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METHOD COMPARISON AND REFERENCE INTERVAL DETERMINATION IN ANIMAL MEDICINE

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Method comparison and reference interval determination in animal medicine

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Summary- Numerous new techniques or tools are in the process of being developed in veterinary medicine with the aim of finding cheaper, non-invasive, more convenient and safer method to test animals. Before implementation in clinical practice, the new tool or method of measurement should demonstrate to be as accurate as the current reference standard method. Therefore it is important to measure the agreement of the new method with the standard method. An acceptable agreement permits interchangeability of the instruments. For this purpose, we have investigated the agreement of several clinical instruments frequently used in clinical practice with their laboratory counterpart. We have estimated the agreement between a point-of-care blood gas analyzer (i-Stat, Abaxis) and a bench-top blood gas analyzer (Nova, Biomedical) in venous samples from Hermann's tortoises. We have estimated the agreement between a point-of-care chemistry analyzer (VetScan VS2, Abaxis) and a laboratory analyzer (Olympus AU400, Olympus Co.) in venous samples from Hermann's tortoises. We have estimated the agreement between portable blood glucose meters (Accu-Chek, Aviva; AlphaTrak 2, Abbott) and a laboratory analyzer (Dimension EXL, Siemens) in venous samples from client-owned rabbits. We have estimated the agreement between point-of-care bench-top glucose measurement (VetScan VS2, Abaxis) and a laboratory analyzer (Dimension EXL, Siemens) in venous samples from client-owned rabbits.

Beyond method comparison and validation, reference interval determination for common laboratory testing is required to allow the clinician to discriminate individuals that are different from the remaining population for a certain parameter. We have calculated reference intervals for blood gas in Hermann's tortoises. We have calculated reference intervals for protein electrophoresis in Hermann's tortoises. We have described normal hematology in Hermann's tortoises. We have calculated reference intervals for clinical

chemistry in Hermann's tortoises. We have calculated reference intervals for aldosterone in ferrets. Based on our results, animal species requires individual validation of laboratory methods and reference intervals. Lack of consideration of these findings may result in clinical misdiagnosis and improper treatment of animals.

INTRODUCTION

Several critical variables in medicine are measured in numerical forms or continuous data, such as blood pressure, glucose and oxygen concentrations. In any clinical situation, we are expected to have accurate readings of these variables. Numerous new techniques or tools are in the process of being developed in veterinary medicine with the aim of finding cheaper, non-invasive, more convenient and safer methods to test animals. Before implementation in clinical practice, the new tool or method of measurement should demonstrate to be at least as accurate as the current reference standard method. Therefore, it is important to measure the agreement of the new method with the standard method. An acceptable agreement permits interchangeability of the instruments.

Beyond method comparison and validation, reference interval determination for common laboratory testing is a further critical procedure. Reference interval determination allows the clinician in discriminating individuals that are different from the remaining population for a certain parameter. Gräsbeck introduced the concept of population-based reference values in 1969 to describe the variation in blood analyte concentrations in groups of healthy individuals (Gräsbeck, 1969). These concepts were then applied to veterinary species by Lumsden and colleagues (Lumsden 1978; Lumsden et al., 1980). Reference values are typically reported as reference intervals (RI) comprising 95% of the healthy population. Since their introduction, population-based RI have become one of the most commonly used laboratory tools employed in the clinical decision-making process (Horn and Pesce, 2005). Although use of population-based RI is universally accepted, the optimal

method for their derivation is frequently debated and is a recurring topic in the clinical laboratory literature.

Several steps are required when performing a method-comparison study or when reference intervals are determined: a first step requires the definition of the population of interest, as well as the criteria used to confirm health in individuals selected from this population (selection, inclusion, exclusion, and partitioning criteria). The demographics of the reference population should be representative of the patient population for which the RI will be used in making clinical decisions. In method comparison studies, a population that includes both healthy and diseased individuals is ideal, because this permits to test the instrument on a wider range of the analyte. Therefore, such studies are ideally performed in clinical practice, where individuals are representative of the target population and either healthy and diseased animals are presented. Given our final clinical purposes, we opted to include three species, Hermann's tortoise, rabbit and ferret that are common in clinical practice.

Hermann's tortoises

Hermann's tortoise (*Testudo hermanni*) is one of the most popular reptiles kept as pets in Europe. The small size (carapace rarely exceeds 210 mm in length in

wild populations), herbivorous habits, and toughness make these tortoises an exceptional garden inhabitant. Hermann's tortoises are herbivorous chelonians, endemic along the Mediterranean coasts of Europe (Fritz et al. 2006; Mazzotti et al. 2004). Hermann's tortoise is listed by the International Union for Conservation of Nature as a near-threatened species, but the number of such tortoises in the wild continues to decrease (IUCN 2014). This species is protected by the Bern Convention and by the European Habitats Directive, while

international trade of this species is regulated by the Washington Convention (IUCN 2014). In order to provide proper veterinary care to captive and wild tortoises, hematologic and biochemical blood analyses are essentials. They permit to assess the health status also of animals that present few clinical signs that can be assessed during a standard physical exam, as compared to most mammals. Standardization of blood collection techniques, environmental factors and the physiologic status must be considered in assessing normal reference intervals. Although reference intervals for selected chemical analytes of Hermann's tortoises have been published (Scope et al. 2013), certain analytes have not been evaluated or have an unclear variation secondary to sex and season. Besides reference interval determination, there are two novel point-of-care (POC) instruments that deserves further investigations in tortoises. For POC determination of blood gases, a cartridge that combines analysis of blood gases (pH, PCO_2, PO_2, HCO_3^-, tCO_2, BEecf, AnGap) with hematocrit, hemoglobin, selected electrolytes (iCa, Na, K) and glucose has been recently produced (CG8+ Testing cartridge, 03P88-25; Abbott, Birmingham, UK). For POC determination of blood biochemical values a reagent disc specifically designed for reptiles that analyzes glucose, uric acid, biliary acids, AST, CK, K, Ca, Na, phosphorus, total proteins and albumin is commercially available (VetScan Avian Reptilian Profile Plus, Abaxis, Inc., Union City, CA). Both these analyzers require approximately 100 µl of whole blood, a volume that can be harvested from most juvenile to adult Hermann's tortoises.

Therefore, the aims were (1) to describe reference intervals for hematology, biochemistry and protein electrophoresis, (2) to assess agreement of two point-of-care analyzers with their respective laboratory equivalent, and (3) to evaluate an automatic instrument for blood cell count.

The data are reported in:

1) Di Girolamo N, Nardini G, Ferlizza E, Isani G. Evaluation of point-of-care analyzers for blood gas and clinical chemistry in Hermann's tortoises (*Testudo hermanni*). In draft.
2) Andreani G, Carpenè E, Cannavacciuolo A, Di Girolamo N, Ferlizza E, Isani G. Reference values for hematology and plasma biochemistry variables, and protein electrophoresis of healthy Hermann's tortoises (*Testudo hermanni ssp.*). Vet Clin Pathol. 2014, 43(4):573-83.
3) Bielli M, Nardini G, Di Girolamo N, Savarino P. Hematological values for adult eastern Hermann's tortoise (*Testudo hermanni boettgeri*) in semi-natural conditions. J Vet Diagn Invest. 2015 Jan;27(1):68-73

Rabbits

Rabbits are among the most common pet animals in Europe and the United States (AVMA 2012). Clinicians treating rabbits should be familiar with several factors unique to these animals including hind-gut fermentation, unusual calcium metabolism, very small thoracic size compared with body mass, relatively high metabolic rate, and the fact that rabbits are a prey animal that succumbs to stress easily (Schepers et al. 2009). Because of these physiological peculiarities, the clinical approach to rabbits is extremely different as compared to the clinical approach to dogs and cats. Probably, the more peculiar difference in the clinical approach to rabbits is that they tend to conceal illness. Therefore, ancillary diagnostic techniques are often required after physical examination. Nevertheless, reference blood gas analysis values and their variations during the course of diseases have

never been properly reported in pet rabbits. The absence of previous studies focusing on blood gas, electrolytes and chemistries in rabbits are possibly due to the cost related with an in-house reference analyzer and the difficulty to obtain adequate volume samples.

The aim of the research was (1) to assess the agreement between one point-of-care blood gas analyser and its laboratory counterpart, (2) to assess the performance of two portable blood glucose meters and one bench-top analyser for measurement of blood glucose.

The obtained data are reported in:

1) Selleri P, Di Girolamo N. Point-of-care blood gases, electrolytes, chemistries, hemoglobin, and hematocrit measurement in venous samples from pet rabbits. J Am Anim Hosp Assoc. 2014 Sep-Oct;50(5):305-14.
2) Selleri P, Di Girolamo N, Novari G. Performance of two portable meters and a benchtop analyzer for blood glucose concentration measurement in rabbits. J Am Vet Med Assoc. 2014 Jul 1;245(1):87-98.

Ferrets

The popularity of ferrets (*Mustela putorius furo*) as household pets and their value in the world of science dramatically increased the request for veterinary medical care of these mustelids in the last years (Shepherd 2008; Belser et al. 2011). Although ferrets are carnivores like dogs and cats, they have striking differences with such species, especially concerning the type of disorders that affect them. One of the more common presenting complaint of pet ferrets are seizures. Seizures commonly occur in ferrets and may be consequent to a wide variety of causes. However, true spontaneous epileptic seizures have never been described in ferrets and seizures are usually reactive, i.e. caused by metabolic

or toxic conditions (Donnelly 2011). Hypoglycemia is considered the most common condition subsequent to seizures in ferrets, however other conditions including electrolyte disorders, intoxication, hepatic encephalopathy, hypothyroidism, uremic encephalopathy, hypoxia and hyperglycemia may be the cause of seizures (Brauer et al. 2011). Hypoglycemic seizures are an extremely common emergency presentation in ferrets. Typically, hypoglycemia in ferrets is the consequence of hyperinsulinemia caused by pancreatic beta-cell tumors (i.e., insulinomas)(Caplan et al. 1996). Other causes that should be included in the differentials for hypoglycemia include inanition, liver disorders, hypoadrenocorticism (Syme et al. 1998). A less common cause of seizures in ferrets is severe hypocalcemia (Wilson et al. 2013; De Matos et al. 2014). For the diagnosis a complete blood analysis, including ionic calcium is indicated. Although no proper reference ranges for ionic calcium in ferrets are established, values higher than 1.1 mmol/L may be considered normal based on dog and cats reference ranges and unpublished data. Differential diagnoses for hypocalcemia should include hypoparathyroidism, pseudohypoparathyroidism, hypomagnesemia, renal failure, acute pancreatitis, hypoalbuminemia, puerperal tetany, ethylene glycol intoxication, intestinal malabsorption, nutritional secondary hyperparathyroidism, and tumor lysis syndrome. Among these causes, primary hypoparathyroidism has been recently diagnosed in two ferrets and pseudohypoparathyroidism in a further ferret (Wilson et al. 2013; De Matos et al. 2014). Diagnosis of these conditions is based on a combination of low serum calcium concentration, high serum phosphorus concentration, and appropriate renal function in the face of low PTH concentrations (hypoparathyroidism) or high PTH concentrations (pseudohypoparathyroidism)(Wilson et al. 2013; De Matos et al. 2014). Other disorders

that occasionally cause central neurological signs and seizures in ferrets include brain tumors, intoxication and viral, fungal and protozoan infections, including distemper, rabies, Aleutian disease, systemic coronavirus, *Cryptococcus,* and *Toxoplasma gondii* infection. Another common condition, peculiar to ferrets is hyperestrogenism-related anemia. Hyperestrogenism may be secondary to prolonged estrus in female ferrets (either intact or neutered with ovarian remnant), adrenal disorders or, rarely, estrogen-producing tumors. The mechanism behind anemia in intact female ferrets relies on the lack of spontaneous ovulation (Kociba 1981). In absence of copulation, ovulation does not occur and the maintained production of estrogens by the follicles may result in bone marrow aplasia, with consequent pancytopenia. Clinical signs of hyperestrogenism in females include swelling of the vulva, alopecia and pruritus, among others. In males, gynecomastia and dysuria as a consequence of prostatic cysts are occasionally observed during hyperestrogenism. Transfusions are empirically indicated for hematocrit values lower than 25%. Ferrets do not have clinically significant blood groups, therefore any healthy adult male is an appropriate donor (Manning and Bell 1990). Ferret blood stored using citrate-phosphate-dextrose-adenine should not be used for transfusion after seven days of storage at 4°C (Pignon et al. 2014). Another emerging disorder in ferrets is systemic hypertension associated with an increase or with normal aldosterone levels. Systemic hypertension may be associated with CHF and blood pressure should be closely monitored in cardiopathic ferrets. The practitioner needs to know that non-invasive blood pressure measurement (with manual sphygmomanometer and Doppler probe) underestimate blood pressure in ferrets. However, it remains an affordable technique for blood pressure monitoring. As a rule of thumb, 30 mmHg may be added after Doppler measurement (based on published

data, mean difference: -28 mmHg, 95% CI approximately: 4 to -60 mmHg)(Olin et al. 1997). Systemic hypertension in ferrets may be secondary to causes similar to other domestic carnivores, including aldosterone-secreting adrenal tumors (Desmarchelier et al. 2008). In cases of hyperaldosteronism, administration of spironolactone and amlodipine mitigates the hypertension. An uncontrolled hypertension may result in dissecting aortic aneurism and sudden death.

Given these considerations, it would be useful to determine preliminary reference intervals for serum aldosterone in healthy ferrets and to observe the variation of such analyte in clinically healthy and diseased ferrets.

The data are reported in:

1) Di Girolamo N, Fecteau K, Carnimeo A, Bongiovanni L, Fracassi F, Isani G, Selleri P. Variability of serum aldosterone concentrations in ferrets. Vet Clin Pathol, under review.

Evaluation of point-of-care analyzers for blood gas and clinical chemistry in Hermann's tortoises (*Testudo hermanni*)

Background

Point-of-care (POC) analyzers have become common in emergency and critical care settings in both human and veterinary hospitals due to their versatility (Grosenbaugh et al. 1998): the POC analyzer decreases sample processing time (Adams et al. 1995; Bingham et al. 1999), thus possibly decreasing the effect of delay in estimation of vital analytes (Woolley and Hickling 2003). Although a minimal training is mandatory to adhere to standard operating procedures, previous studies have found that the operator independence of POC analyzers renders them easy to use even by personnel untrained in laboratory techniques (Grosenbaugh et al. 1998; Jacobs et al. 1993). Due to their portable nature, they may be especially suited for field evaluation of wildlife, including free-ranging chelonians (Atkins et al. 2010).

Hermann's tortoises (*Testudo hermanni*) are herbivorous, medium-sized chelonians that are endemic along the Mediterranean coasts of Europe (Fritz et al. 2006; Mazzotti et al. 2004). Hermann's tortoise is listed by the International Union for Conservation of Nature as a near-threatened species, but the number of such tortoises in the wild continues to decrease (IUCN 2014). Although reference intervals for selected chemical analytes of Hermann's tortoises have been recently provided (Scope et al. 2013; Andreani et al. 2014), to the author's knowledge no data evaluating the performance of POC analyzers in this species are currently available.

For POC determination of blood gases, a cartridge that combines analysis of blood gases (pH, PCO_2, PO_2, HCO_3^-, tCO_2, BEecf, AnGap) with hematocrit, hemoglobin, selected electrolytes (iCa, Na, K) and glucose has been produced (CG8+ Testing cartridge, 03P88-25; Abbott, Birmingham, UK). For POC determination of blood biochemical values a reagent disc specifically designed for reptiles that analyzes glucose, uric acid, biliary acids, AST, CK, K, Ca, Na, phosphorus, total proteins and albumin is commercially available (VetScan Avian Reptilian Profile Plus, Abaxis, Inc., Union City, CA). Both these analyzers require approximately 100 µl of whole blood, a volume that can be harvested from most juvenile to adult Hermann's tortoises.

The purpose of the present study was to assess the agreement between POC analyzers and clinical laboratory analyzers routinely used in veterinary medicine to measure biochemical and blood gas variables in venous samples from Hermann's tortoises. In addition, preliminary reference intervals for blood gas profiles of Hermann's tortoises are reported.

Materials and methods

Study design and animals

A prospective observational cross-sectional method-comparison study was planned composed by two phases. A first phase included Hermann's tortoises (n = 24) undergoing blood sampling in the Clinica per Animali Esotici, Rome for unrelated diagnostic reasons in May 2012. The primary outcome of such phase was the agreement between a portable blood gas and electrolyte analyzer (PGA) and a reference bench-top blood gas and electrolyte analyzer (RGA). The second phase included Hermann's tortoises (n = 23) undergoing blood sampling in the Clinica Veterinaria Modena Sud for health assessment

before a pharmacokinetic study (Nardini et al. 2014). The primary outcome of the second phase was the agreement between a portable bench-top chemistry analyzer (PCA) and a reference chemistry analyzer (RCA).

