Relationship, difference, and diagnostic discordance between blood ionized and total calcium concentrations in client-owned chelonians

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OBJECTIVE
To determine (1) the relationship, (2) the difference, and (3) the diagnostic discordance between blood total calcium concentration (tCa) and ionized calcium concentration (iCa) in a population of client-owned chelonian patients.

ANIMALS
161 consecutively admitted client-owned chelonians.

PROCEDURES
Results for tCa, iCa, and other variables were extracted from records. Bound calcium concentration (tCa – iCa) was calculated. General linear models were developed to evaluate factors affecting tCa, iCa and bound calcium concentrations. Diagnostic discordance between tCa and iCa was assessed.

RESULTS
iCa decreased with increasing albumin concentration, it was not influenced by sex, and it was lower in chelonians with disorders of the reproductive tract than in those with disorders of the endocrine/hemopoietic and urinary systems. Total calcium and bound calcium concentrations increased with increasing albumin concentration; they were higher in females and in chelonians with disorders of the reproductive tract. Of the 161 chelonians, 93 (57.7%; 95% CI, 49.7% to 65.5%) would have had a different diagnosis of calcium status based on tCa and iCa results (ie, diagnostic discordance). A 2.2 mmol/L cutoff value for bound calcium could differentiate chelonians with and without disorders of the reproductive tract with a sensitivity (95% CI) of 81.8% (64.5% to 93.0%) and specificity of 76.4% (68.0% to 83.5%).

CLINICAL RELEVANCE
tCa and iCa were related and were associated with albumin concentrations in chelonians. Evaluation of tCa or iCa alone is likely to result in underdiagnosis of alterations of the calcium status. Calculation of bound calcium concentration could help identify chelonians with reproductive disorders.

Studies of blood calcium concentrations in chelonians date back to the early 20th century.1 Measurements of blood calcium concentrations are now widely used in clinical settings to monitor calcium homeostasis in humans and animals, including reptiles.2,3 Changes in blood calcium concentrations may have serious clinical implications in reptiles, as in other animals.4 Blood calcium concentrations may be affected by several parameters including diet, exposure to ultraviolet B radiation, sex, season, physiological status, and medications, among others. For example, lower calcium concentrations are observed in reptiles exposed to limited ultraviolet B radiation or administered diets with insufficient levels of available calcium.5 Higher total calcium concentrations are observed in female reptiles during their reproductive activity,6 and increases in total calcium concentrations are dependent on circulating estrogens.7 Plasmatic calcium is present in three different states: ionized or free (approx 56% in dogs and 45% in humans), protein bound (approx 34% in dogs and 41% in humans), and bound to other anions (approximately 10% in dogs and 14% in humans).6,9 The entire amount of calcium present in plasma, serum, or whole blood is often referred to as total calcium (tCa) concentration. The free form of calcium, customarily defined as ionized calcium (iCa), has been historically more difficult to analyze.10 Ionized calcium is now regarded as the reference standard for evaluating calcium homeostasis in dogs.11-14 This is based on the physiological concept that iCa is the most biologically active form10 and on research that
Materials and Methods

Study design and inclusion criteria
Consecutively admitted chelonians examined by a board-certified specialist in herpetological medicine (ND) from May 2017 to July 2018 at the Tai Wai Small Animal & Exotic Hospital, Hong Kong, were eligible for inclusion in the study. As part of the standard bloodwork offered for chelonians presented to the hospital, owners were offered the following at their own expense: (1) analysis with a point-of-care biochemistry analyzer; (2) analysis with a portable blood gas, electrolyte, and Hct analyzer; (3) manual CBC; and (4) manual PCV and total solids (TS) measurement via refractometry.

Results for chelonians were not included in the study if blood was not obtained as a part of the clinical assessment, the minimum amount of blood necessary for analysis was not collected, or the owner declined the analysis. Only the first available result for each individual animal was eligible for inclusion in the analysis. The owner of each chelonian signed an informed consent form for the diagnostic procedures as per hospital policy.

Data extraction
For this study, the following variables were retrieved from the records of each chelonian: identification number, species (male, female, or unknown), organ system affected, plasma TS concentration, and blood iCa, tCa, and albumin concentrations. The organ system affected was determined at the end of data collection on the basis of the clinical diagnosis reported in the medical records at the time of hospital discharge and based on available clinical, diagnostic, surgical, and pathological findings.

The organ system affected was selected from the following categories: cardiovascular, endocrine/hemopoietic, gastrointestinal/liver, nervous, ophthalmic, renal/urinary, respiratory, reproductive, skin/musculoskeletal, and no specific organ system. The reproductive system was considered affected when abnormalities in organs involved in sexual reproduction were detected (ovaries, oviducts, testes, or phallicus, including ectopic eggs). The endocrine and hemopoietic systems were considered affected when abnormalities were detected in secretions of internal glands, including as a consequence of metabolic and nutritional disturbances, or anemia without other underlying causes. Since most animals had multiple organ system affected, the primary organ system affected was defined as the organ affected that resulted in the current reason for presentation. For instance, if a chelonian had long-term history of nutritional secondary hyperparathyroidism, but the animal recently developed intestinal impaction and it was presented for a prolapse of the intestinal tract, the primary organ system affected would have been gastrointestinal/liver rather than endocrine/hemopoietic, and endocrine/hemopoietic would have been listed as a secondary organ system affected.

