moderate changes [ $10\%$  to  $50\%$ ]; and 3, marked changes [ $> 50\%$ ]). Parameters scored were (1) proximal tubule degeneration, inflammation, and necrosis; (2) distal tubule degeneration, inflammation, and necrosis; (3) severity of intraluminal mineral deposition; and (4) intraluminal urate deposition. Tubular degeneration was characterized by pale, swollen, vacuolated epithelial cells with a loss of cytoplasmic detail, while necrosis was defined by cytoplasmic hypereosinophilia, a loss of cellular borders, and nuclear pyknosis/karyorrhexis. The median value of the 5 evaluated fields was then reported as the scored result for each parameter for the specimen.

Sections of liver from each animal harvested at necropsy were processed and stained as described above to check for evidence of hepatic degeneration/necrosis. A total of 5, randomly selected hepatic fields (magnification, X200) were evaluated and scored with a standardized semiquantitative scale: (0, within normal limits/absent; 1,

mild changes [ $< 10\%$  of the cells or field affected]; 2, moderate changes [ $10\%$  to  $50\%$ ]; and 3, marked changes [ $> 50\%$ ]. Parameters scored were (1) steatosis, (2) inflammation, (3) necrosis, (4) gout tophi, and (5) fibrosis. The median of the scored result was reported for each parameter for the specimen.

In addition to the experimental study, an adult female corn snake was kept in the same environmental conditions and was euthanized with the same protocol. A normal health status was confirmed by necropsy and a plasma biochemistry panel within reference ranges.<sup>19</sup> A wet weight of 0.58 grams from each organ (kidney, liver, heart, pancreas, small intestine, ovary, and skeletal muscle) was sampled using a scale with a precision of 0.1 mg (Sartorius Canada Inc). Each tissue was homogenized for 10 minutes in a microtube with glass beads and 1 mL of ultrapure water (Mini-Beadbeater 96; Biospec Products Inc). The content was centrifuged at  $15,060 \times g$  for 10 minutes (Centrifuge 5425; Eppendorf Canada Ltd), and the supernatant was submitted for NAG concentration measurement. The same NAG commercial assay kit was adapted to run on an automated chemistry analyzer (Beckman Coulter).

Data were analyzed using a statistical analysis software (R version 4.2.1, R Core Team [2022]. R: A language and environment for statistical computing. R Foundation for Statistical Computing. <https://www.R-project.org/>). For each blood parameter, the initial value measured on each individual and the values obtained after 3 and 11 injections of gentamicin (time points called T3 and T11 for all snakes) were compared using paired Wilcoxon tests. When a parameter had a value below the detection limit of a test, the lowest measurable value of the test was used for statistical analysis. A Benjamini-Hochberg correction was applied to the  $P$  values. To evaluate the correlation of biochemical parameters with proximal and distal tubular necrosis lesions, a linear mixed model was created with each blood parameter as the dependent variable (including uric acid, lactate, sodium, total calcium, iCa, calcium:phosphorus ratio, ALT, AST, and urea), the histologic score as an independent variable, and the snake specimen as a random variable. The R package 'lme4' was used for this analysis. The significance of the variable was tested via a likelihood ratio test. Data distribution of the residues of each model was evaluated using a Shapiro-Wilk normality test. A logarithmic transformation of the ALT variable was used to achieve normality of the model residues. The level of statistical significance was set at .05.