All experimental procedures were approved by the Ethical and Scientific Committee of the University of Bologna, and were carried out in accordance with European legislation regarding the protection of animals used for experimental and other scientific purposes. The owners gave written informed consent to the inclusion of samples of their animals in the study.

Sample collection and analysis

Portable gas analyzer-Two experienced operators performed venipuncture and analysis of samples. The tortoises were restrained in lateral recumbence and venipuncture site over one jugular vein was cleaned with a povidone-iodine swab. The same operator performed venipuncture and the subsequent analyses in order to minimize procedure time due to sample handling. In the meanwhile the second operator prevented bleeding and hematoma formation applying gentle digital pressure on the venipuncture site. Blood samples were collected with a 23-Gauge needle into 1-mL plastic syringes containing 25 international units of dry balanced heparin (Westmed 1cc 25 U Balanced Heparin, 3100-25; Westmed Inc., Tucson, AZ) and were immediately analyzed with the PGA (i-STAT 1 Analyzer, SN-703112; Abbott Point of Care Inc., Abaxis Inc., Union City, CA). The whole blood sample was introduced into the cartridge via the heparinized syringe, and a portion of the same blood sample was analyzed with the RGA (Stat Profile Critical Care Xpress 12; Nova Biomedical Corporation, Waltham, MA) immediately thereafter. To ensure the minimum delay in blood

analysis the analyzers were located adjacent to each other and adjacent to the table where blood collection was performed.

Sample collection and handling were performed according to the manufacturers' guidelines to avoid pre-analytical errors. The most important of these was the prevention of contamination of syringe sample with air, and the immediate and proper mixing of blood syringes before introduction of each sample. Appropriate measures were taken to avoid touching the contact pads, which could have interfered with data transmission, or exerting pressure over the center of the cartridge, which could have caused premature release of the calibrating solution. PGA cartridges were stored in a refrigerator (3° to 8°C) and were only used prior to the expiration date provided by the manufacturer. The temperature of each cartridge was allowed to equilibrate with the ambient temperature of the room (20°C) before the cartridge pouch was opened for 5 minutes, although a study proved evidence that results of analyses were not substantially affected by use of cold, rather than warm, test cartridges (Looney et al. 1998). The PGA aqueous control solutions were used to assess 1 cartridge out of each cartridge batch as recommended by the manufacturer. The ampule containing the control solution was equilibrated for 4 hours at room temperature. Plain syringes were used to transfer the aqueous control from the ampule to the cartridge as recommended. Calibration verification of the PGA was not performed as it is explicitly not recommended by the manufacturer.

The RGA was maintained according to manufacturer's recommendations: the analyzer used an internal 2-point calibration to measure sensor slopes and to verify sensor performance. The 2-point calibration was automatically performed at 2 hour intervals. The RGA was in Mode A so all analytes were checked with a 1-point calibration every each sample analysis.

The 1-point calibration is performed by exposing the electrodes to a known standard and comparing the new value to the value obtained during the 2-point calibration. All samples were handled by a licensed veterinarian trained to use the instruments (ND).

Analytes measured- Analytic variables measured from both the PGA with the specific cartridge and RGA during analysis included pH, PCO_2, PO_2, Na, K, iCa, glucose and Hct. The PCA calculates hemoglobin from Hct. Therefore hemoglobin was not included in the analysis.

Portable chemistry analyzer-Venipuncture was performed as described for blood gas analysis. Blood samples were collected with a 23-Gauge needle into 1-mL plastic syringes without anticoagulant. The blood was transferred from the syringe into tubes containing lithium-heparin and 0.1 mL of blood from the lithium-heparin tube was immediately inserted in a reagent disc for analysis with the PCA (VetScan VS2, Abaxis, Inc., Union City, CA). The lithium-heparin tubes were then centrifuged at 1,300 X *g* for 10 minutes, and plasma was harvested within 12 minutes after collection. Plasma samples were shipped to the clinical laboratory of the University of Bologna for paired analyses with the RCA. All the plasma samples were analyzed within 24 hours after collection.

The reagent discs, specific for reptile biochemical analyses, were stored in their sealed pouches at 4°C and were never exposed to direct sunlight or temperatures above 26°C. Reagent discs were unsealed just prior to use. The device was serviced by the local provider few days before the beginning of the study. All samples were handled by a licensed veterinarian trained to use the instrument (GN).

An automated biochemical analyzer (Olympus AU400; Mishima Olympus Co. Ltd, Shizuoka, Japan) was used as the reference analyzer. The selected analytes were determined using commercially available kits (Olympus Systems Reagents; Olympus life and Material Science Europe GmbH, Hamburg, Germany). Standard procedures, including assay of control material and calibration of the laboratory analyzer were performed daily before starting the analysis session.

Analytes measured-Blood chemistry variables measured included glucose, uric acid, AST (EC 2.6.1.1), CK (EC 2.7.3.2), K, Ca, Na, P, total proteins and albumin.

Plasma protein electrophoresis-After analysis via PCA and RCA, n = 12 plasma samples (10 μL) were separated on a 0.8% high-resolution agarose gel (Hydragel HR, Sebia, Lisses, France). The electrophoresis was run at a constant 255 V on an automated system (Hydrasis, Sebia, Lisses, France) for 20 minutes. Gels were stained by Acid Violet. Stained gels were digitalized with a scanner yielding the densitometric profile (software: Phoresis 6.1.2, Sebia). The relative percentage and absolute concentration of each protein fraction were calculated based on densities determined by a densitometer. The separation between α and β zones was set at the midpoint of the pherogram as reported by Andreani et al. (2014).

Statistical analysis

Summary statistics were compiled for measured variables. Data were analyzed for non-normality by means of the Kolmogorov-Smirnov test. Reference intervals of pH, PO_2 and

PCO_2 were calculated with the robust method and 90% confidence interval for upper and lower limits were provided (ASVCP, 2012).

Sample size estimation – Although in method comparison studies a sample size of 30-40 individuals is generally indicated, financial, time and animal availability were of concern during the planning of the study. Therefore, a pilot analysis of data was planned after the first 13 blood gas results. The purpose of the analysis was to calculate the sample size necessary to obtain correlation coefficients (prerequisite for Passing-Bablok regression) presenting alpha error < 0.01 and beta error < 0.01. The variables which were accounted in this pilot analysis were pH, PO_2 and PCO_2. The lowest coefficient of correlation was 0.804 (r range: 0.804 - 0.994) for the PCO_2. Thus, the minimum sample size was calculated to be of 23 individuals.

Missing data-In case of system failures of single or multiple values, results of the remaining variables of those cartridges were included in the statistical analysis.

Results outside analytic range-In case of values outside the analytic range of the instrument the minimal or maximal value of the analytical range was employed for the analysis: CK <35, the number 35 was used for purpose of the analysis. Ca >20, the number 20 was used for purpose of the analysis.

Method comparison-Method comparison was performed with Bland-Altman bias plots and Passing-Bablok regression analysis. The Bland-Altman analysis estimates how much two methods differ in the quantitative measurement and therefore aids in making the decision if one method can be substituted for another. Bland-Altman plots were constructed for each variable by plotting the difference between the measurements on the vertical axis against the respective mean of those measurements on the horizontal axis. The limits of

agreement were determined by ±1.96 SD centered on the mean difference. In addition, the association between the difference and the analyte concentration was examined by standard regression analysis of the difference between the two methods on their average. Briefly, a change in bias related to analyte concentration (proportional bias) is shown by the significant slope of the regression line (Bland and Altman 1986; Bland and Altman 1999).

Passing-Bablok regression is a nonparametric model, which allows measurement error (imprecision) in both methods, does not require the measurement error to be normally distributed, and is insensitive to outliers. By first, linearity of data was examined by visual inspection of the scatter plot (Bablok and Passing 1985) and the coefficient of correlation *r* was calculated as a prerequisite of the Passing-Bablok regression (Bablok et al. 1988). Secondly, the Cusum linearity test was performed (Bablok and Passing 1985). The null hypothesis tested by means of the Cusum test is a random distribution of residuals around the fitted regression line (Bablok and Passing 1983). Lastly, the regression equation was calculated. Constant bias was considered present if the 95% confidence interval for the y-intercept did not include the value 0. Similarly, proportional bias was considered present if the 95% confidence interval for the slope did not include the value 1 (Bablok and Passing 1985).

Data were analyzed using commercial softwares (MedCalc 12.2.1; MedCalc Software bvba, Mariakerke, BE; SPSS statistics v22.0; IBM, Chicago, IL). Two tailed *P* values of less than 0.05 were considered significant.

Results

Tortoises ranged from 550 to 2300 grams of body weight (median weight, 1011 grams). Twenty-seven of them were males and 20 females. Data obtained with the blood gas analyzers and the chemistry analyzers are reported in Table 1 and Table 3, respectively. Reference intervals for pH, PO_2 and PCO_2 are reported in Table 2.

Portable analyzer for blood gas analysis – Analysis of pH, PCO_2, PO_2, Na, K, iCa, glucose and Hct provided results in all 24 cartridges (100%). The difference between PGA and RGA measurements of each variable were displayed graphically as Bland-Altman agreement plots in Figure 1. Mean difference ± SD (95% CI) between RGA and PGA were for pH: 0.017 ± 0.03 (0.005 to 0.03); PO_2: 18.82 ± 4.32 (16.99 to 20.64); PCO_2: -8.41 ± 6.09 (-10.98 to -5.83); glucose: 6.08 ± 7.04 (3.11 to 9.06); Hct: 0.71 ± 2.03 (-0.15 to 1.57); iCa: -0.14 ± 0.10 (-0.18 to -0.10); K: 0.09 ± 0.21 (0.001 to 0.18); Na: 0.57 ± 1.58 (-0.10 to 1.24). Limits of agreement for each variable were reported in Table 4. In the plots of pH, PO_2, PCO_2, glucose, iCa and K the line of equality was not included in the 95% CI of mean of differences (Figure 1), indicating a constant difference. The slope of the regression fit of the difference versus average (coefficient ± SE; P value) was significant in the Bland-Altman plots of pH (-0.15 ± 0.07; P = 0.048); PO_2 (0.09 ± 0.03; P = 0.025); PCO_2 (-0.20 ± 0.09; P = 0.04); glucose (0.11 ± 0.03; P < 0.001). Thus, a proportional bias was present between the PGA and the RGA for those analytes.

Results of the Passing-Bablok regression were listed in Table 4. The analysis demonstrated an at-least-proportional difference (slope $\neq 1$) in pH, glucose and Hct. An at-least-constant difference (y-intercept $\neq$ 0) was present in pH, PO_2, glucose and Hct. The Cusum test for linearity and Pearson *r* confirmed that all compared variables fitted a linear model.

Portable analyzer for clinical chemistry – Analysis of glucose, uric acid, AST, CK, K, Ca, Na, P, total proteins and albumin provided results in all 23 reagent discs (100%). Three samples had a CK of 0 U/L with the PCA, while corresponding values of 18, 24 and 104 U/L were measured with the RCA. The difference between PCA and RCA measurements of each variable was displayed graphically as Bland-Altman agreement plots in Figure 2. Mean difference ± SD (95% CI) between RCA and PCA were for AST: 14.6 ± 8.38 (10.99 to 18.23) U/L; CK: -9.78 ± 67.87 (-39.13 to 19.57) U/L; glucose: -12.78 ± 3.60 (-14.34 to -11.22) mg/dL; Na: 1.09 ± 4.78 (-0.98 to 3.15) mmol/L; K: 0.17 ± 0.19 (0.09 to 0.26) mmol/L; P: -0.22 ± 0.2 (-0.31 to -0.13) mg/dL; Ca: -0.19 ± 1.21 (-0.71 to 0.33) mg/dL; Uric acid: -0.06 ± 0.43 (-0.24 to 0.13); albumin: 0.17 ± 0.27 (0.05 to 0.28) g/dL; total protein: 0.15 ± 0.17 (0.08 to 0.22) g/dL. Limits of agreement for each variable were reported in Table 5. In the plots of albumin, AST, K, P, glucose and total protein the line of equality was not included in the 95% CI of mean of differences (Figure 2), indicating a constant difference. The slope of the regression fit of the difference versus average (coefficient ± SE; P value) was significant in the Bland-Altman plots of albumin (-0.49 ± 0.22; P = 0.04); AST (0.13 ± 0.06; P = 0.04), Na (-0.54 ± 0.13; P < 0.001); Ca (0.25 ± 0.08; P = 0.006). Thus, a proportional bias was present between the PCA and the RCA for those values.

Results of the Passing-Bablok regression were listed in Table 5. The analysis did not find an at-least-proportional difference in any of the analytes. An at-least-constant difference was present in glucose and K (Figure 3). Cusum test for linearity and Pearson *r* confirmed that all compared variables fitted a linear model with the exception of CK.

Albumin quantification – In twelve samples albumin was determined with the PCA, the RCA and agarose gel electrophoresis. Albumin values presented a mean difference (LoA) of -0.23 (-0.87 to 0.42) g/L between the PCA and electrophoresis and of -0.29 (-0.78 to 0.20) g/L between the RCA and electrophoresis. In both Bland-Altman plots the line of equality was not included in the 95% CI of mean of differences, indicating constant bias.

Discussion

In the present study we evaluated the use of a PGA and a PCA in tortoises by comparing them to analyzers routinely used in clinical laboratories. Furthermore, we provided preliminary reference intervals for venous blood gas analysis in Hermann's tortoises in summer. Although several benefits are associated with the use of POC analyzers, one of the major concerns remains the validity of their measurements compared to primary, bench-top instruments used in the laboratory (Jacobs et al. 1993; Klein et al. 1999; Sediame et al. 1999; Papadea et al. 2002). Only an adequate agreement between instruments ensures comparability of test results (Papadea et al. 2002).

Method comparison studies testing analytical performances of PGAs have been done in domestic, laboratory and wild animals (Grosenbaugh et al. 1998, Tinkey et al. 2006, Steinmetz et al. 2007, Wenker et al. 2007, Peiró et al. 2010, Burdick et al. 2012, Selleri and Di Girolamo 2014). In reptiles, despite the employment of this PGA in previous studies (Harms et al. 2003, Lewbart et al. 2014), agreement with reference analyzers has been only evaluated for Cl, glucose, K, Na, and Hct measurements (Wolf et al. 2008; McCain et al. 2010). In the present study, the PGA had constant and/or proportional bias for each of the analytes except Na.

Regarding acid-base and blood gases evaluation, the PGA underestimated pH and PO_2, while PCO_2 was overestimated. Therefore, there is the potential risk to overdiagnose some conditions (e.g., respiratory acidosis characterized by decreased pH and increased PCO_2). The poor agreement of PO_2 is unlikely to be clinically significant given the lack of clinical importance of venous PO_2 in tortoises (Khan et al. 2010).

Both POC analyzers underestimated blood K concentrations. POC analyzers may be especially useful for evaluation of K in the field, as in chelonians late separation of red cells from serum or plasma after blood collection may interfere with the reliability of K measurements (Abou-Madi and Jacobson 2003). Underestimation of K may potentially lead to an underdiagnosis of conditions that result in hyperkalemia, e.g., acute renal failure (Divers 2000). Sodium did not present constant bias with both POC analyzers, but with the PCA there was increasing bias at increasing Na values. A great advantage of the PGA is the measurement of the iCa that may have an important role in tortoise medicine (Eatwell 2009a; Eatwell 2009b). iCa was significantly overestimated by the PGA. Decreased iCa is typically observed in chelonians suffering nutritional secondary hyperparathyroidism (Stringer et al. 2010) and renal disease (Selleri and Hernandez-Divers 2006). Therefore, the overestimation of iCa by the PGA may potentially result in underdiagnosis of such disorders.

The direction of bias for glucose was different in the two POC devices. The PGA underestimated the glucose, while the PCA overestimated the glucose as compared to the reference analyzers. This last finding is in agreement with data reported for other reptiles (Atkins et al. 2010; McCain et al. 2010). Differently, in mammals no significant bias was found between the same instruments (Selleri et al. 2014). The overestimation of blood

glucose in reptiles with the PCA is unexpected, because both the PCA and the RCA use a similar, hexokinase-based reaction. Clinicians should take into account this finding, and further investigations are needed to improve the analysis of glucose.

CK and AST, enzymes associated with muscular (CK) and liver metabolism (AST), had wide LoA, between -142.81 to 123.25 U/L and -1.81 to 31.02 U/L, respectively. Due to the wide range of normality of these enzymes compared with electrolytes, wider LoA are not necessarily related with clinical misinterpretation. In fact, allowable total error of these enzymes is greater than most analytes (ASCVP 2012). Besides the wide LoA, values of CK provided by the PCA did not fit a linear model when compared to values provided by the RCA. The Passing-Bablok regression has the assumption that the values are correlated (Bablok et al. 1988). In general, objective assessment of acceptability is very difficult in cases of non-linear correlation (Jensen and Kjelgaard-Hansen 2006).