Sample collection and analysis
Veni puncture and sample processing were performed following current hospital standards. The total volume of blood collected would never exceed 0.5% of the body weight of the animal. Chelonians were restrained in lateral recumbency, and a blood sample was obtained from a jugular vein. One operator performed venipuncture and sample processing, while the second operator held the animal in lateral recumbency and applied gentle digital pressure on the venipuncture site. To minimize delay in analysis, venipuncture was performed in proximity to the portable blood gas analyzer (i-STAT 1 analyzer, Abbott), and the analyzer was turned on and it was ready for the cartridge to be inserted. To avoid exposure to room air, the blood sample was introduced into the cartridge (CG8+, Abbott) directly from the syringe. The remaining blood in the syringe was transferred into blood collection lithium-heparin tubes and inverted 8 to 10 times to ensure anticoagulation. From the lithium-heparin tube, 0.1 mL of blood was inserted in a reagent disc (avian/reptilian Profile Plus, Abaxis) for analysis with a biochemistry analyzer (Vetscan VS2 chemistry, Abaxis) using a 100-μL pipette.
Analyze the text to extract the main points and convert it into a coherent, natural language text.

Statistical analysis

Summary statistics, including the Shapiro-Wilk normality test, were performed for continuous variables. Due to nonnormality, all continuous variables were reported as median, quartiles, and range. All continuous variables were reported with 1 decimal place, except for tCa values, which were reported with 2 decimal places. For the purpose of the analysis, tCa results, provided by the analyzer in mg/dL, were transformed to mmol/L by use of the conversion factor 0.2495 (eg, 10 mg/dL = 2.495 mmol/L). The minimum or maximum thresholds were used for those data points that were recorded as beyond the reportable range of the analyzer. The difference in calcium (bound calcium) was calculated as tCa (mmol/L) minus iCa (mmol/L) for each chelonian. Only the primary organ system affected for each chelonian was used for the purpose of statistical analysis.

Diagnostic discordance—Each chelonian was categorized as having a normal, low, or high iCa concentration and normal, low, or high tCa concentration based on a priori determined cutoffs. The primary cutoffs for ionized and tCa concentrations were arbitrarily decided before performing the analysis based on a number of previous studies on nonmarine chelonians, and they reflect the current reference intervals used by the author for chelonians. The primary cutoffs were 1.2 to 1.6 mmol/L and 10 to 14 mg/dL for iCa and tCa, respectively.

A sensitivity analysis was carried out using wider cutoffs (1.0 to 1.8 mmol/L and 7.4 to 17.2 mg/dL for iCa and tCa, respectively) based on a previously published study on a chelonian species that employed the same biochemistry and blood gas analyzers. Similarly to previous publications concerning dogs and cats, cases where tCa and iCa results did not provide a similar diagnosis of calcium status based on the predefined cutoffs were classified as having diagnostic discordance. The percentage of diagnostic discordance with its 95% CI was calculated for all chelonians.

Modeling—Generalized linear models were built to explore the relationship between tCa and iCa concentrations and the effect of previously known confounders on this relationship and to explore the effect of multiple variables on the difference between tCa and iCa concentrations. Dependent variables were iCa (mmol/L), tCa (mg/dL) and the difference between tCa and iCa (mmol/L). Predictor variables for potential inclusion in the models were as follows: tCa or iCa (only in the first 2 models), TS, albumin, sex (male/female/unknown), species, habitat, or organ system affected. In each model, to evaluate the potential effect of species, all chelonians excluding the 4 most represented species (Trachemys scripta elegans, Centrochelys sulcata, Stigmochelys pardalis, and Cuora trifasciata) were united under a single category and used as the comparator in the model. To evaluate the potential effect of organ system affected, the organ systems ophthalmic and cardiovascular, which included 2 and 1 individuals each, respectively, were united together with animals with no diagnosis in a category named others.

Sex and diagnosis were included in all models based on clinical importance. The inclusion of species rather than habitat and albumin rather than TS depended on the fit of each individual model. Interactions between predictors were included and tested in the final models and retained if they contributed to the fit of the model. Values of the Akaike information criterion (AIC) were used to compare models with different predictors. Residuals of the models were checked for normal distribution. Results were provided as coefficients, 95% CIs, and P values.