## Results

Baseline blood gas analysis results were within published reference ranges<sup>19,21</sup> except for 1 sample with slightly decreased lactate concentration (1.69 mmol/L; reference values, 2.66 to 20.1 mmol/L).<sup>21</sup> Baseline renal histology was within normal limits in all 6 snakes involved in the experimental study, including 2 snakes with mild renal changes considered incidental and insufficient

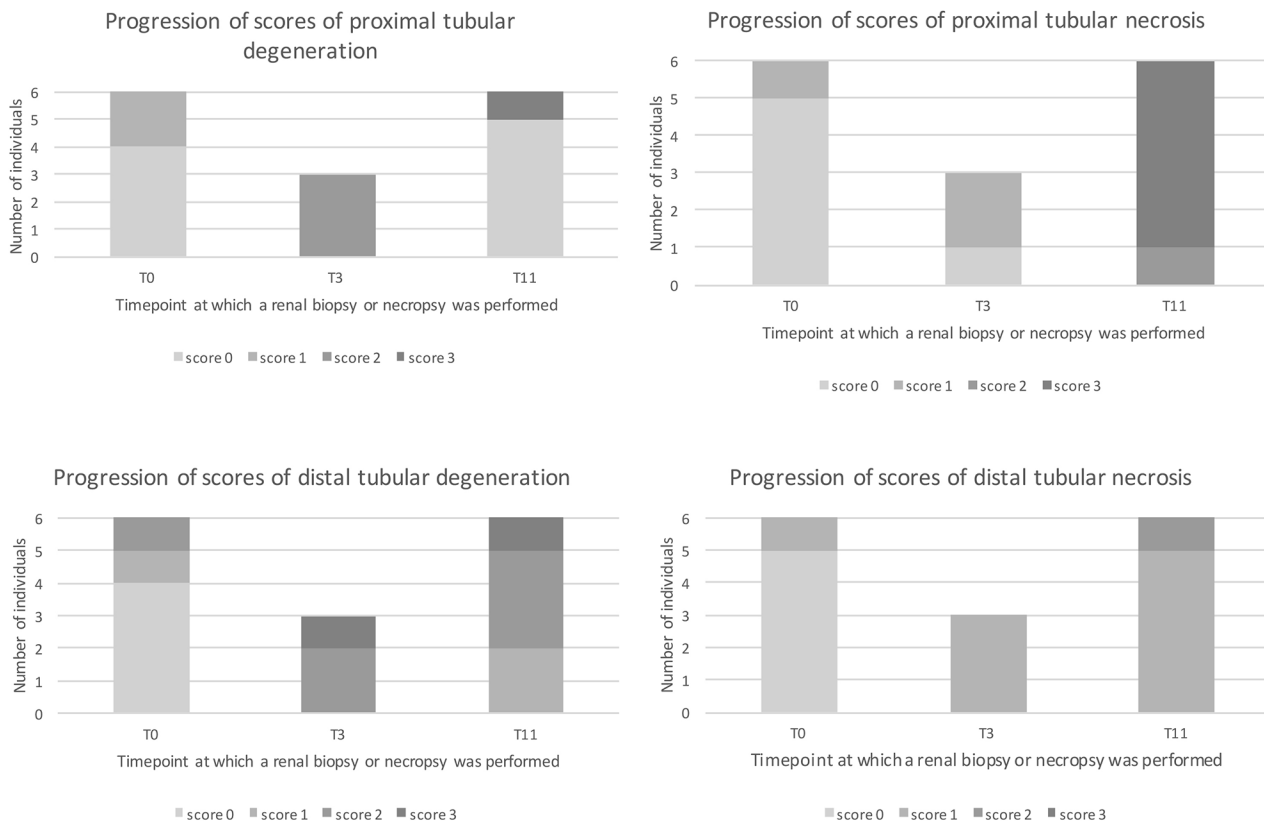
for exclusion from the study. During the gentamicin injections, none of the snakes developed renal lesions that were detectable on coelomic ultrasound. Snakes reached limit points at the 11th injection of gentamicin which justified euthanasia at that time. Clinical signs developing during the second week of the study varied by individual and included diarrhea, polydipsia, polyuria, lethargy, and anorexia. Observed renal pathologic changes are summarized (**Figure 1**). Since all renal biopsies performed at T11 for the 3 snakes had identical scores as the results obtained at necropsy, renal scores at necropsy are displayed for these 3 snakes. As the injections progressed, the proximal tubules displayed a mild to moderate degeneration with more frequent, intense levels of necrosis, while the distal tubules more frequently demonstrated higher levels of degeneration with less frequent, intense necrosis (**Table 2**). Little to no inflammation was associated with the aforementioned tubular degeneration/necrosis in either the proximal or distal segments. Mild to moderate intratubular mineralization was observed in both baseline (T = 0) and biopsy/necropsy samples with no obvious increase in frequency or intensity as the study progressed. Intratubular luminal urate deposition was not observed in any submitted sample at any time point. On necropsy, hepatic steatosis was noted in all snakes, and 1 snake displayed multiple hepatic tophi characterized by foci of urate crystal deposition, hepatocellular necrosis, and a moderate to marked heterophilic infiltrate with macrophages.