The present study demonstrates that bromocresol green dye-binding method for determination of albumin concentration can be inadequate in Hermann's tortoises, as reported for other reptiles (Müller and Brunnberg 2010). The overestimation of albumin by bromocresol green is a common finding due to nonspecific interactions with other plasma proteins (Doumas and Peters 2009). Therefore, we suggest quantifying plasma albumin by agarose gel electrophoresis.

Acceptability of agreement is subjective and largely depends on the use for which the instrument is needed (Jay 2011). In this study both POC devices had wide limits of agreement for most analytes, which may result in inaccurate clinical decisions. Determination of the real clinical impact of a POC device requires randomized controlled trials (e.g., Kendall et al. 1998) and was beyond the scope of our study. Nevertheless, our

finding suggests that either specific reference intervals or a specific calibration for the PGA and for the PCA are required. In addition, the use of different analyzers for multiple assessments of the same tortoise at different times should be discouraged.

Limitations

The number of tortoises included in each of the phases of the study was lower compared to that suggested for method comparison study. Nevertheless, in zoological medicine there is an intrinsic difficulty related with the obtainment of samples (ASVCP, 2012), and previous studies on single reptile species had a similar sample size (Wolf et al. 2010).

While in this study we evaluated the agreement (i.e., "trueness") of the two POC analyzers (Menditto et al. 2007), assessment of precision (i.e., repeatability and reproducibility) was not performed due to financial constraints and lack of sufficient amount of blood samples. Future studies should include multiple analyses with the same instruments and ideally with different instruments of the same models, in order to calculate coefficients of variation and to provide an insight upon the overall accuracy of the instruments.

Finally, the population sampled included clinically healthy specimens, limiting somehow the opportunity to extrapolate data from this study to diseased or injured specimens.

Conclusions

The present study confirms that although the use of POC devices in tortoises is intriguing due to the versatility of the instruments and the small amount of blood required, results provided by POC analyzers may lack agreement with laboratory-based analyzers when used in reptile species.

Table 1. Descriptive statistics of values obtained analyzing venous blood samples from Hermann's tortoises (n = 24) with a portable gas analyzer (PGA, i-STAT 1, Abaxis) and a reference analyzer (RGA, Stat Profile Critical Care Xpress 12, Nova Biomedical Corporation).

	Mean	SEM	Median	Minimum	Maximum	Normal Distr.
pH						
PGA	7.329	0.017	7.336	7.172	7.519	0.99
RGA	7.346	0.015	7.362	7.195	7.482	0.70
***P*O$_2$ (mmHg)**						
PGA	67.79	4.48	60.5	44.0	133.0	0.18
RGA	86.61	4.88	78.1	60.6	155.1	0.06
***P*CO$_2$ (mmHg)**						
PGA	58.51	2.87	55.85	35.7	99.1	0.93
RGA	50.10	2.37	50.25	28.1	72.6	0.84
Ionized Calcium (mg/dL)						
PGA	1.58	0.03	1.6	1.13	1.84	0.73
RGA	1.44	0.03	1.45	1.09	1.69	0.79
Potassium (mmol/L)						
PGA	4.67	0.12	4.75	3.1	5.5	0.93
RGA	4.76	0.13	4.89	3.25	5.72	0.84
Sodium (mmol/L)						
PGA	131.50	0.87	131.0	123.0	143.0	0.44
RGA	132.07	0.81	131.05	126.3	142.5	0.71
Glucose (mg/dL)						
PGA	121.29	7.44	124.5	67.0	221.0	0.83
RGA	127.37	8.35	133.0	65.0	240.0	0.95
Hematocrit (%)						
PGA	22.62	1.05	21.5	14.0	33.0	0.83
RGA	23.33	0.93	23.5	15.0	31.0	0.97

Table 2. Preliminary reference intervals (robust method) for pH, PO_2 and PCO_2 from venous blood samples of Hermann's tortoises in summer (n = 24) measured with portable (PGA) and reference gas analyzer (RGA). The 90% CI for upper and lower limits are provided.

	Reference intervals			
	Lower limit	**90% CI**	**Upper limit**	**90% CI**
pH				
PGA	7.145	7.101, 7.203	7.509	7.457, 7.555
RGA	7.191	7.146, 7.249	7.511	7.469, 7.546
***P*O$_2$ (mmHg)**				
PGA	12.57	0, 32.59	109.05	86.72, 126.73
RGA	24.95	9.26, 46.97	130.67	107.48, 149.80
***P*CO$_2$ (mmHg)**				
PGA	27.17	18.29, 37.79	87.63	76.77, 96.87
RGA	25.83	19.24, 33.60	75.15	68.82, 81.08

Table 3**.** Descriptive statistics of values obtained analyzing venous blood samples from Hermann's tortoises (n = 23) with a portable chemistry analyzer (PCA, VetScan VS2, Abaxis) and a reference analyzer (RCA, (Olympus AU400, Olympus).

	Mean	**SEM**	**Median**	**Minimum**	**Maximum**	**Normal Distr.**
Albumin (g/L)						
PCA	1.80	0.07	1.8	1.3	2.7	0.52
RCA	1.97	0.04	1.99	1.58	2.33	0.91
AST (U/L)						
PCA	54.83	5.19	54.0	23.0	101.0	0.68
RCA	69.43	5.92	77.0	29.0	122.0	0.70
Calcium (mg/dL)						
PCA	13.94	0.51	13.0	11.5	20.0	0.16
RCA	13.75	0.64	12.36	11.0	20.91	0.05
CK (U/L)						
PCA	54.78	9.06	44.0	0.0	192.0	0.23
RCA	45.0	13.54	18.0	5.0	274.0	0.01
Glucose (mg/dL)						
PCA	70.04	4.03	74.0	30.0	102.0	0.85
RCA	57.26	3.73	61.0	18.0	88.0	0.95
Phosphorus (mg/dL)						
PCA	3.28	0.16	3.2	2.2	4.8	0.86
RCA	3.06	0.16	2.9	2.1	4.8	0.60
Potassium (mmol/L)						
PCA	5.99	0.16	6.2	3.9	7.0	0.48
RCA	6.16	0.15	6.4	4.3	7.0	0.63
Sodium (mmol/L)						
PCA	130.0	1.59	131.0	101.0	144.0	0.15
RCA	131.09	0.97	131.0	121.0	138.0	0.62
Total Proteins (g/dL)						
PCA	5.23	0.12	5.2	4.0	6.4	0.68
RCA	5.38	0.11	5.37	4.37	6.6	0.43
Uric Acid (mg/dL)						
PCA	4.16	0.20	4.2	1.8	5.6	0.94
RCA	4.10	0.21	3.95	1.86	5.89	0.88

Table 4. Agreement of a portable gas analyzer (PGA, i-STAT 1, Abaxis) and a reference analyzer (RGA, Stat Profile Critical Care Xpress 12, Nova Biomedical Corporation) in venous samples from Hermann's tortoises (n = 24).

	Bland-Altman analysis		Passing-Bablok Regression			
Analyte	**Mean difference**	**Limits of Agreement**	**Regression Equation***	**95% CI Intercept A**	**95% CI Slope B**	***r*†**
pH	0.02	-0.04 to 0.07	y = -1.47 + 1.20 x	-2.55 to -0.34	1.04 to 1.34	0.94
***P*O$_2$ (mmHg)**	18.82	10.33 to 27.30	y = -12.64 + 0.93 x	-21.92 to -5.70	0.84 to 1.04	0.99
***P*CO$_2$ (mmHg)**	-8.41	-20.35 to 3.53	y = 3.42 + 1.06 x	-11.83 to 11.38	0.89 to 1.36	0.90
Ionized Calcium (mg/dL)	-0.14	-0.34 to 0.06	y = 0.14 + 1.0 x	-0.28 to 0.65	0.65 to 1.28	0.79
Potassium (mmol/L)	0.09	-0.32 to 0.51	y = 0.05 + 0.96 x	-0.22 to 0.81	0.80 to 1.02	0.94
Sodium (mmol/L)	0.57	-2.53 to 3.68	y = -5.46 + 1.04 x	-33.44 to 17.90	0.86 to 1.25	0.93
Glucose (mg/dL)	6.08	-7.73 to 19.89	y = 8.09 + 0.89 x	0.60 to 15.86	0.82 to 0.95	0.99
Hematocrit (%)	0.71	-3.27 to 4.69	y = -5.80 + 1.20 x	-10.50 to 1.0	1.0 to 1.40	0.92

†All P values for r were less than 0.0001.
*None of the analytes had significant deviation from linearity (Cusum test: P > 0.05).

Table 5. Agreement of a portable chemistry analyzer (PCA, VetScan VS2, Abaxis) and a reference analyzer (RCA, (Olympus AU400, Olympus).in venous samples from Hermann's tortoises (n = 23).

	Bland-Altman analysis		Passing-Bablok Regression			
Analyte	**Mean difference**	**Limits of Agreement**	**Regression Equation***	**95% CI Intercept A**	**95% CI Slope B**	***r***
Albumin (g/dL)	0.17	-0.36 to 0.69	y = -1.07 + 1.43 x	-3.07 to 0.003	0.89 to 2.5	0.56†
AST (U/L)	14.61	-1.81 to 31.02	y = -4.05 + 0.85 x	-15.0 to 2.83	0.76 to 1.0	0.96††
Calcium (mg/dL)	-0.19	-2.56 to 2.18	y = 1.57 + 0.91 x	-0.15 to 2.88	0.81 to 1.06	0.93††
CK (U/L)	-9.78	-142.81 to 123.25	y = 25.25 + 1.16 x	-26.0 to 33.7	0.79 to 4.0	0.27**
Glucose (mg/dL)	-12.78	-19.84 to -5.72	y = 8.07 + 1.07 x	3.43 to 12.0	1.0 to 1.14	0.98††
Phosphorus (mg/dL)	- 0.22	-0.61 to 0.17	y = 0.2 + 1.0 x	-0.19 to 0.7	0.86 to 1.14	0.97††
Potassium (mmol/L)	0.17	-0.21 to 0.56	y = -0.88 + 1.1 x	-1.78 to -0.2	1.0 to 1.25	0.97††
Sodium (mmol/L)	1.09	-8.27 to 10.45	y = -1.0 + 1.0 x	-73.22 to 32.0	0.75 to 1.55	0.81††
Total proteins (g/dL)	0.15	-0.18 to 0.49	y = -0.88 + 1.13 x	-1.67 to 0.02	0.96 to 1.28	0.96††
Uric Acid (mg/dL)	-0.06	-0.90 to 0.78	y = 0.006 + 0.97 x	-0.40 to 0.49	0.87 to 1.07	0.91††

**P values for r higher than 0.05.
*None of the analytes had significant deviation from linearity (Cusum test: P > 0.05).

†P values for r less than 0.01.
††P values for r less than 0.0001.

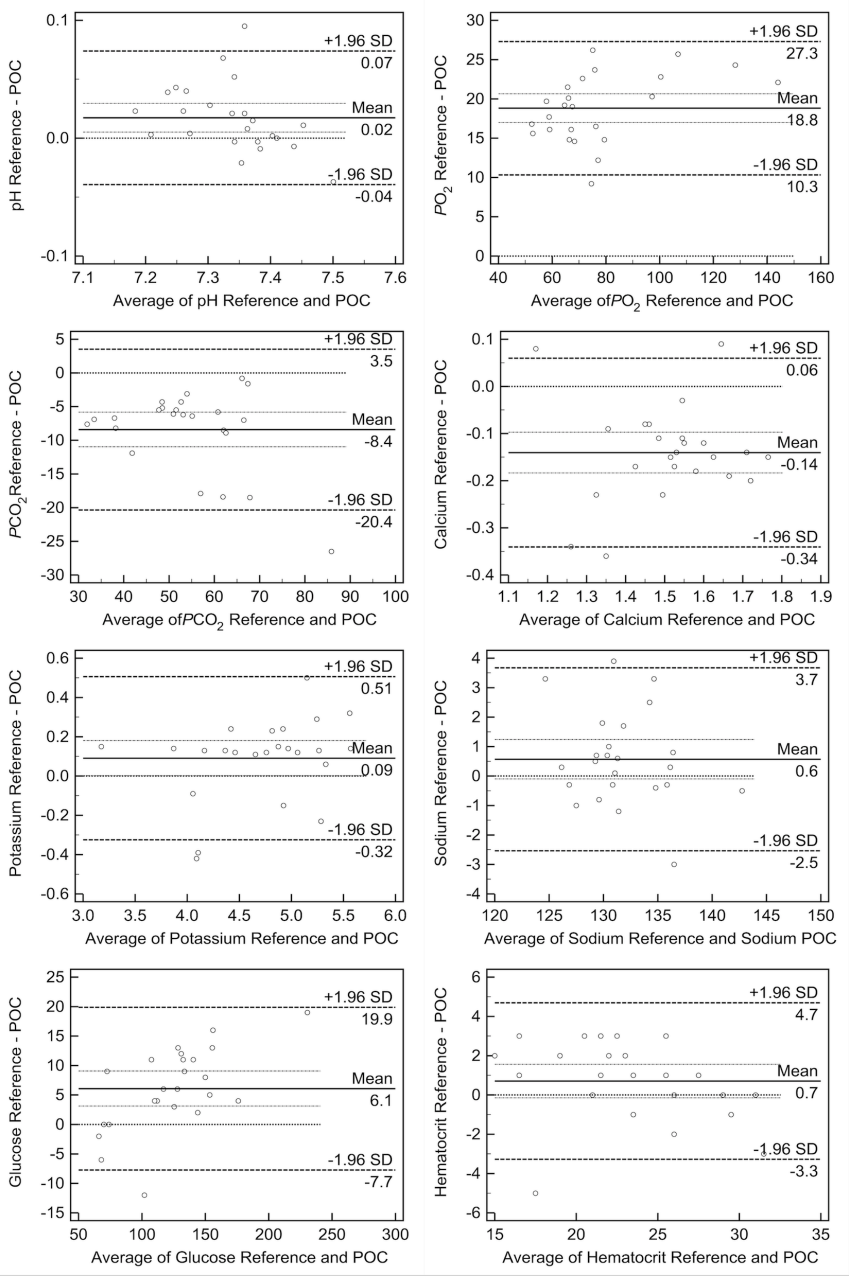

Figure 1. Bland-Altman agreement plots for analytes measured with a POC blood gas analyzer and a reference blood gas analyzer in blood samples from Hermann's tortoises. The middle solid horizontal line represents the mean difference between the pairs of measurements, representing the mean 'measurement error'. The upper and lower horizontal dashed lines represent the 95% limits of agreement, i.e. the range of 'measurement errors' that would occur on 95% of occasions. The 95% confidence intervals of mean of differences are depicted as dashed and dotted lines. If the 95% confidence intervals of mean of differences do not included the 0 value, there is a significant constant bias.

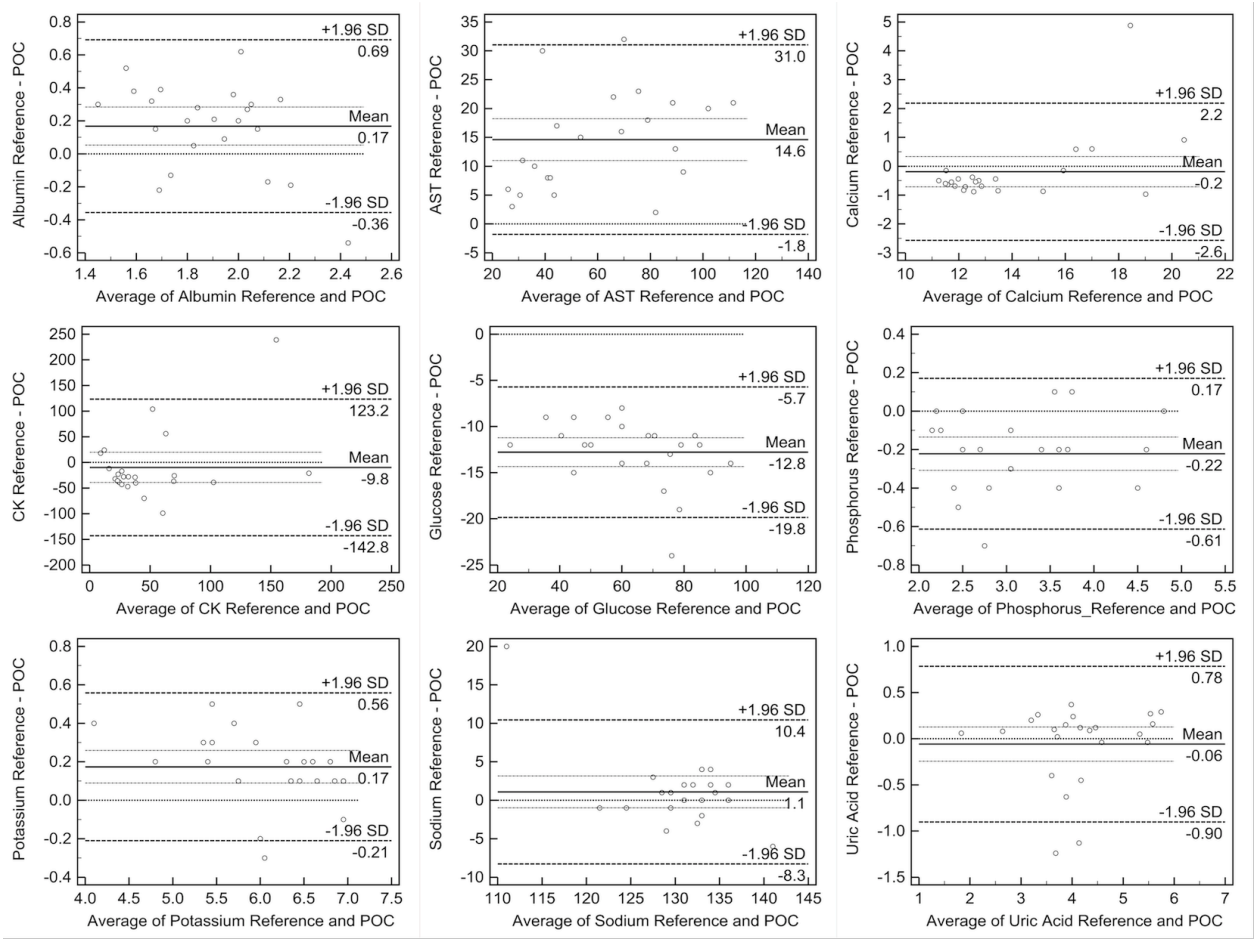

Figure 2. Bland-Altman agreement plots for analytes determined with a POC chemistry analyzer and a reference chemistry analyzer in blood samples from Hermann's tortoises. See Figure 1 caption for details.