Since the analysis highlighted a difference in bound calcium between chelonians with reproductive disorders and other chelonians, the diagnostic accuracy of this finding was further investigated. A receiver operating characteristic curve was built to identify cutoffs for bound calcium concentration that could be effective to discriminate chelonians with and without reproductive disease. Sensitivity, specificity, positive predictive value, and negative predictive value were provided with their 95% CIs for the selected cutoff.

Analyses were performed with commercial software (SPSS statistics, version 22.0, IBM Corp). Two-tailed P values < 0.05 were considered significant.

Results

Samples

At total of 192 whole blood samples were obtained from chelonians during the study period. After multiple samples obtained from the same chelonians were removed, 161 independent samples were included in the final analysis.

Patient-related variables

Species, habitat, and sex—Of the 161 chelonians included, 61 (37.9%) were red-eared sliders (Trachemys scripta elegans), 23 (14.3%) were African spurred tortoises (Centrochelys sulcata), 14 (8.7%) were Leopard tortoises (Stigmochelys pardalis), 14 (8.7%) were Chinese three-striped box turtles (Cuora trifasciata), 7 (4.3%) were Indian star tortoises (Geochelone elegans), 7 (4.3%) were Chinese stripe-necked turtles (Ocadia sinensis), 6 (3.7%) were Reeves turtles (Mauremys reevesii), 6 (3.7%) were pancake tortoises (Malacochersus tornieri), and the remaining species were represented by ≤ 3 individuals. Overall, 104 (64.6%) chelonians were classified as predominantly aquatic species and 57 (35.4%) as predominantly terrestrial species.
predominantly terrestrial species. Animals included 65 (40.4%) sexually intact females, 4 (2.5%) spayed females, 26 (16.1%) males, and 66 (41.0%) chelonians of undetermined (unknown) sex.

**Organ system diagnosis**—Seventeen chelonians had no specific organ system alteration diagnosed. There was a total of 209 organ system diagnoses for 161 chelonians, ranging from a minimum of 0 to a maximum of 3 per individual chelonian. The organ systems affected were endocrine/hemopoietic (including metabolic and anemia; n = 46; 22.0% of the total organ system affected), cutaneous/musculoskeletal (45; 21.5%), reproductive (39; 18.7%), renal/urinary (26; 12.4%), gastrointestinal/hepatic (26; 12.4%), respiratory (17; 8.1%), ophthalmic (8; 3.8%), neurologic (1; 0.5%), and cardiovascular (1; 0.5%).

**Total solids and albumin**—Results for plasma TS concentrations were available for 156 chelonians, and values were nonnormally distributed (Shapiro-Wilk test, \( P < 0.03 \)). Blood albumin concentrations were available for all 161 chelonians and were normally distributed (Shapiro-Wilk test, \( P = 0.07 \)). The median TS concentration was 5.0 mg/dL (interquartile [25th to 75th percentile] range [IQR], 3.6 to 7.0 mg/dL; range, 0.8 to 11.4 mg/dL), and the median albumin concentration was 1.6 mg/dL (IQR, 1.1 to 2.2 mg/dL; range, 0.1 to 3.1 mg/dL).

**Blood tCa and iCa concentrations**

Values for both blood tCa concentration and blood iCa concentration were available for all 161 chelonians included and were nonnormally distributed (Shapiro-Wilk test, \( P < 0.001 \) and \( P = 0.01 \), respectively). Overall, the median blood tCa concentration was 12.8 mg/dL (IQR, 10.5 to 17.3 mg/dL; range, 0.8 to 11.4 mg/dL), and the median albumin concentration was 1.6 mg/dL (IQR, 1.1 to 2.2 mg/dL; range, 0.1 to 3.1 mg/dL).

### Table 1—Summary statistics for ionized calcium (iCa), total calcium (tCa), and bound calcium (tCa - iCa) concentrations for 161 chelonians stratified by species, habitat, sex, and organ system affected.