Evolution of the plasma biochemistry panel parameters is illustrated (**Figure 2**). Uric acid concentration did not increase significantly between T0 and T3 ( $P = .22$ ), although there was a tendency toward an increase between T0 and T11 ( $P = .06$ ) and 4 out of 6 snakes displayed hyperuricemia at T11 (reference, 168 to 1,194  $\mu\text{mol/L}$ <sup>19</sup>).

Plasma concentrations of ALP, BUN, CK, LDH, and total proteins did not change significantly over time and remained within reference intervals for corn snakes.<sup>19</sup> Plasma CK concentrations remained similar in both groups. No significant differences in SDMA, NAG, AST, ALT, and GGT concentrations were found between baseline and any time point. All measured AST concentrations remained within reference intervals<sup>19</sup> at all time points except for 2 samples at T11.

Total calcium concentrations and the calcium:phosphorus ratio decreased significantly between T0 and T3 ( $P = .03$ ) and between T0 and T11 ( $P = .03$ ). Two out of 6 snakes had a slightly decreased total calcium at T3 (range, 3.16 to 3.29 mmol/L; reference, 3.38 to 4.90 mmol/L). No significant changes were noted in plasma phosphorus concentrations overtime. All 6 snakes displayed calcium:phosphorus ratios within reference values at T3.<sup>19</sup> Four out of 6 snakes displayed a decreased calcium:phosphorus ratio at T11 (range, 1.4 to 2.1; reference, 2.2 to 9.7).<sup>19</sup>

On blood gas analysis, iCa decreased significantly between T0 and T3 ( $P = .03$ ) and between T0 and T11 ( $P = .03$ ) (**Figure 3**). All snakes had baseline iCa concentrations within the published reference range



**Figure 1**—Histogram of individual median scores of tubular lesions in 6 corn snakes (*Pantherophis guttatus*) undergoing renal biopsies before the beginning of the study (T0) and in 3 corn snakes after 3 injections (T3) and after 11 injections (T11) of gentamicin 50 mg/kg, SC, in the caudal third of the body (renal biopsy or necropsy results).

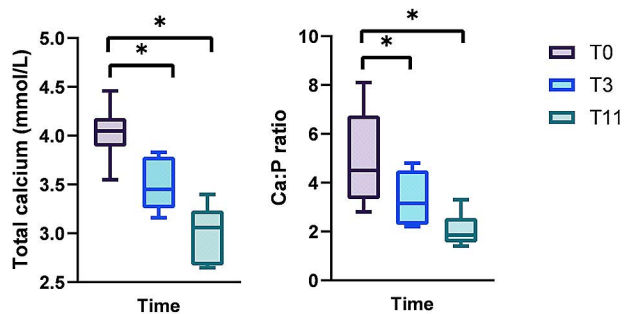
**Table 2**—Histologic median score values regarding renal lesions in 6 corn snakes (*P. guttatus*) undergoing renal biopsies before the beginning of the study (T0), in 3 corn snakes after 3 injections (T3) and after 11 injections (T11) of gentamicin, and in all 6 corn snakes at necropsy.