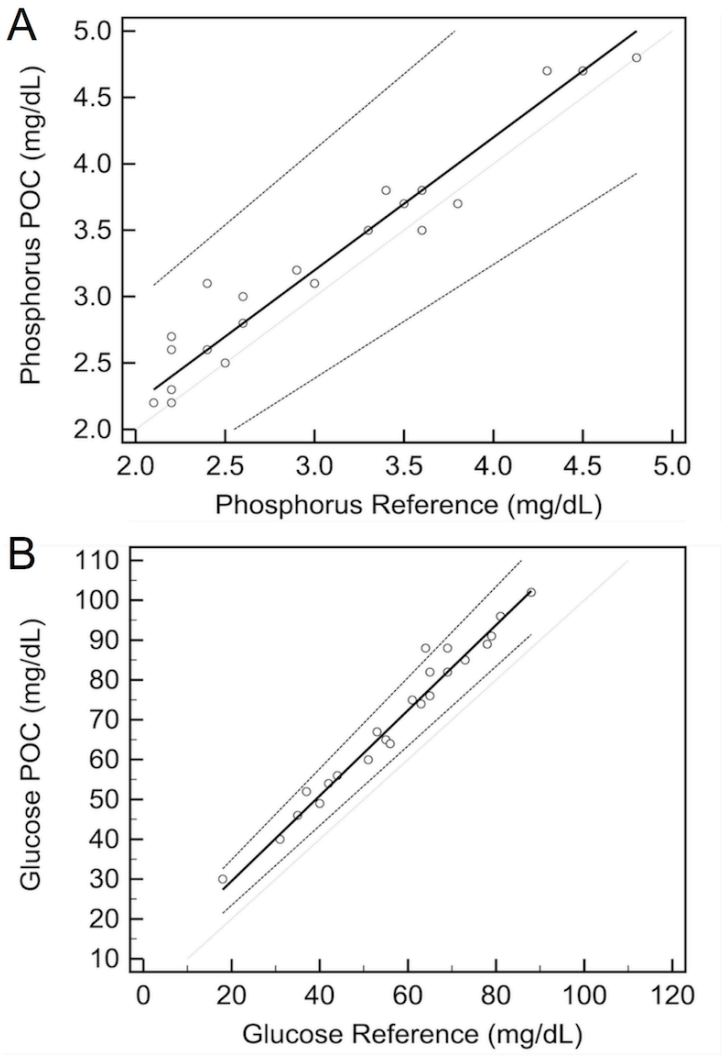

Figure 3. Passing-Bablok regression for two representative analytes measured with a POC chemistry analyzer and a reference chemistry analyzer. The regression line is indicated by the solid line, with the confidence intervals marked as dashed lines. A. Phosphorus; lack of constant or proportional bias. B. Glucose; presence of constant bias. Notice that the POC analyzer significantly overestimated blood glucose concentration.

Reference values for hematology and plasma biochemistry variables, and protein electrophoresis of healthy Hermann's tortoises (*Testudo hermanni* ssp)

Background

Chelonians face serious threats due to destruction of their habitat, and overcollection for human consumption and the pet trade which have led to the extinction of some species. In addition, tortoises and sea turtles are considered of increasing interest as potential biomonitors for environmental pollution.[1,2] *Testudo hermanni* ssp is currently considered globally endangered.[3,4] In particular, *T. hermanni* is endangered in France[5] and Italy[6], and has been listed since 2002 on the Turtle Conservation Fund's "Extinction Row".[7] Moreover, increasing numbers of reptiles, mainly tortoises, are kept as pets, and captive animals are vulnerable to diseases caused by infectious agents, inappropriate diet and overall living conditions. In the management of captive and wild tortoises, hematologic and biochemical blood analyses are essential to assess the health status as these animals present few clinical signs that can be assessed during a standard physical exam. In addition, many intrinsic factors like sex, age and physiologic status, and extrinsic factors including season, and site and method of blood sample collection, make it difficult to determine reference intervals in reptiles like tortoises.[8] Standardization of blood collection techniques, environmental factors and the physiologic status must be considered in assessing normal reference intervals. Few studies have investigated the blood biochemistry of *T. hermanni* [9–11] even though this species is frequently kept as a pet, and reference intervals for most of the clinical analytes are lacking.

The purposes of this work were to 1) collect baseline data on hematology, blood biochemistry and plasma protein electrophoresis of *T. hermanni* ssp for the definition of normal reference ranges to facilitate the interpretation of laboratory data, and 2) determine the correlation of the observed variability of these analytes with sex and the seasonal time of sampling.

Materials and Methods

Animals and blood sampling

Thirty-four captive adult tortoises (*Testudo hermanni* ssp) were evaluated in this study. They were kept in 3 separate groups living in the province of Bologna, (latitude 44° 29′ 38″ N and longitude 11° 20′ 34″ E, Emilia-Romagna, Italy). The animals were all born and raised in captivity. Tortoises were kept free, maintained under natural sunlight, having access to spontaneous autochthonous plants and to a limited amount of vegetables (mainly *Cichorium* ssp). Blood samples were collected at the end of September before hibernation, and at the beginning of July 2010. All these tortoises were client-owned since hatching, allowing relatively reliable information on age. All tortoises were handled with disposable gloves, discarded after each tortoise, to minimize the probability of infection among animals. During the clinical visit, tortoises were physically examined for disease or trauma; eyes, nostrils, tympanic area, oral cavity, skin, cloaca, plastron and carapace were inspected. The coelomic cavity was palpated through the prefemoral region. Following the examination to exclude diseased animals, the curved carapace length (CCL) was measured and tortoises were weighed. Blood samples were collected only from animals with a weight > 450 g. The sex was determined by plastron indentation and tail morphology.[12]

For venipuncture, animals were manually restrained, without sedation. Blood samples for biochemical and hematologic profiles were collected from the jugular vein and/or from the dorsal cervical sinus using disposable sterile plastic syringes with 23 gauge needles carefully avoiding blood dilution by lymph. A maximum blood volume of approximately 0.5% of the actual individual body weight was taken (eg 0.5-1 ml). Three peripheral blood smears were prepared immediately with a drop of blood directly from syringes without any anticoagulant. The remaining blood samples were then transferred into lithium–heparinized tubes and stored in a refrigerator for a maximum of 6 h until HCT, HGB concentration and the biochemical profiles were performed.

All experimental procedures were approved by the Ethical and Scientific Committee of Bologna University, and were carried out in accordance with European legislation regarding the protection of animals used for experimental and other scientific purposes.

Hematology

Hematologic analyses were made in duplicate and the average of the 2 measurements was used for data analysis. HCT and HGB concentrations were determined with a microhematocrit centrifuge (mod. 4203, ALC International, Milano, Italy) at 3,000*g* for 5 min and by an automated cell counter (cyanmethemoglobin method, Abbott Cell Dyn 3500, Abbott, Illinois, USA), respectively. Smears were air–dried and stained with 3 different staining protocols, 1) a rapid stain (Bio-optica, Milano, Italy), 2) a May Grünwald-Giemsa-Romanowsky stain (Merck, Darmstadt, Germany) and 3) a Wright stain (Sigma-Aldrich, St. Louis, USA). WBC were classified as heterophils, lymphocytes, eosinophils or basophils. RBC and platelet morphology were also evaluated.

Clinical biochemistry

Plasma was separated after centrifugation of samples at 3000*g* for 10 min (centrifuge mod. 5702, Eppendorf, Hamburg, Germany) and stored at 4°C for up to 24 h prior to biochemical analysis. A standard biochemical profile was determined, including concentrations of glucose, urea, creatinine, uric acid, total protein, albumin, A/G ratio, calcium (Ca), phosphate (P), sodium (Na), potassium (K) and chloride (Cl); and activities of AST (EC 2.6.1.1), ALT (EC 2.6.1.2), ALP (EC 3.1.3.1), CK (EC 2.7.3.2), and LDH (EC 1.1.1.27). The selected analytes were determined using commercially available kits (Olympus Systems Reagents, Olympus life and Material Science Europe GmbH, Hamburg, Germany) with an automated biochemical analyzer (Olympus AU400, Mishima Olympus Co. Ltd, Shizuoka, Japan), including total protein and albumin concentration measured by the biuret and the bromocresol green methods, respectively.

Plasma protein electrophoresis

Plasma samples (10 microlitri) stored for up to 24 h at 4°C were separated on 0.8% high resolution agarose gel (Hydragel HR, Sébia, Lisses, France). The electrophoresis was run at a constant 255 V on an automated system (Hydrasis, Sébia, Lisses, France). Gels were stained by Acid Violet. Stained gels were digitalized with a scanner yielding the densitometric profile (software Phoresis 6.1.2, Sébia, Lisses, France). The relative percentage and absolute concentration of each protein fraction was calculated based on densities determined by a densitometer. For fraction identification, the midpoint of the pherogram was arbitrarily determined between the alpha and beta peaks.

Statistics

Reference ranges The normality of the variables was tested with the D'Agostino-Pearson test. A P>.05 was considered indicative of a normal distribution. Hematology and plasma protein electrophoresis data were reported as range (min-max), mean and median. Reference intervals for biochemistry variables were calculated using the robust method suggested by the CLSI document C28-A3. Data were displayed graphically using frequency histograms. To highlight the uncertainty in the upper and lower reference limits resulting from the small sample sizes of this study, 90% confidence intervals (CI) were calculated. For the determination of interindividual variability the coefficient of variation (CV, standard deviation [SD] expressed as a percentage of the average)) was determined. Normally distributed variables were analyzed by a 2-way ANOVA to determine the impact of 1) sex and blood sampling procedures for hematologic data, 2) sex, blood sampling procedures and sampling time point for biochemical analytes, 3) blood sampling procedures and sampling time point for protein electrophoresis.

Non-normality was tested with the D'Agostino-Pearson test, and data were transformed using the power transformation as needed. Arcsine transformation was applied to variables measured as percentages (HCT, differential count). Levene's test for equality of variance was performed prior to the ANOVA test. If Levene's null hypothesis was rejected using raw data or logarithmic and/or square transformed data, the normally-distributed data were analysed using a t-test, and non-normally distributed data were analysed using the non-parametric Mann-Whitney test.

The null hypothesis was that no differences were present between sexes, and blood sampling procedures and timepoints of the year. If variances were heterogeneous, as determined by F-test, data were analysed using a 2-tailed t-test corrected for unequal

variances, and P ≤ .05 was considered significant. Pearson's coefficient of correlation (*r*) was used to measure the degree of the relationship between biochemical variables and age, between biochemical variables and body weight, and between colorimetric (bromocresol green) and electrophoretic methods for albumin quantification. The latter was assessed by Bland-Altman plots to test for agreement. The limits of agreement were determined by ±1.96 SD centered on the mean difference. In addition, the association between the difference and the analyte concentration was examined by regression analysis in the frame of Bland-Altman plots. Data were analyzed using MedCalc 12.2.1 (MedCalc Software, Mariakerke, Belgium).

Results

Animals

Thirty-four healthy tortoises, 14 males and 20 females (overall males to females ratio 0.6 - 0.8) were included in this study. Biometric data on age, body mass and CCL in all animals, or partitioned by sex and sampling timepoint are listed in Table 1. There were no significant differences between data on samples collected from the jugular vein (n=11) or the dorsal cervical sinus (n=23) for all hematologic and biochemical variables, including plasma protein electrophoresis (data not shown).

Ambient temperature at the end of September and before hibernation (n=20, 7 males and 13 females) ranged from 13 - 21°C, and at the beginning of July (n=14 specimens, 7 males and 7 females) from 18 - 30°C. There were 12 h of daylight in September and 15 h in July.

Hematology

Hematology data were collected on 14 animals. Comparison among smear stains revealed that with the rapid stain some WBC, especially heterophils, presented smoky cytoplasm with unstained areas (Figure 1A), while RBC had a purple nucleus and pink cytoplasm. By contrast, with the May Grünwald-Giemsa and the Wright stains all blood cells were more easily identified based on morphology characteristics (Figure 1B and 1C). Mature RBC appeared oval with rounded poles and a homogeneous blue cytoplasm. Thrombocytes were smaller, round to oval with similarly shaped centrally located nuclei. There were 3-6 thrombocytes/100x microscopic field. Heterophils were among the largest WBC, with a peripheral round or oval nucleus characterized by clumped blue chromatin; the cytoplasm contained variable numbers of granules that appeared oval and bright orange with May Grünwald-Giemsa or Wright stains. Eosinophils were similar in size to heterophils but could be recognized by the eccentric purple nucleus and a cytoplasm with darker, redder and rounder granules. Lymphocytes presented small diameters with a thin rim of basophilic cytoplasm and a compact nucleus containing clumped chromatin that was lighter in color than in thrombocytes. Finally, a few basophils were observed and identified by the presence of round, large granules with a color varying from deep purple to deep blue that sometimes obscured the nucleus. No monocytes were observed in any smear. No pathologic cells or parasites were observed.

For HGB concentrations and HCT reference ranges but not actual reference intervals were calculated due to the small number of animals in the group (Table 2). The ranges were normally distributed and values were significantly higher in males than females.

Clinical biochemistry

Plasma biochemical analytes were measured in 34 healthy tortoises and reference intervals were calculated (Table 3). The D'Agostino-Pearson normality test showed that 6 of 17 variables were normally distributed (ALP, total protein, albumin, Cl, Na, K). Highest interindividual variability was deteremined for LDH (CV=84%), ALT (CV=100%), AST (CV=120), and CK (CV=140%) activity, and urea (CV=78%) and uric acid (CV=57%) concentrations, while for total protein, albumin (CV=25% and 28% respectively) and electrolyte concentrations (both Na and Cl, CV=6%) it was the lowest.

Sex and sampling at 2 different times of the year were identified as major factors correlating with blood chemistry in *T hermanni* (Table 4). Females had significantly lower concentrations of urea and uric acid, and lower AST and ALT activity, and higher Ca and Cl concentrations. Significantly higher concentrations were determined in July for glucose, uric acid, albumin and P concentration, and AST activity, while lower concentrations of urea and Cl were measured in September. A significant inverse relationship was found for ALP activity and both age (r = 0.548, p<.01) and body weight (r = 0.542, p<.01), while glucose concentration was inversely correlated with age (r = 0.463, p<.01) (Figure 2).

Plasma protein electrophoresis

Plasma protein electrophoresis was performed in 24 healthy tortoises. In the experimental conditions reported here, 4 fractions were always present in all specimens, including albumin, alpha globulins (frequently appearing in 2 peaks), beta globulins (sometimes appearing in 2 peaks) and a small proportion of gamma globulins (Table 5). All protein fractions presented a normal distribution with the exception of alpha globulins in the September specimens. These latter specimens presented statistically significantly higher

levels of albumin (p<.01) and beta globulins (p<.05) (Figure 3B), while the July specimens had higher proportions of gamma globulins (p<.05) (Figure 3A). There was a significant positive relationship (p<.01) between albumin measured by the bromocresol green dye reaction and the electrophoretic separation and densitometric assessment (Figure 4A). The bias between the 2 techniques is displayed in a Bland-Altman agreement plot in Figure 4B. The mean difference ± standard deviation (SD) (95% CI) was 0.03 ± 0.26. No systematic bias was found: the slope of the regression line of the difference versus the average was not significantly different from zero (y = -0.053x + 0.107, r = -0.05, p = .60) and the line of equality was included in the 95% CI of mean differences.