<table>
<thead>
<tr>
<th>Species</th>
<th>Total calcium (mg/dL)</th>
<th>IQR</th>
<th>n</th>
<th>Median</th>
<th>Range</th>
<th>IQR</th>
<th>n</th>
<th>Median</th>
<th>Range</th>
<th>IQR</th>
<th>n</th>
<th>Median</th>
<th>Range</th>
<th>IQR</th>
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<tbody>
<tr>
<td>African spurred tortoises</td>
<td>23</td>
<td>11.1</td>
<td>5.3–20.0</td>
<td>9.3–12.2</td>
<td>23</td>
<td>1.76</td>
<td>0.72–3.7</td>
<td>1.45–1.85</td>
<td>23</td>
<td>1.03</td>
<td>0.60–2.98</td>
<td>0.78–1.27</td>
<td>23</td>
<td>1.68</td>
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<td>Chinese three-striped box turtles</td>
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<td>12.5</td>
<td>7.1–19.5</td>
<td>11.6–14.7</td>
<td>14</td>
<td>1.41</td>
<td>0.63–2.04</td>
<td>1.20–1.61</td>
<td>14</td>
<td>1.05</td>
<td>0.84–3.15</td>
<td>0.42–4.61</td>
<td>14</td>
<td>1.22</td>
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<tr>
<td>Leopard tortoises</td>
<td>14</td>
<td>10.1</td>
<td>6.2–15.3</td>
<td>9.5–11.2</td>
<td>14</td>
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<td>1.55–1.76</td>
<td>14</td>
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<td>0.64–1.54</td>
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<td>Red-eared sliders</td>
<td>61</td>
<td>17.0</td>
<td>6.2–22.0</td>
<td>13.4–20.0</td>
<td>61</td>
<td>1.58</td>
<td>0.95–2.13</td>
<td>1.12–3.12</td>
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<td>0.59</td>
<td>0.39–0.97</td>
<td>0.72–1.47</td>
<td>61</td>
<td>1.31</td>
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<tr>
<td>Other species</td>
<td>49</td>
<td>12.2</td>
<td>4.5–20.0</td>
<td>10.4–15.4</td>
<td>49</td>
<td>1.46</td>
<td>0.67–2.08</td>
<td>1.22–1.69</td>
<td>49</td>
<td>1.53</td>
<td>0.42–3.71</td>
<td>1.02–5.21</td>
<td>49</td>
<td>1.30</td>
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<td>Habitat</td>
<td>104</td>
<td>15.4</td>
<td>4.5–22.0</td>
<td>11.9–19.7</td>
<td>104</td>
<td>1.49</td>
<td>0.63–2.33</td>
<td>1.34–1.66</td>
<td>104</td>
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<td>0.42–3.97</td>
<td>1.48–3.22</td>
<td>104</td>
<td>1.04</td>
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<td>Aquatic</td>
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<td>9.5–12.2</td>
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<td>0.66–2.37</td>
<td>1.49–1.85</td>
<td>57</td>
<td>1.02</td>
<td>0.60–2.98</td>
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<td>1.10</td>
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<td>1.35–1.72</td>
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<td>0.84–1.54</td>
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<td>1.20</td>
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<tr>
<td>Female</td>
<td>72</td>
<td>17.7</td>
<td>6.0–22.0</td>
<td>14.1–20.0</td>
<td>72</td>
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<td>0.66–2.33</td>
<td>1.39–1.76</td>
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<td>2.72</td>
<td>0.58–3.97</td>
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<td>11.7</td>
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<td>10.4–13.2</td>
<td>26</td>
<td>1.57</td>
<td>0.75–2.04</td>
<td>1.20–2.78</td>
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<td>1.37</td>
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<td>Unknown</td>
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<td>9.5–12.7</td>
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<td>0.63–2.37</td>
<td>1.35–1.72</td>
<td>63</td>
<td>1.20</td>
<td>0.42–3.70</td>
<td>0.84–1.54</td>
<td>63</td>
<td>1.20</td>
</tr>
</tbody>
</table>

**Diagnosis**

Based on the final generalized linear model, blood iCa concentration increased by 0.25 mmol/L for each unit of increase in blood tCa concentration (0.25; 95% CI, 0.19 to 0.30; \( P < 0.001 \)), decreased of 0.20 mmol/L for each unit of decrease in blood albumin concentration (-0.20; 95% CI, -0.28 to -0.12; \( P < 0.001 \)), and was similar between females

**Factors affecting blood iCa concentration**

Based on the final generalized linear model, blood iCa concentration increased by 0.25 mmol/L for each unit of increase in blood tCa concentration (0.25; 95% CI, 0.19 to 0.30; \( P < 0.001 \)), decreased of 0.20 mmol/L for each unit of decrease in blood albumin concentration (-0.20; 95% CI, -0.28 to -0.12; \( P < 0.001 \)), and was similar between females
or males and animals unknown sex (Figure 1). Ionized calcium concentration was 0.29 mmol/L higher in leopard tortoises (0.29; 95% CI, 0.12 to 0.45; \( P = 0.001 \)), 0.24 mmol/L higher in African spurred tortoises (0.24; 95% CI, 0.11 to 0.38; \( P = 0.001 \)), and 0.12 mmol/L higher in red-eared sliders (0.12; 95% CI, 0.01 to 0.24; \( P = 0.038 \)) than in other species, and it was 0.18 mmol/L (0.18; 95% CI: 0.01 to 0.36; \( P = 0.038 \)) and 0.17 mmol/L (0.17; 95% CI, 0.02 to 0.33; \( P = 0.026 \)) higher in chelonians with endocrine/hemopoietic and urinary organ systems affected than in chelonians with reproductive organ system affected (Supplementary Table S1). This final model had an AIC of 71.0 and normally distributed residuals. Inclusion of interaction terms did not significantly improve the final model.