Snake ID	Sample	Histology median score values							
		PTD	PTI	PTN	DTD	DTI	DTN	ITM	IUD
1	BIOPSY 1	0	0	0	0	0	0	0	0
1	NECROPSY	0	0	3	1	0	1	0	0
2	BIOPSY 1	0	0	0	0	0	0	0	0
2	BIOPSY 2	2	0	1	3	0	0	1	0
2	BIOPSY 3	0	0	3	2	0	1	0	0
2	NECROPSY	0	0	3	2	0	1	0	0
3	BIOPSY 1	0	0	0	0	0	0	0	0
3	NECROPSY	0	0	3	1	0	1	1	0
4	BIOPSY 1	0	0	0	0	0	0	0	0
4	NECROPSY	3	0	2	2	0	1	0	0
5	BIOPSY 1	2	0	0	2	0	0	0	0
5	BIOPSY 2	2	0	0	2	0	1	0	0
5	BIOPSY 3	0	0	3	2	0	1	0	0
5	NECROPSY	0	0	3	3	0	2	0	0
6	BIOPSY 1	2	0	0	1	0	0	2	0
6	BIOPSY 2	2	0	1	2	0	0	1	0
6	BIOPSY 3	0	1	3	3	0	1	1	0
6	NECROPSY	0	1	3	2	1	0	1	0

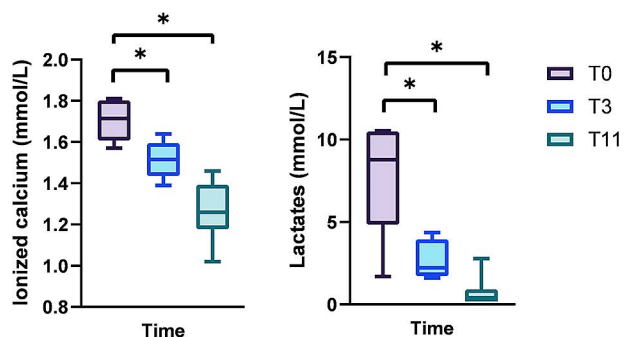
DTD = Distal tubule degeneration. DTI = Distal tubule inflammation. DTN = Distal tubule necrosis. ITM = Intratubular mineral deposition. IUD = Intraluminal urate deposition. PTD = Proximal tubule degeneration. PTI = Proximal tubule inflammation. PTN = Proximal tubule necrosis.

for ball pythons (1.67 to 1.91 mmol/L; n = 14).<sup>22</sup> iCa concentrations fell below reference values in 5 out of 6 snakes at T3 (range, 1.39 to 1.64 mmol/L) and

in all 6 snakes at T11 (range, 1.02 to 1.46). Lactate concentration decreased significantly between T0 and T3 ( $P = .03$ ) and between T0 and T11 ( $P = .03$ ).



**Figure 2**—Evolution of plasma concentration of total calcium and calcium:phosphorus ratio in 6 corn snakes (*P. guttatus*) during induction of experimental tubular necrosis using gentamicin 50 mg/kg, SC, in the caudal third of the body. The boxes represent the quartiles, whiskers represent the minimum and maximum, and the line inside the box represents the median. T0 is the baseline before starting gentamicin injection, T3 is the second time point obtained after 3 injections of gentamicin, and T11 is the final time point obtained after 11 injections of gentamicin. \*Significant variations with  $P < .05$ .

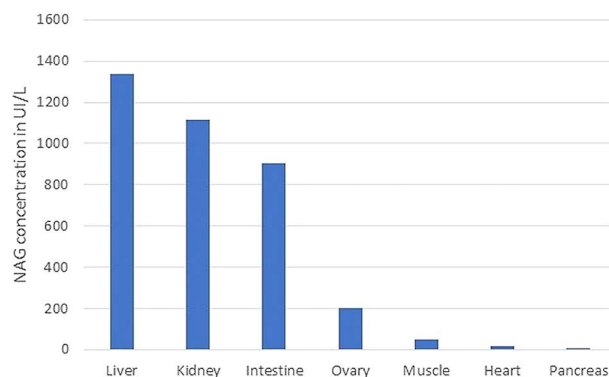


**Figure 3**—Evolution of whole blood concentrations of ionized calcium (iCa) and lactate measured on blood gas panel (Heska Element POC; Epocal Inc) during induction of experimental tubular necrosis using gentamicin in 6 corn snakes (*P. guttatus*). See Figure 2 for the remainder of the key.