Discussion

The clinical chemistry of reptiles and other lower vertebrates has not attracted the same level of attention as it has for mammals. The present study offers practitioners a survey of the most important hematologic and biochemical analyses to facilitate the interpretation of laboratory data. Results of the present study confirm that the influence of environmental and physiological factors should be considered when evaluating hematology, plasma biochemistry and protein electrophoresis of this species.

Hematology

Many intrinsic and extrinsic factors in reptiles complicate the evaluation of hematologic data, including species, age, gender, nutrition, physiologic status, hibernation, habitat, season, temperature, captive or wild. Manual counting techniques and evaluation of blood smears are necessary for accurate interpretation of the leukogram, because reptiles have nucleated erythrocytes, which interfere with automated analysis. Different protocols for

reptile blood smear evaluation recommend Romanowsky-type stains, such as Wright, Wright-Giemsa, May Grünwald-Giemsa.[13,14] Giemsa. Wright is considered the best stain, but Wright-Giemsa[14] and May Grünwald-Giemsa staining are also recommended. On the contrary, rapid staining can cause degranulation artefacts affecting the proper differentation of lymphocytes from granulocytes. Our data confirm the reliability of Romanowsky-type staining also for *T. hermanni*.

The level of lymph-induced hemodilution differs depending on the sampling site and can therefore affect different hematologic measurements. An earlier study suggested that blood should be collected from the jugular vein to reduce abnormal background staining of smears due to lymphodilution.[15] We did not find significant differences between the 2 blood sampling sites on HGB concentration and HCT, which is in agreement with a study on chameleons.[16] Recently, venipuncture of the subcarapacial vein has been linked to adverse neurologic events (eg, transient to permanent paresis of tail, hind limbs, fore limbs).[17] Although these observations are anecdotal, clinicians are advised to use caution in venipuncturing the subcarapacial vein and to prioritize less invasive sites. *T. hermanni* HCT and HGB concentration were significantly higher in males than in females, as reported in *Gopherus agassizii.*[18] In analogy with mammals, we hypothesize that this is related to an effect of testosterone.

Blood chemistry

Though calculated on a small number of tortoises, the reference intervals for select plasma biochemical analytes were generally similar to those reported previously for *Testudo* ssp [8,10,11] and *Geochelone radiata.*[19]

With the exception of creatinine, metabolites were significantly influenced by sex and the time point of sampling, for instance urea and uric acid concentrations were lower in females, which is in accordance with another study.[19] Glucose and uric acid concentrations were higher in specimens collected in September shortly before hibernation, whereas urea concentration was lower than in July, which is unlike *G. radiata* for which no differences were observed. [19] Tortoises, like other reptiles and birds, excrete uric acid which is derived metabolic product from purine and protein catabolism. The presence of plasma urea is indicative of protein catabolism via the urea cycle. Depending on environmental factors such as temperature, water availability and diet, tortoises can modulate the production of uric acid and urea.[20] Clearly, differences in the concentrations of final products of protein metabolism observed in *T. hermanni* are related to biochemical adaptations to the environment, however the exact mechanism is not known.

Total protein concentrations determined in this study are similar to those reported for the desert tortoise *G. radiata.*[19] Ranging from 2.2 to 5.5 g/dL, the normal total plasma protein concentration in reptiles is generally lower than that of mammals.[8,10,19] Chelonians typically have lower albumin and higher globulin concentrations resulting in an overall lower A/G ratio.[19,21]

Factors expected to have a significant influence on plasma transaminase activity include liver or muscle diseases, but while AST activity can be elevated in a wide spectrum of clinical disorders, increased plasma ALT is considered a more specific indicator of hepatocellular disease in some species. In the specimens analyzed in this study low ALT activity (1-16 U/L) contrasted with a wide range for AST (18-628) activity, which is in accordance with the data reported earlier for healthy tortoises.[8,10,19] In mammals, ALP

plays an important role in bone mineralization.[22] In reptiles ALP is widely distributed in several tissues, including bones and reproductive tract.[23] Presumably, tissue pathology can result in increased serum/plasma ALP activity. The present study also found a negative association between age, body mass and ALP (p<.01), in accordance with data reported in wild juvenile *Chelonia mydas*[24]. In general, higher ALP activity levels can be associated with increased osteoblastic activity and hence growing animals, as reported also in mammals.[25] CK is considered a muscle-specific enzyme and its increase in plasma is used to diagnose acute striated muscle necrosis, frequently in association with an increase in LDH and AST. On the other hand, sampling after extensive physical activity, especially males, can be characterized by transiently increased activity of those enzymes. In this study, AST activity was higher in males than in females, which could be due to an increased activity and aggressive behavior associated with mating or fighting in early summer as reported in other tortoises.[26,27]

Minimal interindividual variations were observed for Ca, P, Na, Cl and K concentrations, overall the ranges were comparable to ranges reported for other species of tortoises.[10,19,27] Generally, electrolyte concentrations are remarkably constant due to the tight homeostatic control related to their basal biochemical role common to low and high vertebrates. The higher levels of Ca in *T. hermanni* females may be related to the reproductive cycle as Ca is mobilized during vitellogenesis and egg formation, as observed in *T. horsfieldi*[28], *G. agassizii*[27,29] and other *Testudo* ssp[30]. Mediterranean tortoises (*T. hermanni* and *T. graeca*) are generally monestrous species laying a single clutch of eggs each year in August.

Plasma protein electrophoresis

Serum/plasma protein electrophoresis is the current standard technique in veterinary medicine to investigate dysproteinemias, and to identify and monitor specific pathologies. Few basic studies have investigated the electrophoretic profile of plasma proteins in tortoises.[9,19] Two main difficulties arise when performing protein electrophoresis with specimens from exotic and wild animals such as chelonians. First, there are no defined criteria for the identification of different fractions, and protein separation is optimized for human protein fraction separation. For instance, presetting the albumin peak skewed the identification of the main globulin sub-fractions in some specimens; therefore, we preferred to define only one alpha and one beta globulin fraction, based on the suggestion in an earlier report on *Trachemys.* Second, considerable inter- and intraspecific variation, enhanced by environmental factors including habitat, season and diet, complicated the lineup of study data with previously reported data. Overall, the data in this study was in reasonable agreement with previous studies on tortoises.[19,21] Interestingly, albumin was reported higher in postprandial specimens of green turtles [31], accordingly, the higher albumin concentrations in the July specimens of this study may be related to an abundance of metabolic substrates.

The Bland-Altman difference plot indicated an agreement between albumin quantification obtained by bromocresol green dye (automated colorimetric method) and the electrophoretic methods (densitometric analysis), suggesting that these methods produce reliable results and hence both can be used in *T. hermanni*. However, another study found that bromocresol green overestimated albumin concentration in plasma of turtles compared to electrophoresis on cellulose acetate membranes.[32] These different results can be due either to a suboptimal electrophoretic separation on acetate gels and/or to the use

of a longer reaction time. Information on the proteins present in the different globulin fractions is anecdotal in chelonians, but we can hypothesize that they are generally comparable to mammals due to the essential role played in vertebrate metabolism. The significantly higher amount of alpha globulins (mainly the putative alpha2 fraction) present in *T. hermanni* sampled in September before hibernation could be related to the abundance of 2 specific alpha zone protein, alpha2 macroglobulin (alpha2M) and alpha lipoproteins (HDL and VLDL). Alpha 2M is an important circulating protease inhibitor involved in regulating a number of steps in the clotting cascade and in complement activation. Increased alpha 2M levels would attenuate/prevent clot formation when heart rate decreases and blood viscosity increases, as suggested in hibernating mammals.[33] Proteins migrating in the alpha zone also include lipoproteins, though in lower amounts than alpha 2M; mammals present alpha 1 HDL responsible for transporting cholesterol from peripheral tissues to the liver, and alpha 2 VLDL responsible for triglyceride delivery to peripheral organs. Accordingly, the increase in the putative alpha 2 fraction in *T. hermanni* in September could indicate a switch to a lipid-based metabolism, as reported for hibernating mammals.[34]

Conclusions

This study confirms the reliability of Romanowsky-type (May Grünwald-Giemsa and Wright) stains for morphologic analysis of peripheral blood cells in *T. hermanni*. Most of the measured biochemical analytes in *T. hermanni* were in accordance with those reported for other healthy tortoises and can be used for the calculation of a normal reference interval. There were significant differences for some hematologic and biochemical variables related

to sex and sampling timepoint Therefore, physiological and environmental factors should to be taken into account when evaluating tortoise health status including laboratory data analysis. The reference ranges defined in the present study for hematologic and biochemical blood values can be considered a useful tool for clinical pathologists, clinicians and researchers working in tortoise medicine and conservation. However, more interaction among laboratories working with non-conventional species would be useful to minimize inter-laboratory differences and to devise standard criteria and analysis protocols in protein electrophoresis.

Table 1. Biometric parameters.

	Parameter	Range (min-max)	Mean (±SE)	Median
Total	**Age** y *(n=34)*[a]	6-35	15(±1)	15
	Body mass g *(n=34)*[b]	466-2192	886(±69)	800
	CCL cm *(n=24)*[a]	18.0-2.0	22.0(±1.0)	22.0
Males	**Age** y *(n=14)*	6-25	16(±2)	15
	Body mass g *(n=14)*	466-2122	907(±131)	774
	CCL cm *(n=10)*	18.0-28.0	22(±0.5)	21.0
Females	**Age** y *(n=20)*	6-35	14(±2)	15
	Body mass g *(n=20)*	500-2192	876(±92)	815
	CCL cm *(n=14)*	18.0-28.0	21.9(±0.4)	22.6
Autumn	**Age** y *(n=20)*	9-35	18(±1)	16
	Body mass g *(n=20)*	466-2192	970(±108)	834
	CCL cm *(n=20)*	18.0-28.0	22(±1.0)	22
Summer	**Age** y *(n=14)*	6-20	10(±2)	8
	Body mass g *(n=14)*	500-1500	774(±111)	650
	CCL cm *(n=14)*	21.0-25.0	23.0(±1.0)	22

[a] Parameter normally distributed (P>0.05, D'Agostino-Pearson test)
[b] Parameter non-normally distributed (P<0.05, D'Agostino-Pearson test)

Table 2. Hematological parameters in *T. hermanni*.

Analyte		Range (min-max)	Mean(±SE)	Median
Hemoglobin[a] **(g dL^{-1})**	**Total** *(n=9)*	4.1-13.5	8.8(±0.6)	8.4
	Males *(n=4)*	9.8-13.5	12.1(±0.8)	13.0
	Females*** *(n=5)*	4.1-8.4	6.2(±0.9)	5.0
Hematocrit[a] **(%)**	**Total** *(n=14)*	11-40	23(±2)	23
	Males *(n=6)*	25-40	31(±2)	32
	Females * *(n=8)*	11-28	17(±2)	14

*Significance P<0.05 **Significance P≤0.01 ***Significance P≤0.001
[a] Analyte normally distributed (P>0.05, D'Agostino-Pearson test)

Table 3. Reference intervals for plasma biochemical parameters in specimens of *T. hermanni* (*n*=34); reference intervals were calculated as mean±2σ for normally distributed parameters and as minimum-maximum for non-normally distributed parameters.

	Reference interval	Mean (±SE)	Median	95% CI
Glucose [b] **(mg dL^{-1})**	37-177	73 (±5)	68	51-81
Urea [b] **(mg dL^{-1})**	1.78-16.67	5.59 (±0.67)	3.75	3.01-6.36
Creatinine [b] **(mg dL^{-1})**	0.08-0.35	0.17 (±0.01)	0.16	0.14-0.17
Uric Acid [b] **(mg dL^{-1})**	0.69-7.86	2.72 (±0.27)	2.62	2.12-3.26
Total proteins [a] **(g dL^{-1})**	1.9-6.1	4.0 (±0.2)	4.3	3.6-4.4
Albumin [a] **(g dL^{-1})**	0.6-2.5	1.6 (±0.1)	1.8	1.4-1.8
A/G ratio [b]	0.26-0.92	0.64 (±0.02)	0.66	0.61-0.69
AST [b] **(U L^{-1})**	18-628	134 (±28)	74	49-94
ALT [b] **(U L^{-1})**	1-16	4 (±0.6)	1	1-4
ALP [a] **(U L^{-1})**	91-645	368 (±24)	392	320-417
CK [b] **(U L^{1})**	19-1346	284 (±57)	168	136-227
LDH [b] **(U L^{-1})**	166-1556	476 (±43)	395	317-458
Ca [b] **(mg dL^{-1})**	10.7-28.1	14.7 (±0.7)	12.9	12.1-14.3
P [b] **(mg dL^{-1})**	1.8-8.7	3.4 (±0.2)	3.1	2.9-3.5
Na [a] **(mmol L^{-1})**	114-146	130 (±1)	130	127-133
K [a] **(mmol L^{-1})**	3.4-8.2	5.8 (±0.2)	5.6	5.4-6.2
Cl [a] **(mmol L^{-1})**	91-112	101(±1)	101	100-103

[a] Parameter normally distributed (P<0.05, D'Agostino-Pearson test)
[b] Parameter non-normally distributed (P>0.05, D'Agostino-Pearson test)

Table 4. Effect of sex and season on plasma biochemical parameters in *Testudo hermanni.* The minimum-maximum ranges are reported. Males, *n*=14; females *n*=20; summer (*n*=14).

Analyte		Range (min-max)	Mean (±SE)	Median
Glucose c* **(mg dL^{-1})**	**Males** (*n*=14)	47-108	77 (±5)	80
	Females (*n*=19)	37-177	70 (±8)	53
	Summer (*n*=19)	37-99	58 (±4)	50
	Autumn* ** (*n*=14)	53-177	92 (±0.10)	84
Urea d **(mg dL^{-1})**	**Males** (*n*=13)	2.78-14.20	6.47 (±0.99)	6.81
	Female** (*n*=20)	1.78-16.67	5.02 (±0.90)	3.24
	Summer (*n*=19)	2.94-16.67	7.07 (±1.04)	5.67
	Autumn * ** (*n*=14)	1.78-6.95	3.58 (±1.08)	2.96
Creatinine a,d **(mg dL^{-1})**	**Male** (*n*=14)	0.13-0.29	0.17 (±0.01)	0.16
	Females (*n*=20)	0.08-0.35	0.18 (±0.02)	0.15
	Summer (*n*=20)	0.08-0.35	0.18 (±0.02)	0.16
	Autumn (*n*=14)	0.08-0.23	0.16 (±0.02)	0.16
Uric acid c** **(mg dL^{-1})**	**Males** (*n*=13)	1.77-7.86	3.55(±0.43)	3.19
	Females** (*n*=19)	0.69-4.92	2.17(±0.30)	1.75
	Summer (*n*=19)	0.69-4.43	1.92(±0.25)	1.70
	Autumn * ** (*n*=14)	1.77-7.86	3.80(±0.36)	3.60
Total proteins a,d **(g dL^{-1})**	**Males** (*n*=14)	2.0-5.5	4.3(±0.2)	4.4
	Females (*n*=20)	1.9-5.5	3.8(±0.2)	4.2
	Summer (*n*=20)	1.9-5.5	3.7(±0.3)	4.1
	Autumn* (*n*=14)	3.6-5.5	4.5(±0.3)	4.4
Albumin a **(g dL^{-1})**	**Males** (*n*=13)	1.3-2.2	1.8(±0.1)	1.8
	Females (*n*=20)	0.5-2.0	1.5(±0.1)	1.8
	Summer (*n*=19)	0.5-2.2	1.4(±0.1)	1.6
	Autumn* (*n*=14)	1.3-2.2	1.8(±0.1)	1.8
A/G ratioe	**Males** (*n*=13)	0.51-0.90	0.67(±0.03)	0.66
	Females (*n*=20)	0.26-0.92	0.63(±0.04)	0.64
	Summer (*n*=19)	0.26-0.81	0.62(±0.03)	0.66
	Autumn (*n*=14)	0.51-0.92	0.68(±0.04)	0.67
AST d* **(U L^{-1})**	**Males** (*n*=14)	48-628	190(±50)	107
	Females ** (*n*=19)	18-628	88(±30)	58
	Spring (*n*=19)	18-295	79(±15)	58
	Autumn *	48-628	203(±35)	84
ALT b **(U L^{-1})**	**Males** (*n*=14)	1-16	5(±1.1)	4
	Females* (*n*=19)	1-10	2(±0.5)	1
	Summer (*n*=19)	1-7	3(±0.5)	1
	Autumn (*n*=14)	1-16	5(±1.0)	3
ALP **(U L^{-1})**	**Males** (*n*=14)	160-558	351(±33)	375
	Females (*n*=20)	90-695	380(±34)	397
	Summer (*n*=20)	90-499	350(±26)	401
	Autumn (*n*=14)	160-695	395(±40)	363
CK f **(U L^{-1})**	**Males** (*n*=13)	38-969	305(±72)	188
	Females (*n*=20)	19-1346	270(±84)	146
	Summer (*n*=20)	19-1346	339(±95)	167
	Autumn (*n*=14)	81-630	213(±97)	168
LDH d	**Males** (*n*=13)	195-1556	509(±97)	453

(U L^{-1})	**Females** (*n*=20)	166-590	360(±30)	354
	Summer (*n*=20)	166-1556	426(±69)	395
	Autumn	195-754	409(±36)	380
Ca [a,g]	**Males** (*n*=14)	11.3-19.2	12.9(±0.5)	12.2
(mg dL^{-1})	**Females*** (*n*=20)	10.7-28.2	15.9(±1.1)	14.2
	Summer (*n*=20)	10.7-21.7	14.0(±0.8)	12.3
	Autumn (*n*=14)	11.3-28.2	15.6(±1.3)	13.2
P [d**]	**Males** (*n*=14)	2.4-4.6	3.4(±0.2)	3.2
(mg dL^{-1})	**Females** (*n*=20)	1.8-8.7	3.5(±0.4)	3.2
	Summer (*n*=20)	1.8-4.3	2.8(±0.2)	2.9
	Autumn*** (*n*=14)	2.4-8.7	4.3(±0.5)	3.8
Na [a]	**Males** (*n*=14)	119-138	125(±1)	124
(mmol L^{-1})	**Females***** (*n*=20)	120-150	134(±2)	135
	Summer (*n*=20)	119-139	130(±1)	131
	Autumn (*n*=14)	120-150	130(±2)	127
K*	**Males** (*n*=14)	4.9-8.7	6.3(±0.4)	6.0
(mmol L^{-1})	**Females** (*n*=20)	3.5-7.4	5.5(±0.2)	5.5
	Summer (*n*=20)	3.5-8.7	5.8(±0.3)	5.8
	Autumn(*n*=14)	4.9-8.5	5.8(±0.3)	5.4
Cl*	**Males** (*n*=14)	94-104	99(±1)	99
(mmol L^{-1})	**Females*** (*n*=20)	94-113	103(±1)	104
	Summer (*n*=20)	94-113	104(±1)	104
	Autumn* (*n*=14)	94-109	99(±2)	98

The effect of sex and physiological status on parameters was evaluated by a two-way ANOVA. *Significance P<0.05 **Significance P≤0.01 ***Significance P≤0.001

[a]Analyte presenting *P*-value of the Levene's test for equality of variance less than 0.05, thus assumptions for two-way ANOVA were not met.