**Factors affecting blood tCa concentration**

Blood tCa concentration increased by 5.49 mg/dL for each unit increase in blood iCa concentration (5.49; 95% CI, 4.23 to 6.75; \( P < 0.001 \)), increased by 2.3 mg/dL for each unit increase of albumin (2.30; 95% CI, 1.57 to 3.03; \( P < 0.001 \)), and was 2.25 mg/dL higher in females (2.25; 95% CI, 1.13 to 3.38; \( P < 0.001 \)) than in animals of unknown sex, while it was similar between males and animals of unknown sex (0.18; 95% CI, –1.03 to 1.40; \( P = 0.76 \)). Blood tCa concentration was also 3.03 mg/dL lower in leopard tortoises (–3.03; 95% CI, –4.56 to –1.50; \( P < 0.001 \)) and 1.82 mg/dL lower in African spurred tortoises (–1.82; 95% CI, –3.12 to –0.53; \( P = 0.006 \)) than in other species, and was between 1.54 and 2.68 mg/dL higher in chelonians with reproductive organ system affected than in chelonians with any other organ systems affected (Supplementary Table S2). This final model had an AIC of 725.6 and normally distributed residuals. Inclusion of interaction terms did not significantly improve the final model.

**Factors affecting blood bound calcium concentration**

The difference between blood tCa and iCa concentrations (ie, bound calcium concentration) increased by 0.54 mmol/L for each unit increase in blood albumin concentration (0.54; 95% CI, 0.36 to 0.72; \( P < 0.001 \)), was 0.59 mmol/L higher in females (0.59; 95% CI, 0.30 to 0.87; \( P < 0.001 \)) than in animals of unknown sex, and was similar between males and animals of unknown sex (0.02; 95% CI, –0.28 to 0.33; \( P = 0.88 \)). Bound calcium concentration was also 0.70 mmol/L lower in leopard tortoises (–0.70; 95% CI, –1.1 to –0.32; \( P < 0.001 \)) and 0.38 mmol/L lower in African spurred tortoises (–0.38; 95% CI, –0.70 to –0.06; \( P = 0.02 \)) than in other species, and was between 0.40 and 0.68 mmol/L higher in chelonians with reproductive disorders than in chelonians with any other organ system affected (Table 2; Figure 2). This final model had an AIC of 323.7 and normally distributed residuals. An alternative model that included habitat as predictor instead of species as predictor had a slightly lower AIC (320.0) but was considered less informative due to the lack of species (Supplementary Table S3). Inclusion of interaction terms did not significantly improve the final model.

**Diagnostic accuracy of tCa and iCa concentrations to determine calcium status**

When chelonians were categorized on the basis of a priori determined tCa cutoffs of 10 to 14 mg/dL, 67 (41.6%) were normocalcemic, 31 (19.3%) were hypocalcemic, and 63 (39.1%) were hypercalcemic. When chelonians were categorized on the basis of a priori determined iCa cutoffs of 1.2 to 1.6 mmol/L,
70 (43.5%) were normocalcemic, 22 (13.7%) were hypocalcemic, and 69 (42.9%) were hypercalcemic (Figure 3). Both for tCa and iCa, the use of wider cutoffs for definition of hypo- and hypercalcemia resulted in a higher proportion of chelonians categorized as normocalcemic (Supplementary Figure S1).

Of the 161 chelonians, 93 (57.7%; 95% CI, 49.7% to 65.5%) showed diagnostic discordance using the primary cutoffs (Figure 3), and 59 (36.6%; 95% CI, 29.2% to 44.6%) using the wider cutoffs (Supplementary Figure S1). Considering the primary cutoffs, 5 (7.4%) and 38 (56.7%) chelonians with total normocalcemia (n = 67) had ionized hypocalcemia and hypercalcemia. Twelve (38.7%) and 3 (9.7%) chelonians with total hypocalcemia (n = 31) had ionized normocalcemia and hypercalcemia. Thirty-four (54.8%) and 1 (1.6%) chelonians with total hypercalcemia (n = 63) had ionized normocalcemia and hypocalcemia. Thirty-four (54.8%) and 1 (1.6%) chelonians with total hypercalcemia (n = 63) had ionized normocalcemia and hypocalcemia. Thirty-four (54.8%) and 1 (1.6%) chelonians with total hypercalcemia (n = 63) had ionized normocalcemia and hypocalcemia.

### Diagnostic accuracy of bound calcium for reproductive disorders

Receiver operating curve analysis (AUC: 0.83; 95% CI, 0.74 to 0.91) revealed that the cutoff value for blood-bound calcium concentration at 2.2 mmol/L could differentiate chelonians with and without reproductive disorders with a sensitivity of 81.8% (64.5% to 93.0%), specificity of 76.4% (68.0% to 83.5%), a positive predictive value of 47.4% (38.8% to 56.1%), and negative predictive value of 94.2% (88.6% to 97.7%).