At T3, 4 out of 6 snakes displayed lactate concentrations below reference values.<sup>21</sup> At T11, lactate concentrations fell below the limit of detection of 0.3 mmol/L in 5 out of 6 snakes. No significant changes were observed among time points for blood pH ( $P = .31$ ) or partial pressure of carbon dioxide ( $P = .09$ ). No significant changes were noted for potassium, sodium, and chloride concentrations. At the third time point (T11), sodium was below the reference values in 4 out of 6 snakes (range, 141 to 148 mmol/L; reference, 153 to 180 mmol/L).<sup>21</sup>

Proximal tubular necrosis was significantly correlated with uric acid ( $P < .001$ ), total calcium ( $P < .001$ ), iCa ( $P < .001$ ), lactate ( $P < .001$ ), ALT ( $P < .001$ ), BUN ( $P < .001$ ), sodium ( $P < .001$ ), and AST ( $P = .008$ ) concentrations and calcium:phosphorus ratio ( $P = .006$ ). Distal tubular necrosis was significantly correlated with decreased total calcium ( $P = .002$ ), decreased lactate ( $P = .003$ ) concentrations, and decreased calcium:phosphorus ratio ( $P = .001$ ).

Tissue concentrations of NAG detected in the control snake are depicted (**Figure 4**). The liver displayed the highest NAG concentrations among the tested organs, although renal tissue accounted for the second highest concentration of NAG.



**Figure 4**—Tissue concentration of *N*-acetyl-beta-D-glucosaminidase (NAG) in a 4-year-old healthy adult female corn snake (*P. guttatus*).

## Discussion

The model used in this study was successful in inducing renal tubular necrosis in all snakes tested. Alternatively, a contribution of the initial renal biopsy to the histologic lesions cannot be ruled out. Regardless, this model enabled evaluation of the effects of acute tubular lesions on blood parameters.

Parameters found to vary significantly as renal tubular pathology progressed were uric acid, ALT, AST, total calcium, lactate, sodium, and iCa concentrations, as well as the calcium:phosphorus ratio. Interpretation of these parameters remains challenging in a clinical setting as it relies on suboptimal reference values published in textbooks.<sup>24,25</sup> To be diagnostically useful, blood parameters of snakes with renal disease should have minimal overlap with those of healthy animals. Thus, defining narrower reference intervals for corn snakes would be very relevant to minimize this overlap. At this point, no reference intervals complying with the guidelines of the American Society of Veterinary Clinical Pathology<sup>26</sup> have been published for corn snakes. Using a single analyzer to establish reference intervals and better defining the seasonality and site of venipuncture would result in narrower reference intervals. Despite this limitation, multiple blood parameters were found to deviate from current reference values used for corn snakes in this model of induced tubular lesions.

Absolute hyperuricemia was detected in 4 out of 6 snakes after 11 days of daily gentamicin injections, although this change was not statistically significant compared to baseline concentration. One of these snakes had not eaten during the previous 10 days so its hyperuricemia was not attributed to postprandial changes. In the other 3 hyperuricemic snakes, a contribution of postprandial hyperuricemia can not