[b]Analyte presenting non-normal distribution after power transformation, thus non parametric Mann-Whitney test was performed.

[c]Box-Cox transformation. Non-normality after transformation was tested with the D'Agostino-Pearson test.

[d]Power transformation Lambda=-0.5. Non-normality after transformation was tested with the D'Agostino-Pearson test.

[e]Power transformation Lambda=2. Non-normality after transformation was tested with the D'Agostino-Pearson test.

[f]Power transformation Lambda=1. Non-normality after transformation was tested with the D'Agostino-Pearson test.

[g]Power transformation Lambda=-3. Non-normality after transformation was tested with the D'Agostino-Pearson test.

*Significant interactions between sex and season were identified for the following parameters: glucose, uric acid, AST, P, K and Cl. *Significance P<0.05 **Significance P≤0.01

Table 5. Reference intervals for plasma protein fractions for healthy *T. hermanni* ssp (N=24).

	Summer (n=6)		**Autumn (N=18)**	
	Range (min-max)	**Median (±SD)**	**Range (min-max)**	**Median (±SD)**
Albumin[b] **(g dL^{-1})**	1.4-2.4	1.92 (±0.39)	0.69-1.94	1.41 (±0.38)
α-Globulin (g dL-1)	0.9-1.35	1.05(±0.18)	0.56-1.62	1.29(±0.38)
β-Globulin (g dL^{-1})	0.2-1	0.3 (±0.38)	0.09-1.53	0.87 (±0.47)
γ–Globulin (g dL^{-1})	0.2-0.3	0.28 (±0.05)	0.04-0.55	0.2 (±0.14)
A/G ratio	0.54-0.86	0.75 (±0.14)	0.45-0.74	0.59 (±0.09)
Total proteins[a] **(g dL^{-1})**	4.06-5.52	4.38 (±0.54)	1.91-5.14	4.10 (±1.12)

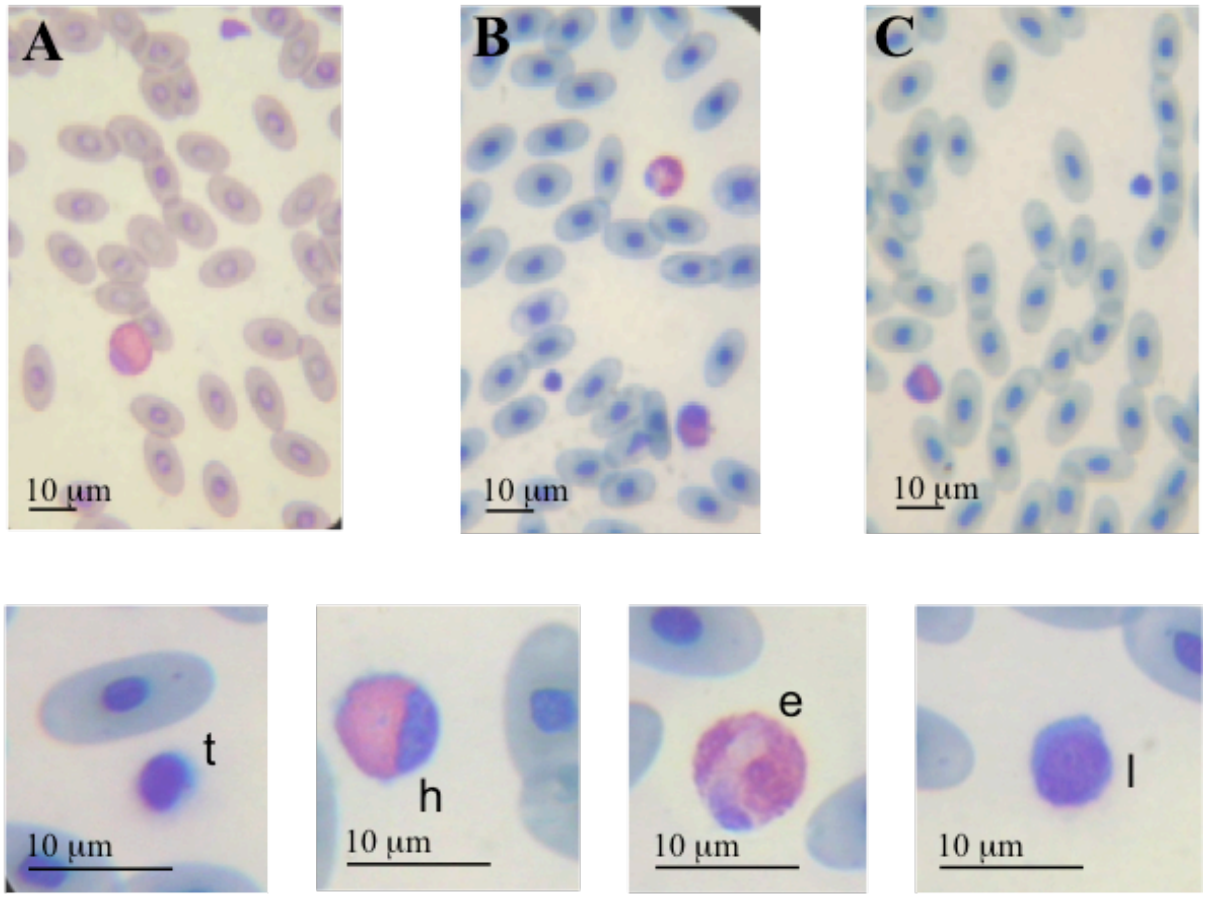

Figure 1. Representative peripheral blood smears from a tortoise (*Testudo hermanni)* stained with 3 different staining protocols, (A) Rapid stain, (B) May Grünwald-Giemsa stain, and (C) Wright stain. Most of the blood cells are mature nucleated RBC with rounded poles and a homogeneous cytoplasm. There are rare WBC.
T indicates thrombocyte (with a round, dense homogenous nucleus); h, heterophil (with peripheral oval nucleus and a granulated cytoplasm); e, eosinophil (with an eccentric purple nucleus and a cytoplasm with darker, redder and rounder granules); l, lymphocyte with pale blue basophilic cytoplasm and a compact nucleus. X 400 magnification. Bars = 10 µm.

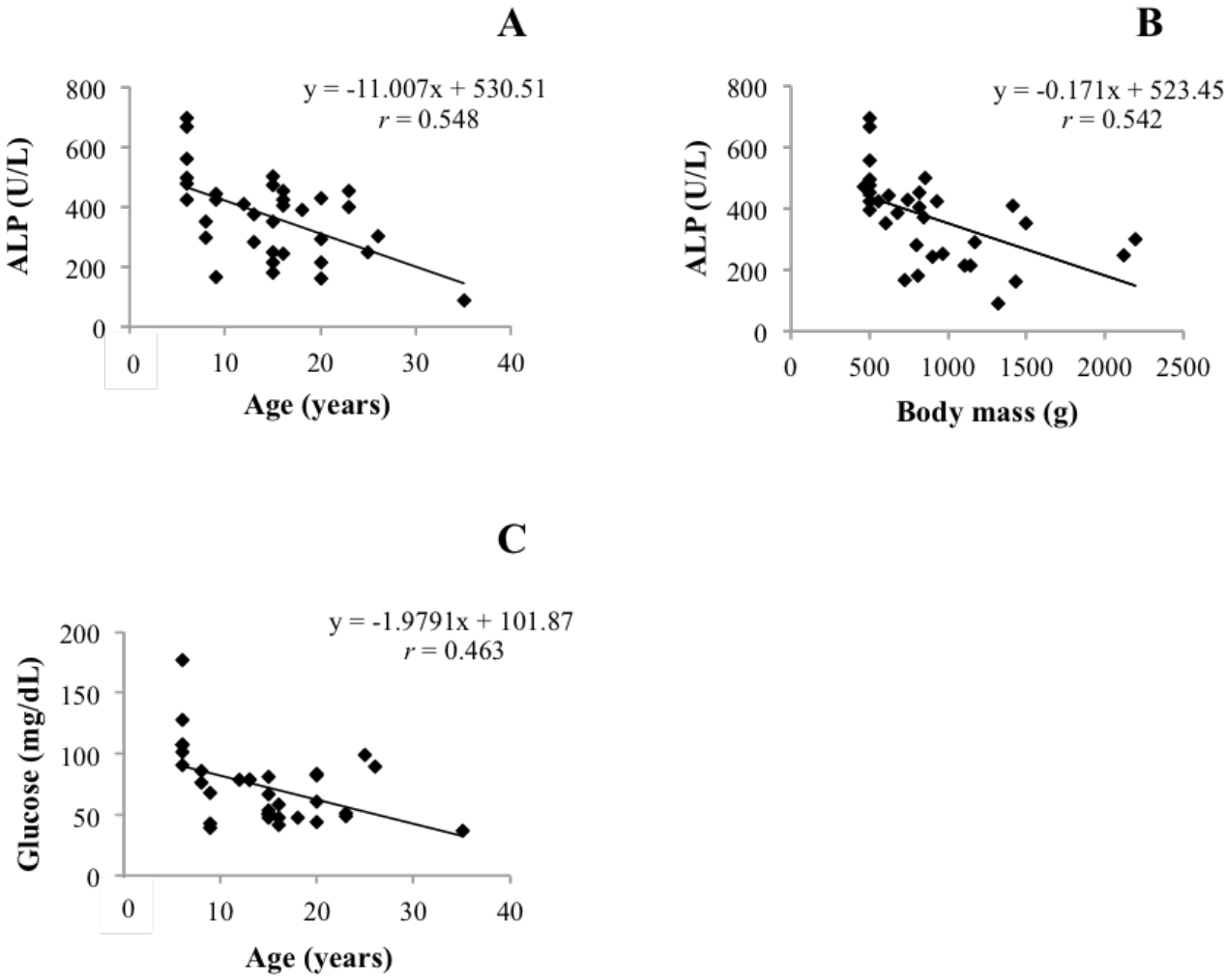

Figure 2. Linear regression between (A) ALP activity and age, (B) ALP activity and body weight, and (C) between glucose and age. The *p*-value related to *r* is significant ($p<.01$).

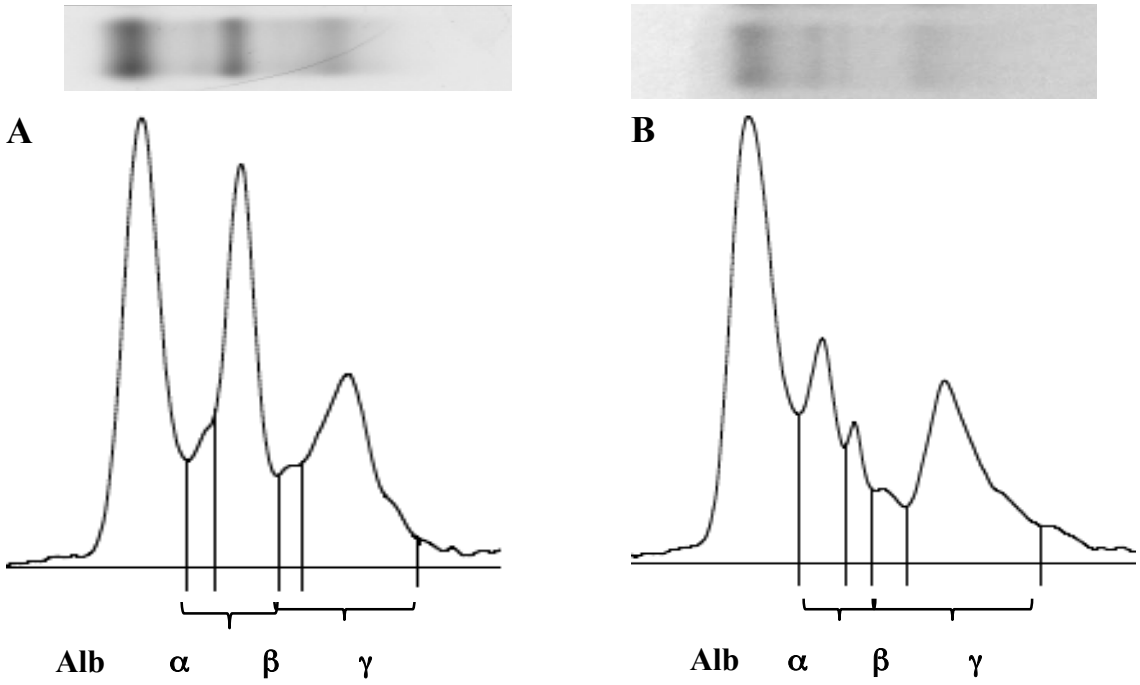

Figure 3. Representative protein electropherogram using agarose gel for protein electrophoresis of *Testudo hermanni. (A)* Sampling at the end of September), (B) sampling at beginning of July. Albumin (Alb), ✔, 🚲 and ■ fractions are indicated.

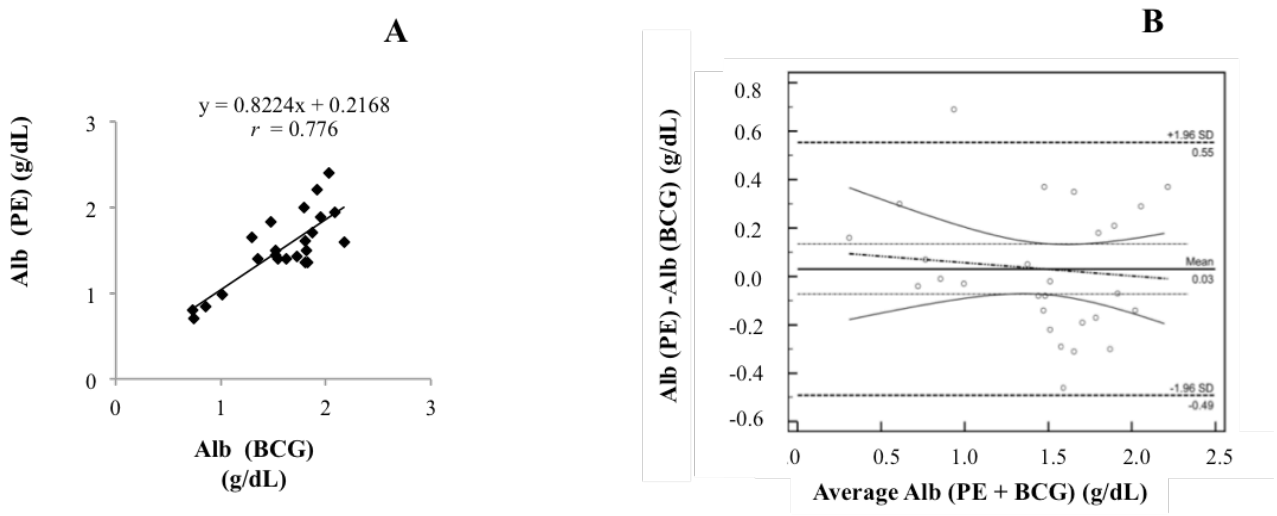

Figure 4. Plasma albumin concentration in *Testudo hermanni* was determined by bromocresol green (BCG, colorimetric method) and protein electrophoresis (PE, densitometric method). (A) There is a linear regression between albumin plasma concentrations determined by BCG and PE methods; (B) Bland-Altman plot of the difference between albumin concentration measured by BCG and PE. Limits of agreement (95% CI) were -0.49 (-0.67 to -0.31) to 0.55 (0.37 to 0.73).