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**Table 2—Results of the generalized linear model built to explore factors associated with changes in blood bound calcium (tCa – iCa) concentration in 161 chelonians.**

<table>
<thead>
<tr>
<th>Change in bound calcium (mmol/L)</th>
<th>Lower 95% CI</th>
<th>Upper 95% CI</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>0.54</td>
<td>0.36</td>
<td>0.72</td>
</tr>
<tr>
<td>Sex</td>
<td>0.59</td>
<td>0.31</td>
<td>0.87</td>
</tr>
<tr>
<td>Female</td>
<td>0.02</td>
<td>-0.28</td>
<td>0.33</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
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<td></td>
</tr>
<tr>
<td>Species</td>
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<tr>
<td>African spurred tortoises</td>
<td>-0.38</td>
<td>-0.7</td>
<td>-0.06</td>
</tr>
<tr>
<td>Chinese three-striped box turtles</td>
<td>-0.06</td>
<td>-0.45</td>
<td>0.33</td>
</tr>
<tr>
<td>Leopard tortoises</td>
<td>-0.7</td>
<td>-1.09</td>
<td>-0.32</td>
</tr>
<tr>
<td>Red-eared sliders</td>
<td>0.03</td>
<td>-0.25</td>
<td>0.31</td>
</tr>
<tr>
<td>Other species</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organ system affected</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endocrine/hemopoietic</td>
<td>-0.56</td>
<td>-0.92</td>
<td>-0.20</td>
</tr>
<tr>
<td>GI/liver</td>
<td>-0.46</td>
<td>-0.85</td>
<td>-0.07</td>
</tr>
<tr>
<td>Other systems</td>
<td>-0.4</td>
<td>-0.78</td>
<td>-0.03</td>
</tr>
<tr>
<td>Renal/urinary</td>
<td>-0.45</td>
<td>-0.86</td>
<td>-0.04</td>
</tr>
<tr>
<td>Respiratory</td>
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<td>-1.14</td>
<td>-0.23</td>
</tr>
<tr>
<td>Skin/musculoskeletal</td>
<td>-0.67</td>
<td>-1.03</td>
<td>-0.31</td>
</tr>
<tr>
<td>Reproductive</td>
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</table>

See Table 1 for key.
to 97.1%). To maximize sensitivity, using a cutoff value for bound calcium of 1.66 mmol/L had a sensitivity of 90.9% and specificity of 65.0%. To maximize specificity, using a cutoff value for bound calcium of 3.0 mmol/L had a sensitivity of 60.6% and a specificity of 90.6%.

Discussion

In the present study, the relationship, difference, and diagnostic discordance were determined between blood tCa and iCa concentrations in a population of client-owned chelonians. Diagnostic discordance between tCa and iCa in chelonians was common (between 57.7% and 36.6%, depending on the cutoffs used). The relationship between blood iCa and tCa concentrations in chelonians was also found to be complex and affected by multiple factors. The difference between blood tCa and iCa concentrations (ie, the bound calcium) varied, depending on blood albumin concentration and chelonian sex and species, and was especially high in chelonians diagnosed with reproductive disorders. Based on these results, evaluation of 3 measurements, namely tCa, iCa, and bound calcium, could be useful to properly evaluate the calcium status of chelonians.

The concept of diagnostic discordance between tCa and iCa concentrations has been employed in small animal medicine for almost 2 decades. Research estimating diagnostic discordance for tCa and iCa values in dogs and cats has involved calculation of the overall discordance or specific discordance for hypocalcemia and hypercalcemia, using either tCa or iCa as a referent. Regardless of the values reported, the calculations were similar and involved classification of each subject as hypo-, hyper-, or normocalcemic based on tCa cutoffs and iCa cutoffs independently and then calculation of the percentage over the total number of animals or a specific subgroup of them (eg, hypocalcemic and hypercalcemic subjects). The use of one or the other analyte as a referent does not change the result when the value is reported over the total of the animals.

In dogs, diagnostic discordance of tCa and iCa was 18.5% and 27% based on two studies, indicating that in a fifth to a quarter of dogs, standalone tCa and iCa assessments would have resulted in a different clinical interpretation. Diagnostic discordance is higher in cats, reaching 40% in one study. In dogs, there was a modest improvement in diagnostic discordance (from 18.5% to 15.4%) when using a predictive model to estimate iCa concentration and a modest worsening when using albumin-corrected tCa concentration and total protein-corrected tCa concentration (19.5% and 25%, respectively). In the present study, diagnostic discordance in chelonians was 57.7% using the primary cutoffs employed by the author in clinical practice, and it was 36.6% using wider cutoffs. This indicates that over a third, and possibly over half, of chelonians presented to clinical practice may be differently categorized as hypo-, hyper-, or normocalcemic depending on whether tCa or iCa concentration is evaluated. This discordance in chelonians included also certain extremes such as subjects that had ionized hypercalcemia and total hypocalcemia and subjects that had ionized hypocalcemia and total hypercalcemia. While previous findings suggested that, based on the discordance, iCa concentration should be directly measured to assess calcium status, the author believes that these results rather indicate that both tCa and iCa concentration should be measured and critically assessed for a proper evaluation of the calcium status, at least in chelonians.