be ruled out as uric acid can be increased for 8 days after a meal in corn snakes<sup>27</sup>, and these snakes had eaten 4 days prior. However, postprandial plasma uric acid concentrations up to 2,550  $\mu\text{mol/L}$  were reported in a previous study,<sup>27</sup> while the snakes of the present study reached uric acid concentrations between 13,338 and 17,778  $\mu\text{mol/L}$ . Thus, hyperuricemia secondary to induced renal disease was suspected. As expected, uric acid proved to be a poorly sensitive marker of renal tubular lesions as hyperuricemia was detected in only some individuals and at T11 only. Plasma BUN concentrations did not significantly increase demonstrating a low sensitivity in snakes. In addition, BUN is a very nonspecific marker of renal disease as it increases after brumation and in cases of dehydration in reptiles.<sup>28,29</sup> Similarly, blood sodium concentration was a poorly sensitive biomarker of renal disease. Renal tubular lesions can be associated with decreased active sodium reabsorption leading to hypovolemic hyponatremia.<sup>30</sup> However, blood sodium concentration would only be a terminal marker of renal tubular disease in snakes.

When evaluating snakes 3 days after initiating gentamicin injections, lactate, total calcium, and iCa concentrations and the calcium:phosphorus ratio were found to be significantly decreased compared to baseline values. In addition, the calcium:phosphorus ratio and total calcium and lactate concentrations were significantly correlated with the score of proximal and distal tubular lesions. Among these parameters, only blood iCa and lactate concentrations were below published reference ranges<sup>22</sup> in a majority of the study snakes at T3. Although 1 snake had a lactate concentration below reference values at the beginning of the study, this snake also showed decreasing blood lactate concentrations over time. As significant decreases in blood lactate concentrations were noted as early as 3 days after the beginning of gentamicin injections, lactate might serve as an early biomarker of tubular lesions. Recently, lactate concentrations have gained interest in reptile medicine as a prognostic indicator in rehabilitated chelonians.<sup>31,32</sup> Increasing lactate concentrations have been associated with a guarded prognosis,<sup>31</sup> while other studies<sup>33</sup> have suggested improved nesting in sea turtles with hyperlactatemia. Reptiles produce lactate during anaerobic metabolism and are particularly prone to perform anaerobic exercise compared to mammals. Chelonians are able to sustain high lactate concentrations during hibernation,<sup>34</sup> and their renal tubular epithelium can use lactate as a metabolite for glucose production. In the present study, it is unknown if an increased uptake of lactate could have occurred at the level of renal tubular cells, as previously described in colubridae<sup>35</sup> or if a decreased reuptake of lactate from the primary urine could have occurred in the proximal tubules.<sup>36</sup> Alternatively, a possible explanation for lactate changes is that snakes' reactivity to handling during induction and venipuncture decreased as renal disease progressed. Since lactate concentration is positively correlated with muscle activity in reptiles,<sup>37</sup> decreased resistance to handling might

explain the decreased lactate concentration in later time points. On the other hand, hyperlactatemia has been described in box turtles with a quiet mentation, compared to healthy conspecifics.<sup>37</sup> Thus, lethargic snakes would have been expected to display higher lactates whereas the opposite was observed in the present study. Alternatively, lactate may decrease over time during anesthesia when snakes are ventilated.<sup>38</sup> However, it took 6 hours of anesthesia in rattlesnakes to reach lactate concentrations similar to those noted at T11,<sup>38</sup> while blood samples were obtained within 10 minutes of induction in all instances in the present study. In addition, acid-base disturbance was restored after only 12 hours in a previous study.<sup>39</sup> Thus, the sole effect of ventilation under anesthesia to explain hypolactatemia seems unlikely, especially in a context where blood pH and carbon dioxide remain within normal limits. Regardless of the cause, further studies should aim to evaluate the prevalence of hypolactatemia in reptiles with renal disease. Assessing the agreement between various handheld lactate meters would also be relevant, as previously performed in sea turtles.<sup>40</sup>

Previous publications have listed the calcium:phosphorus ratio as the earliest affected parameter in cases of renal disease in snakes.<sup>41,42</sup> iCa and lactate concentrations may in fact be more sensitive parameters using currently available reference intervals.<sup>22</sup> This previous statement regarding the high sensitivity of the calcium:phosphorus ratio was based on a retrospective study<sup>43</sup> in 12 green iguanas published in a conference proceeding. It should be noted that iCa concentration is not affected by egg laying in females<sup>44</sup> and remains within similar reference intervals in Indigo snake (*Drymarchon couperi*),<sup>45</sup> which might make it a more specific indicator of renal disease than total calcium concentration. However, iCa may be decreased secondary to hyperparathyroidism, and combined interpretation with more specific markers of renal disease is suggested.