Hematological values for adult eastern Hermann's tortoise (*Testudo hermanni boettgeri*) in seminatural conditions

Background

Among the 323 known species of tortoises 132 of them are listed in the International Union for Conservation of Nature (IUCN) red list as critically endangered, endangered or vulnerable. *T. hermanni* is considered "near threatened" by the IUCN (http://data.iucn.org/dbtw-wpd/edocs/RL-2009-001.pdf.) and it is listed in appendix II of the Convention on International Trade of Endangered Species and in Annex A of the European Council Regulation No 338/79 on the protection of species of wild fauna and flora by regulating their trade. This species occurs in Mediterranean Europe, from coastal northeastern Spain, through southeastern France, Mallorca (Spain), Menorca (Spain), Corsica (France), Sardinia (including Asinara Island) and Sicily (Italy), the coastal plains of peninsular Italy, coastal Croatia, coastal Bosnia-Herzegovina, coastal Montenegro, central and southern Serbia, inland to southwestern Romania, much of Bulgaria, Macedonia, nearly all of Albania, the Greek mainland plus islands from Corfu to Zakynthos, and European Turkey.[5] The subspecies *T. h. hermanni,* is present in northeastern Spain, southeastern France, the Balearic Islands (Spain), Corsica (France), and Sardinia (Italy). *T. h. boettgeri* is the eastern subspecies distributed in the Balkan region[5]. Despite the threatened status in its natural habitats, *T. hermanni* is very popular as a pet animal and it is kept in many gardens and households, especially in Europe. Hematological parameters of reptiles vary

depending to many factors, including physiology of the species and techniques employed for the analysis.

The purpose of this work is to standardize sampling and counting procedures in this species, to provide reference intervals for the main hematological variables and to describe agreement of a semiautomated blood cell counter with manual count.

Methods

All the 23 tortoises (15 males and 8 females) were adult reproductive animals belonging to the specie *T. hermanni*, subspecies *boettgeri*, originally coming from two different locations: location 1 with 13 tortoises (8 males and 5 females) and location 2 with 10 tortoises (7 males and 3 females). The 15 males had a carapace straight line length ranging from 16 to 23 cm and a weight ranging from 735g to 1726g. The 8 females had a carapace straight line length ranging from 19 to 32 cm and a weight comprised between 801g and 2300g. In both the locations the tortoises were kept outdoor in north-eastern Italy under seminatural conditions. They were housed in pens with some other *T. h. boettgeri* and allowed to spontaneously hibernate, usually from the beginning of October until March. Their usual diet was based on native wild grasses (among others: *Plantago lanceolata, P. major, Taraxacum officinalis, Rumex acetosella, Eruca stiva, Sonchus oleraceus, Malva sylvestris, Trifolium repens*) occasionally supplemented with cultivated leafy vegetables (romain lettuce, red radicchio, endive, Belgian and catalogna chicory). Sporadically other vegetables and fruit (*Opuntia ficus-indica,* tomatoes, cucumber, watermelon) were offered. At clinical examination all the tortoises showed a good body condition, all were alert and responsive and any of them presented sign of illness. All the animals were previously serologically tested negative for Chelonian Herpes Virus 1 and 2 as well as for virus X. Fecal

examinations revealed a mild presence of oxyurids in all the tortoises. The samples were taken in September when the tortoises were temporarily housed indoor to serve for a future pharmacokinetic study. The tortoise were housed indoor in a heated room (24°C from h 8.00 to h 20.00, and 21°C from h 20.00 to h 8.00) in individual plastic boxes (80x80 cm) on the floor. The substrate was a mixture of ¾ pine cortex finely minced[a] and ¼ coconut fiber[b] kept moist to maintain humidity in a range comprised between 53 and 66%. Over each block of four boxes was suspended (at 80cm from the floor) a 100W mercury vapor lamp[c] provided with a reflector switched on from h 10.00 to h 17.30. During this time the lamps provided a hot spot in a corner of each box where the temperature reached a peak of 31.7°C. Natural sunlight entered from the windows of the room. All the samples were obtained during the same day after that the tortoises acclimatized for one week. Blood (1.5-2ml) was collected from the jugular vein under manual restraint of the neck using a 2.5 ml syringe with a 25G needle without heparin. The blood was placed in 1.3 ml tubes with Lithium Heparin as anticoagulant[d], inverted several times and immediately refrigerated at 4°C. Two blood films (slide/coverslip technique) were also made from each sample without anticoagulant and air dried[9]. The samples were sent in a styrofoam box with ice to reach the laboratory in the next 24 hours. Blood analysis was performed 24-36 hours after sampling by one of the authors unaware of the origin of the samples nor the compositions of the groups. Each sample tube was taken individually from the fridge, gently inverted several times at room temperature for one minute for mixing and processed immediately. The total red blood cells (RBC) and white blood cells (WBC) counts were performed in a Neubauer hemocytometer[e] using a 1:200 dilution with the Natt and Herrick solution as described by Campbell[4]. When clumps of more than 15 WBC have been

detected, the sample was discarded and a new reading dilution was readily made from the same tube. The packed cell volume (PCV) was measured in heparinized microhematocrit tubes[f] in a centrifuge[g] spun for 5 minutes at 15000 RCF. Hemoglobin (HGB) was measured in a semiautomated blood analyzer[h] after lysis of the RBC and centrifugation of the lysate according to Campbell[4]; erythrocytic indexes were calculated using standard formulae according to the same author[4]. The differential count was done later on the blood films stained with a rapid staining kit[i] counting at light microscope 150/200 cells at 1000X magnification in oil; percentages and absolute numbers are then calculated using mathematical proportions. For comparison RBC, HCT and erythrocytic indexes were also measured using the same semiautomated analyzer used for HGB determination.

Normality of variables was assessed by use of Kolmogorov-Smirnov test. Median and non-parametric statistical tests were performed to account for non-normality. Tukey method for outlier detection was employed. Reference intervals were calculated with the robust method, with 90% CI of upper and lower limit[13]. To study possible variations between genders, a Wilcoxon signed rank test with continuity correction was used considering the following hematological parameters: RBC, WBC, PCV and the WBC differential counts as measured by manual count. To evaluate whether the automated analyzer agreed with the manual count, Bland-Altman analysis and Passing-Bablok regression were performed. Mean difference and limits of agreement (LoA) were provided[2]. Statistical analysis was performed using commercial softwares.[j,k]

Results

The results for the whole population are summarized in tables 1 and 2. Blood sampling was easily accomplished by the chosen technique. The size, the anatomy and the temperament

of the study animals allowed proper restrain and a precise visualization of the jugular vessels for an effective sampling.

The anticoagulant and the counting procedure chosen did not yielded any special problem and WBC clumps were rarely seen in the hemocytometer; the procedure to repeat the reading solution was requested in three cases only. The rapid staining method was adequate for the differential count and at 1000x magnification there were no problems in cell type identification.

Most variables were normally distributed, with the exception of MCV (manual and automatic), monocytes (percentage), lymphocytes (percentage), and basophils (absolute count) that were non-normally distributed. No outliers were detected. A significant statistical difference was found between genders in RBC counts ($p = 0.04$), in PCV ($p < 0.001$), and in HGB values ($p < 0.001$) (Figure 1). No differences were evident in the total WBC count ($p = 0.6$) nor in the WBC differential counts between genders.

Mean difference between manual and semiautomated RBC count was 0.07 $x10^6/\mu l$ (LoA: -0.03 to 0.17) (Figure 2). Mean difference between manual PCV and semiautomated HCT was 3.2% (LoA: 1.5 to 4.9). Mean difference between manual and semiautomated MCV was 15.4 f/l (LoA: -26.5 to 57.5). Mean difference between manual and semiautomated MCHC was 5.9 g/dL (LoA: -0.12 to 11.9). Mean difference between manual and semiautomated MCH was -11.4 p/g (LoA: -31.0 to 8.2). Basing on Passing-Bablok regression, at-least-constant difference (y-intercept ≠ 0) was present between manual and automatic count of RBC, PCV and MCV. An at-least-proportional difference (slope ≠ 1) was present between manual and automatic count of MCV and MCHC.

Discussion

The range values in our study of manually counted RBC and WBC and HGB are wider than those previously reported in the literature for *T.hermanni*[14,16,21]. Possible explanations for the differences found here, can be attributed to the size of sample population, to seasonal variability, to sub-specific differences, to the venipuncture site, to the anticoagulant used or to the counting method[6,12,14-16,20,21]. In fact, some sampling sites are more prone to lead to lymph contamination in chelonians, causing spurious low values of blood cell count. In our experience, lymph contamination is less likely to occur when sampling from the jugular vein.

The stress induced by keeping the animals indoor doesn't seem to have influenced WBC count since our values are not dissimilar to other reported in the literature. The mild parasite load of oxyurids found in some individuals is a typical finding in asymptomatic *Testudo* species.

Differences in RBC count between males and females are commonly reported in reptiles[10]. Among Chelonians, gender-based differences in RBC, HTC, and HGB have been previously reported for *Astrochelys radiata*[22], *Geochelone gigantea*[12], *Gopherus agassizii*[6], *Ocadia sinensis*[7], and *Terrapene carolina*[1]. Gender-based differences have not been found in *Chelonia mydas*[3]. Since erythrocytic indexes are correlated to RBC, HTC and HGB, the same gender-based differences are likely to be found in MCV, MCH and MCHC. The finding of a gender-based difference in selected parameters in *T. h. boettgeri* may have a clinical relevance and suggest the need for gender-specific reference intervals. Unfortunately our results are based on a small population. For this reason data are summarized in table 3 and

graphically represented in figure 2 as suggested by the ASVCP Quality Assurance and Laboratory Standards Committee (QALS) Guidelines[11].

In our study we employed an impedance blood cells counter that yielded comparable results with the manual count for RBC, HTC and erythrocytic indexes. The automated count seems to overestimate the RBC count and to underestimate the HTC determination in a way not likely to affect clinical judgment. A possible explanation is that some small WBC or thrombocytes are counted as RBC and that in the PCV are usually comprised other cells (WBC, thrombocytes) other than RBC only. Even though some authors recommend the use of an automated or semiautomated counting machines for reptile RBC and HTC determinations,[17-19] according to our findings the use of such equipments should be previously validated in order to obtain reliable results.

Clinical evaluation of reptilian species is always challenging and, though more extensive studies are needed, clinical pathology can aid in chelonian's health monitoring[23]. Validation of automated and semiautomated blood counters for reptilian blood count may make clinical pathology more feasible.

SOURCES AND MANIFACTURERS

a Repti Bark® no. 8, Zoo Med Laboratories Inc., San Luis Obispo, CA, USA

b Coco Earth®, Mondialfauna, Monopoli, Italy

c Powersun®, Zoo Med Laboratories Inc., San Luis Obispo, CA, USA

d Micro Tube, Sarstedt®, Numbrecht, Germany

e Spencer Bright Line®, American Optical Co., Buffalo, NY, USA

f NRIS® Vitrex Medical A/S, Herlev, Denmark

g Mod. 4223 ALC International S.r.l., Cologno Monzese, Italy

h Mod.S-8300 Semar S.r.l., Sesto Fiorentino, Italy

i Hemacolor®, Merck, Darmstadt, Germany

j SPSS 22.0, IBM,

k MedCalc

Table 1. Mean values and reference intervals for hematological parameters in *Testudo hermanni boettgeri* (no. 23) obtained by manual method. Standard deviation (SD) is reported within brackets.

	Median (SD)	**Range**	**Reference interval**	**90% CI lower limit**	**90% CI upper limit**
Weight (g)	1117 (378)	735 – 2300	---	---	---
RBC (x10^6/µl)	0.8 (0.15)	0.42 – 1.02	0.43 – 1.05	0.34 to 0.53	0.96 to 1.15
PCV (%)	24 (5.9)	11 – 32	10.9 – 34.3	7.3 to 14.52	30.77 to 38.0
HGB (g/dl)	6.8 (2.5)	2.6 -11.5	1.7 – 12.7	0.3 to 3.3	11.0 to 14.1
MCV (fl)*	310 (34.8)	193 – 350	231.6 – 383.7	206.5 to 266.2	348.4 to 409.4
MCH (pg)	100 (22.2)	54.8 – 125.8	47.0 – 144.3	35.1 to 63.3	132.4 to 158.3
MCHC (g/dl)	32.5 (6.5)	21.6 – 43.3	17.4 – 45.7	12.8 to 21.8	41.6 to 49.4
WBC (x10^3/µl)	9.4 (2.9)	4.1 – 14.0	2.4 – 15.3	0.9 to 4.4	13.4 to 16.7

*Variable did not follow a normal distribution

Table 2. Normal mean values and ranges for differential WBC counts in *Testudo hermanni boettgeri* (no. 23). Standard deviation (SD) is reported within brackets. The robust method was employed to provide reference intervals.

	Median **%**	**Range** **%**	**Reference interval** **%**	**Median x10³ cells/µl**	**Range x10³ cells/µl**	**Reference interval x10³ cells/µl**
Heterophils	26.5(12.5)	11.8-50.9	1.0 – 55.5	2,31	0,79 – 4,74	0 – 4,98
Eosinophils	12.5 (5.6)	5.4-23.4	0.9 – 25.1	0,97	0,36 – 2,4	0 – 2,52
Basophils*	1.2 (0.98)	0-3.8	0 – 3.1	0,085	0 – 0,19	0 – 0,25
Lymphocytes*	54.2 (11.5)	29.3-66.5	24.7 – 76.8	4,44	1,44 – 8,49	0,24 – 8,55
Monocytes*	5.6 (2.82)	2.8-13.7	0 – 11.6	0,48	0,18 – 1,34	0 – 1,22

*Variable did not follow a normal distribution

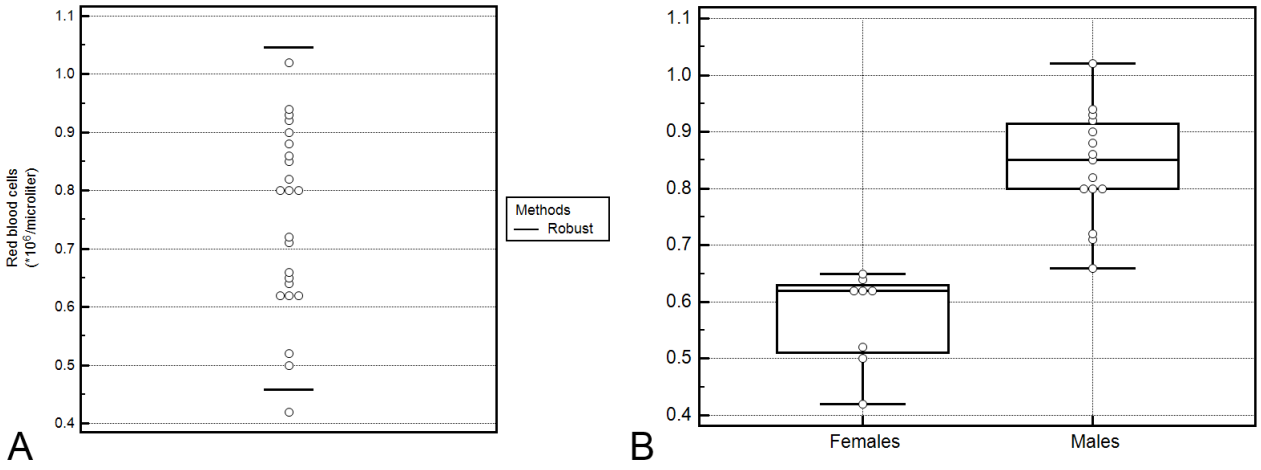

Figure 1. Combined box-and-whisker and dot plots showing the differences in RBC, PCV and RBC between female and male Hermann's tortoises. The boxes represent the values from the first to the third quartile (25th to 75th percentile). The horizontal line in each box represents the median. The whiskers include values of 1.5 times the interquartile range.