This conclusion is further supported by the potential clinical relevance of bound calcium concentration shown in the present study. As circulating calcium is present in 3 major fractions—ionized (also defined free), protein bound, and complexed (ie, bound to other anions), subtracting iCa values from tCa values theoretically provides an estimation of bound calcium, that is calcium that is both protein bound and complexed. Bound calcium is considered to be not biologically active and is about half of the total circulating calcium in mammals. Measurement of iCa concentration is technically more complicated than measurement of tCa concentration, and while measurement of circulating tCa has been in place for over a century, measurement of iCa has been introduced in clinical settings for a few decades.

Based on the physiological concept that iCa is the more biologically active form, several authors and studies have used iCa as the reference standard for assessment of calcium status. However, there is limited evidence to support this concept in reptiles. The present study showed that the values for blood-bound calcium concentration significantly differed based on multiple factors in chelonians presented to a veterinary hospital. Not surprisingly, blood-bound calcium concentration increased by 0.54 mmol/L for each mg/dL increase in blood albumin concentration, likely because the majority of protein-bound calcium is considered to be bound to albumin. Bound calcium concentration was lower in specific tortoise species than in other chelonians, the reason for which remains unclear. More interesting is the fact that protein-bound calcium concentration was 0.59 mmol/L higher in females, and it was between 0.40 and 0.68 mmol/L higher in chelonians with reproductive disorders than in chelonians with any other organ system affected. This finding, in association with the fact that blood iCa concentration remained relatively stable in female chelonians, supports the theory that the increase in tCa concentration in female chelonians during reproductive activity is due to an increase in bound calcium rather than iCa. Considering the large size of this effect, the value of bound calcium could potentially be used as a clinical tool for identifying chelonians reproduc-tively active or with reproductive disorders.

In the present study, blood iCa concentrations were affected by blood total calcium and albumin concentrations and species. Not surprisingly, blood iCa concentration increased of 0.25 mmol/L for each
mmol/L of increase in blood tCa concentration. The fact that a positive relationship was found between tCa and iCa values was not unexpected, and it was observed in a previous study that assessed the relationship between tCa and iCa in chelonians. An interesting finding was that blood iCa concentration decreased by 0.20 mmol/L for each mg/dL of increase of albumin. A potential explanation for this association is that with increasing in albumin concentrations, the amount of circulating calcium bound to proteins may increase and the fraction of free calcium may decrease. An identical finding has been observed in a recent study in dogs, in which serum albumin concentrations were the third most important predictor of iCa concentrations, and iCa concentrations decreased as serum albumin concentration increased. In a previous study in chelonians, there was no correlation between circulating tCa and albumin values. Possible explanations for this difference include a drastically smaller sample size (31 subjects) in the previous study, and the lack of multivariable modeling, among other reasons. Interestingly, while blood tCa concentrations were strikingly higher in female chelonians in the present study, blood iCa concentrations were not affected by sex. This finding is in agreement with several previous studies on chelonians, in which females did not have a statistically significant higher concentration of iCa than males. Although iCa concentration is regarded as less variable in reptile species than tCa concentration, a significant difference was still found in blood iCa concentration between species, with leopard tortoises and African-spurred tortoises having iCa concentrations 0.24 to 0.29 mmol/L higher than in other species, and red-eared sliders having concentrations 0.12 mmol/L higher than in other species. Ionized calcium was relatively unaffected by the primary organ system affected, with the only significant differences being in chelonians with endocrine/hemopoietic and urinary organ systems affected, which had blood iCa concentrations 0.17 to 0.18 mmol/L higher than chelonians with reproductive organ system affected. Overall, blood iCa concentrations in the present study were consistent with values in other aquatic and terrestrial chelonians and higher than in marine chelonians.

Previous studies in chelonians have evaluated the clinical role that iCa concentrations may play in chelonians, as well as the effect that manual and chemical restraint may have on iCa measurements. For example, cold-stunned green sea turtles had lower iCa concentrations than healthy, free-ranging juvenile green turtles from the same region. Also, cold-stunned turtles had a significantly higher iCa concentration during recovery than at admission, and iCa values were correlated with the number of hospitalization days, suggesting an increase over time for the analyte. Not all studies have found that higher iCa concentrations are a positive predictive factor. In cold-stunned Kemp’s ridley turtles (Lepidochelys kempii), iCa concentrations increased over the first 2 or 3 days of hospitalization to a significantly greater degree in turtles that died rather than in those that survived. In terms of restraint, when blood was collected in 7 leatherback turtles right after capture and a mean of 25 minutes later before release, iCa concentrations differ to a statistically significant degree; however, they were between 8% and 25% lower in 6 of the 7 turtles. In 6 desert tortoises anesthetized with sevoflurane, there was a 9% decrease, which was not statistically significant, in iCa concentration over time.