Surprisingly, NAG was not found to be a specific biomarker for corn snake renal tissue as it was detected in high concentrations in the liver and intestine in this species. In pigeons, renal NAG concentration has been shown to be at least 6 times higher than in other tissues, including liver, intestine, and pancreas.<sup>16</sup> This difference highlights the importance of species-specific studies to evaluate enzymatic tissue distribution.

In the present study, SDMA proved to be an insensitive marker of tubular necrosis in corn snakes. In contrast, a similar experimental model using gentamicin to induce acute tubular necrosis in rats resulted in an increased serum SDMA concentration.<sup>46</sup> A previous retrospective study hypothesized that SDMA could be an early indicator of renal disease in chelonians<sup>11</sup>; this conclusion was based on the results obtained in 28 individuals showing a correlation between elevated uric acid and SDMA concentrations. However, no renal histology was available, and it is, therefore, possible that this conclusion was erroneous. Further studies are warranted to determine if SDMA concentrations could

increase in reptiles affected by glomerular disease instead of renal tubular pathology or in reptiles with chronic versus acute renal disease or whether its metabolism differs between chelonians and snakes. Pending more studies, SDMA concentration should not be used as a marker of renal disease in reptiles. Other diagnostic options are recommended such as renal biopsy.<sup>42,47</sup>

Despite hepatic steatosis being noted in all snakes, AST and ALT did not increase significantly, which was surprising as these enzymes have been found in high quantities in colubrid snake renal and hepatic tissue.<sup>13</sup> No significant increase in plasma GGT concentrations was detected at any time point in the present study. This enzyme is thought to be highly specific as it is detected only in the lungs and kidneys of snakes and box turtles.<sup>13,48</sup> However, the presence of an enzyme in renal tissue does not necessarily correlate with its elevation in the bloodstream after tissue damage, as previously demonstrated for glutamate dehydrogenase in birds.<sup>49</sup> Similarly, GGT may be excreted in the urine rather than increasing in the bloodstream. Although no data are available in reptiles, GGT has been detected in bird urine.<sup>50</sup> Further studies should evaluate GGT concentration in urine in healthy versus renal insufficient reptiles.

Limitations of the present study include the small number of individuals included, but ethical considerations precluded enrollment of a large snake cohort. In particular, more healthy individuals could have been used to evaluate the NAG tissue distribution and evaluate the interindividual variability. The variations of parameters observed in this study could be related to renal lesions or could potentially be associated with repeated handling or other factors in the absence of a control group. In addition, significant disagreement has been described for several analytes measured by blood gas analyzers compared to gold-standard techniques in reptiles.<sup>51</sup> Further studies should aim to evaluate the analytical performance of blood gas analyzers in reptiles. In dogs and cats, other novel markers of renal disease include clusterin, cystatin B, and inosine.<sup>8,52</sup> Serum inosine concentration has been shown to decrease in a model of gentamicin-induced renal disease in dogs as it is a marker of tubular epithelial cells.<sup>8</sup> However, this marker was not available at the time of this study. In conclusion, iCa and lactate concentrations were the most sensitive early indicators of renal tubular pathology in corn snakes among tested parameters. Although still within reference values, total calcium concentrations and the calcium:phosphorus ratio decreased significantly 3 days after induction of renal disease compared to baseline values. Further studies are needed to establish reference intervals for iCa and lactate in colubrid snakes.

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## Supplementary Materials

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