In the present study, the author made some methodological choices based on the currently available evidence. First, blood iCa concentration was reported and analyzed in this study without any postanalytical correction. It is currently unclear whether postanalytical correction of iCa measurements in reptiles is important, valid, and accurate. Further studies focused on the impact of correction formulas in chelonians are necessary to answer this clinical question. Second, primary cutoffs were chosen for determination of diagnostic discordance on the basis of the reference intervals currently in use by the author and multiple articles, rather than cutoffs for each individual species. Different individual articles in chelonians provide different reference intervals for circulating analytes. If excluding some obvious examples of interspecific variability, such as indigo snakes (Drymarchon corais) that have strikingly higher calcium concentrations than other reptiles, or sea turtles that have lower calcium concentrations, it is difficult to establish whether different published reference intervals for calcium actually represent interspecific differences. In fact, most reference interval studies in chelonians do not comply with the minimal sample sizes required by the American Society for Veterinary Clinical Pathology for reference interval studies, and some studies include blood sampling from the subcarapacial sinus, which is a site that is known to provide different results than the jugular vein. Studies also differ in methodologies. In addition to these confounding factors, there are pronounced sexual and seasonal differences in most analytes in chelonians, that are not always accounted for. Therefore, the author believes that the approach employed in the present study was adequate to represent the degree of diagnostic discordance between blood tCa and iCa concentrations in chelonians. This is particularly true because the study results were supported by a sensitivity analysis with wider ranges of normality for tCa and iCa values.

One of the limitations of the present study was the lack of phosphorus evaluation. An older study that included 1 chelonian species found that the percentage of circulating phosphorus that was protein bound changed, depending on the calcium concentrations. At increasing tCa concentrations, the concentrations of protein-bound phosphorus increased, likely due to the increase of complexes between calcium and phosphorus (Ca₃[PO₄]₂). This aforementioned study found that in Cuban terrapins (Trachemys decussata [Pseudemys rugosa]), the normal concentration of calcium renders a substantial part of the phosphorus already protein bound.
(ie, nonfiltrable).\textsuperscript{50} Based on the results of such study, total hypercalcemia may result in less ionic phosphorus, which could potentially have a clinical effect in chelonians. Severe hypophosphatemia in other species may result in seizures and focal neurologic findings.\textsuperscript{51,52} It is therefore possible that reptiles presented with seizures and high tCa, normal iCa, and normal total phosphorus concentrations could actually be affected by ionized hypophosphatemia. Further studies on the concentrations of ionized phosphorus in chelonians with total hypercalcemia are therefore warranted.

Another limitation was the lack of proper reference intervals for blood iCa and tCa concentrations in chelonians presented to a veterinary hospital. Although unlikely considering that a sensitivity analysis was performed with wide ranges, it is theoretically possible that normal values for iCa and tCa may lay within a broader range than the cutoffs employed in this study, resulting in a different diagnostic discordance. A potential source of bias was the fact that clinical diagnosis of chelonians also depended on the results of some of the outcomes or predictors included in the present study. Nevertheless, circulation of bound calcium in chelonians was not determined during clinical activity and bound calcium was not a criterion used to categorize chelonians as having reproductive disease, limiting the chance of actual bias. Another limitation was the fact that in smaller chelonians, the blood used for biochemical analysis may have been exposed to an excessive quantity of heparin in the heparinized vials. The number of small chelonians included in the study was, however, limited.

A further limitation was the fact that currently the repeatability of iCa measurement with the portable analyzer in chelonians is unclear. However, a previous study\textsuperscript{20} that included both the blood gas analyzer and the chemistry analyzer included in the present study showed that both iCa and tCa concentrations had acceptable agreement in a chelonian species. Finally, the present study was limited by the measurement of blood albumin concentration via the bromocresol green method. In chelonians, the bromocresol green method has been found to be suboptimal, compared with protein electrophoresis,\textsuperscript{29,53} especially in diseased chelonians.\textsuperscript{55} The author does not believe that potential inaccuracy would have had a substantial impact on the study results, considering that albumin was employed as an independent variable in the models, and results obtained when plasma TS concentration was included in the models were similar overall.

The present study confirmed that in chelonians, as in other veterinary patients, the different fractions of circulating calcium are in a close relationship that involves albumins. Based on our results, values of total and ionized calcium in chelonians have relatively high diagnostic discordance. The diagnostic discordance affected approximately half of the patients and its magnitude resulted in opposite diagnoses (hypocalcemia vs hypercalcemia) in 2.5% of the cases. The idea of regarding iCa concentration as a standalone calcium indicator in chelonians may be incorrect for diagnostic purposes. Instead, concurrent evaluation of blood tCa, iCa, and bound calcium is preferable. Measurement of bound calcium concentration could assist in the diagnosis of chelonians with reproductive disorders.

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References


**Supplementary Materials**

Supplementary materials are posted online at the journal website: avmajournals.avma.org