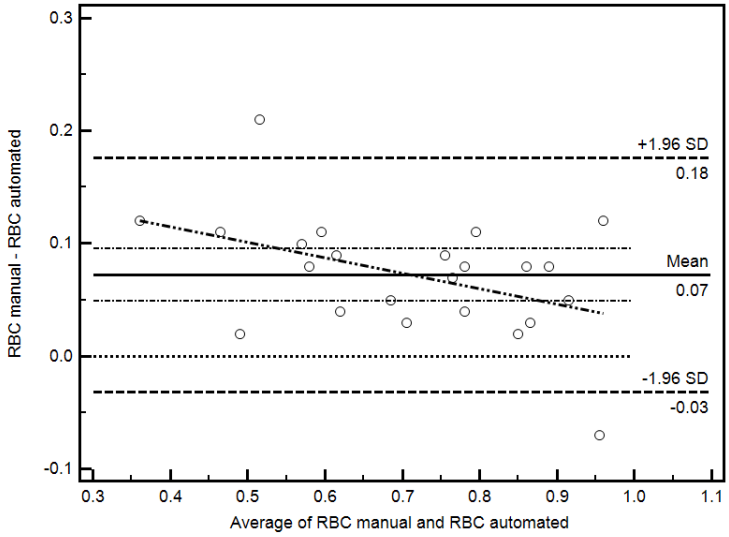

Figure 2. Representative Bland-Altman plot, showing agreement between automated and manual count of red blood cells in blood samples of Hermann's tortoises. Mean difference (solid line) with its 95% CI (dotted and dashed lines) and two SD limits of agreement (dashed lines) are provided. The slope regression line is depicted.

Point-of-care blood gases, electrolytes, chemistries, hemoglobin and hematocrit measurement in venous samples from pet rabbits

Background

Rabbits are among the most common pets in the households of Europe and United States[1,2,3] and represent a significant part of most small animal practices' patients in Great Britain.[4]

Nevertheless, reference blood gas analysis values and their variations during the course of diseases have never been properly reported in pet rabbits. The absence of previous studies focusing on blood gas in rabbits are possibly due to the cost related with an in-house reference analyzer (RA) and the difficulty to obtain adequate volume samples in rabbits for shipping them to a clinical laboratory.

A point-of-care portable clinical analyzer (PCA) could overwhelm those limitations. PCAs have become fairly commonplace in emergency and critical care settings in both human and veterinary hospitals:[5] the PCA decreases sample processing time,[6,7] thus possibly decreasing the effect of delay in estimation of blood gases.[8] Although a minimal training is mandatory to adhere to standard operating procedures, previous studies have found that the operator independence of PCAs renders them easy to use even by personnel untrained in laboratory techniques.[5,9]

Recently, a cartridge that combines analysis of blood gas (pH, PCO_2, HCO_3^-, tCO_2, BEecf, AnGap) and hematocrit, hemoglobin, selected electrolytes (Na^+, Cl^-, K^+) and chemistries

(BUN, Glu) measurement has been produced.[10] The volume of blood required for the analysis is of 65 µl, a volume that can be safely harvested from almost any rabbit. Indeed, a similar cartridge seems a promising diagnostic tool to assist rabbits' clinicians in managing emergencies and in monitoring response to critical care and fluid therapy.[11,12]

One of the major concerns about the use of PCAs is the validity of measurements by point-of-care devices compared to primary, bench-top instruments used in the clinical laboratory.[9,13-16] An adequate agreement between instruments ensures transferability and consistent interpretation of test results.[15] For this purpose method comparison studies testing analytical performances of PCAs have been performed in several species, including dogs,[5,17-19] cats,[5] horses,[17,20] sheep,[20] cattle,[20] mice,[21] northern elephant seals (*Mirounga angustirostris*),[22] viscachas (*Lagostomus maximus*),[23] white-tailed deers (*Odocoileus virginianus*)[24] and chickens.[25] Method comparison studies compare type and magnitude of systematic errors (i.e. constant and proportional bias) between an instrument and a "true value".[26] Unfortunately in veterinary clinical pathology, certified species-specific reference material or reference methods are seldom available, and routinely applied methods are frequently used as the method to which a new method is compared.[26] To the author's knowledge, no data evaluating the performance of a PCA in rabbits are currently available.

The purpose of the present study was to compare the performance of a PCA, using a specific cartridge, to a reference bench-top analyzer for blood gas analysis and selected electrolyte, selected chemistries, Hct and Hb measurement in venous samples collected from pet rabbits. The specific hypothesis was that the PCA and the RA, as in previous comparative researches,[17,20,25] provided interchangeable results.

Materials and Methods

Study design and animals- An observational prospective cross-sectional study was planned. The primary outcome was the agreement between the PCA and the RA.

Pet rabbits (*n* = 30) undergoing blood sampling in the XXX for unrelated diagnostic reasons were planned to be included in the present study. In order to cover a wide range of analyte concentrations were included both *healthy* and *diseased* rabbits. The *healthy* rabbits were rabbits presented to the clinic for the summer pension service or were rabbits undergoing elective surgery (castration/spaying) and underwent blood analysis for a routine preliminary assessment of their clinical status. If those rabbits did not present abnormalities upon physical examination and other clinical pathology results, they were considered *healthy* (*n* = 13). All the other rabbits included in the present study, in which blood collection was preliminary to semi-elective or urgent surgeries or necessary to confirm the diagnosis of a pathological status, were considered *diseased*. Diseased rabbits (*n* = 17) underwent blood sampling for several primary purposes, including hematology and/or biochemistry and/or serologic testing.

The predefined exclusion criterion was that the rabbit did not need blood collection. All the rabbits presented to the clinic in the study period were included in the analysis until the predefined sample size was achieved.

The study was performed in compliance with the directive 2010/63/EU of the European parliament and of the European council. The owners gave written informed consent to the inclusion of samples of their animals in the study.

Instruments used-A PCA[a] with disposable cartridges[b] and a RA[c] were used side-by-side.

For quality control of the output results, PCA cartridges were stored in a refrigerator (3° to 8°C) and cartridges were only used prior to the expiration date provided by the manufacturer. The temperature of each cartridge was allowed to equilibrate with the ambient temperature of the room (20°C) before the cartridge pouch was opened for 5 minutes, although a study proved evidence that results of analyses were not substantially affected by use of cold, rather than warm, test cartridges.[17] The PCA aqueous control solutions[d] were used to assess 1 cartridge out of each cartridge batch as recommended by the manufacturer. The ampule containing the control solution was equilibrated for 4 hours at room temperature. Plain syringes were used to transfer the aqueous control from the ampule to the cartridge as recommended.[e] Calibration verification of the PCA was not performed as it is explicitly not recommended by the manufacturer.[e]

The RA was maintained according to manufacturer's recommendations: the analyzer used an internal 2-point calibration to measure pH, PCO_2, Na^+, K^+, Cl^-, BUN, glucose and Hct sensor slopes and to verify sensor performance.[f] The 2-point calibration was automatically performed at 2 hour intervals. Hb was manually calibrated with 2 external calibrators once daily. The RA was in Mode A so all analytes were checked with a 1-point calibration every each sample analysis. The 1-point calibration is performed by exposing the electrodes to a known standard and comparing the new value to the value obtained during the 2-point calibration.

All samples were handled by a licensed veterinarian trained to use the instruments (ND).

Analytes measured- Analytic variables measured from both the PCA and RA during analysis included pH, PCO_2, Na^+, K^+, Cl^-, BUN, glucose and Hct. Hemoglobin was measured by the RA and calculated by the PCA.

The PCA uses direct (undiluted) electrochemical methods.[e] pH and PCO_2 are measured by direct potentiometry. Na^+, K^+, Cl^- are measured by ion-selective electrode potentiometry. In the calculation of results for pH, PCO_2, Na^+, K^+, Cl^- concentration is related to potential through the Nernst equation. Glucose is measured amperometrically. Oxidation of glucose, catalyzed by the enzyme glucose oxidase, produces hydrogen peroxide. The liberated hydrogen peroxide is oxidized at the electrode to produce a current, which is proportional to the sample glucose concentration. Urea is hydrolyzed to ammonium ions in a reaction catalyzed by the enzyme urease. The ammonium ions are measured potentiometrically by an ion-selective electrode. In the calculation of results for urea, concentration is related to potential through the Nernst Equation. Hematocrit is determined conductometrically. The measured conductivity, after correction for electrolyte concentration, is inversely related to the hematocrit. The PCA provides a calculated hemoglobin result that assumes a normal MCHC in humans: hemoglobin (g/dL) = hematocrit (% PCV) x 0.34.

The RA used in this study uses direct (undiluted) electrochemical methods.[f] pH is measured using a hydrogen ion selective glass membrane. PCO_2 is measured with a modified pH sensor composed by a gas permeable membrane mounted on a combined measuring/reference electrode. Na^+, K^+, Cl^- are measured by ion-selective electrode potentiometry. Glucose is measured basing on the level of hydrogen peroxide produced during the enzymatic reaction among glucose and oxygen in presence of the glucose oxidase enzyme. The hydrogen peroxide is oxidized by means of a constant potential of 0.7 volt. The current produced is proportional to the sample glucose concentration. Urea is hydrolyzed to ammonium ions in a reaction catalyzed by the enzyme urease. The ammonium ions are measured potentiometrically by an ion-selective electrode. In the

calculation of results for urea, concentration is related to potential through the Nernst Equation. The hematocrit is determined by measuring electrical resistance of the blood sample and corrected for the concentration of the sodium ion. Hemoglobin is measured by combining a conductivity measurement and a photometric measurement.

Blood sample type and handling- Two experienced operators performed venipuncture and analysis of samples. The rabbits were restrained in lateral recumbency and venipuncture site over the left saphenous vein was cleaned with a 70% alcohol swab. Venipuncture and the subsequent analyses were performed by the same operator in order to minimize procedure time due to sample handling. In the meanwhile the second operator prevented bleeding and hematoma formation applying gentle digital pressure on the venipuncture site.

Blood samples were collected with a 23-Gauge needle into 1-mL plastic syringes containing 25 international units of dry balanced heparin[g] and were immediately analyzed by use of the PCA. The whole blood sample was introduced into the cartridge via the heparinized syringe, and a portion of the same blood sample was analyzed by use of the conventional RA immediately thereafter. To ensure the minimum delay in blood analysis the analyzers were located immediately adjacent to each other and immediately adjacent to the table where blood collection was performed.

Sample collection and handling were performed according to the manufacturers' guidelines to avoid pre-analytical errors. The most important of these was the prevention of contamination of syringe sample with air, and the immediate and proper mixing of blood syringes before introduction of each sample. Appropriate measures were taken to avoid touching the contact pads, which could have interfered with data transmission, or exerting

pressure over the center of the cartridge, which could have caused premature release of the calibrating solution.

Sample size estimation- No previous report of similar comparisons in rabbits are present in literature. Therefore, a pilot analysis of data was planned after the first 10 samples. The purpose of the analysis was at least to calculate the sample size necessary to obtain correlation coefficients presenting alpha error < 0.01 and beta error < 0.01.[27] The variables which were accounted in this pilot analysis were pH, PCO_2, HCO_3^- and BUN. The lowest coefficient of correlation was 0.947 (r range: 0.947 - 0.983) for the PCO_2. Thus, the minimum sample size was calculated to be of 11 individuals. Sampling was planned on thirty individuals in order to include healthy and diseased animals.

Statistical analysis- Summary statistics were compiled for rabbits' body temperature and measured variables. Data were analyzed for non-normality by means of the D'Agostino-Pearson test.

Missing data-In case of system failures of single or multiple values, results of the remaining variables of those cartridges were included in the statistical analysis.

Method comparison-Method comparison was performed with Bland-Altman bias plots and Passing-Bablok regression analysis.

The Bland-Altman analysis estimates how much two methods differ in the quantitative measurement and therefore aids in making the decision if one method can be substituted for another. Bland-Altman plots were constructed for each variable by plotting the difference between the measurements on the vertical axis against the respective mean of those measurements on the horizontal axis. The limits of agreement were determined by ±1.96 SD centered on the mean difference. In addition, the association between the

difference and the magnitude of the measurements was examined by standard regression analysis of the difference between the two methods on their average. Briefly, an increase in bias with magnitude (proportional bias) is shown by the significant slope of the regression line. If proportional bias are present, the analysis described above will give limits of agreement which will include most differences, but they will be wider apart than necessary for small values, and rather narrower than they should be for large values.[28,29] Under these circumstances, logarithmic (log) transformation of both measurements before analysis will enable the standard approach to be used. The limits of agreement derived from log transformed data can be back-transformed to give limits for the ratio of the actual measurements. [29] The log transformation is the only transformation giving back-transformed differences which are easy to interpret.[28]

Passing-Bablok regression is a nonparametric model, which allows measurement error (imprecision) in both methods, does not require the measurement error to be normally distributed, and is insensitive to outliers. By first, linearity of data was examined by visual inspection of the scatter plot[30] and the coefficient of correlation *r* was calculated as a prerequisite of the Passing-Bablok regression.[31] Secondly, the cusum linearity test was performed.[30] The null hypothesis tested by means of the cusum test is a random distribution of residuals around the fitted regression line.[32] Lastly, the regression equation was calculated. Constant bias was considered present if the 95% confidence interval for the y-intercept did not include the value 0. Similarly, proportional bias was considered present if the 95% confidence interval for the slope did not include the value 1.[30]

Data were analyzed using commercial software.[h] Values are reported as mean ± SD (range), unless otherwise specified. Two tailed *P* values of less than 0.05 were considered significant.

Results

Mean body temperature of the rabbits was 38.8 ± 0.7 °C (37.1 - 40.2 °C). Rabbits ranged from 1 to 10.5 years of age (median age, 3.0 years). There were 15 males and 15 females. Tentative diagnoses for diseased rabbits included dental disease (n = 4), encephalitozoonosis (2), gastrointestinal stasis of unknown cause (2), uterine tumor (1), coccidiosis (1), corneal perforation (1), epiphora secondary to dental disease (1), grade III pododermatitis (1), intestinal obstruction (1), liver lobe torsion (1), open pneumothorax (1), syphilis (1).

All samples were analyzed in both analyzers within 20 seconds after blood collection. Analysis of pH, PCO_2, Hb, Na^+, K^+, BUN, glucose and Hct provided results in all 30 cartridges (100%), whereas system failures were noted in 3 cartridges (10%) for the Cl^- and consequently for the calculated Anion Gap. The system failures were observed at Cl^- values (as determined by the RA) of 108.5, 103.5 and 102 mmol/L. The ranges over which the variables were evaluated via the RA and PCA are presented in Table 1 and 2, respectively.

The difference between PCA measurements and RA measurements of each variable are displayed graphically as Bland-Altman agreement plots in Figure 1. Mean difference ± SD (95% CI) between RA and PCA were for pH 0.007 ± 0.023 (-0.001 to 0.016), PCO_2 -8.87 ± 2.42 (-9.78 to -7.97), Hb 0.06 ± 0.92 (-0.28 to 0.40), Na^+ 0.19 ± 2.85 (-0.87 to 1.26), K^+ 0.015 ± 0.55 (-0.19 to 0.22), Cl^- 1.17 ± 1.85 (0.44 to 1.91), BUN 0.43 ± 3.34 (-0.81 to 1.68), glucose

13.46 ± 13.13 (8.56 to 18.37) and Hct 1.8 ± 2.59 (0.83 to 2.76). Limits of agreement for each variable are reported in Table 3.

In the plots of PCO_2, Cl^-, glucose and Hct the line of equality was not included in the 95% CI of mean of differences (Figure 1), indicating a constant difference.

The slope of the regression fit of the difference versus average was significant in the Bland-Altman plots of pH (-3.16 ± 0.61; $P < 0.001$), PCO_2 (-2.21 ± 0.40; $P < 0.001$), Hb (-1.50 ± 0.17; $P < 0.001$), K^+ (-0.95 ± 0.19; $P < 0.001$), Cl^- (-1.04 ± 0.33; $P = 0.005$), glucose (2.09 ± 0.42; $P < 0.001$) and Hct (-1.51 ± 0.21; $P < 0.001$). Thus a proportional bias was present between the PCA and the RA for those values.

Results of the Passing-Bablok regression are listed in Table 3. This analysis demonstrated an at-least-proportional difference (slope ≠ 1) between pH, PCO_2, Hb, Cl^-, glucose and Hct (Figure 2). An at-least-constant difference (y-intercept ≠ 0) was present between pH, Hb, K^+, Cl^-, BUN, glucose and Hct. The cusum test for linearity confirmed that all compared variables fitted a linear model.

Discussion

In the present study, agreement of blood gas analysis, selected electrolyte values, selected chemistries values, Hct and Hb between the PCA and the RA was poor, the only exception made by pH.

The inadequate agreement found in the present study it is not totally unexpected. In the veterinary literature a poor agreement between PCAs and RAs for certain assays has already been described.[16,21] Although the need to evaluate point-of-care units in exotic species has already been pointed out,[11] to the best of the authors' knowledge the present is

the first report employing a point-of-care analyzer in the rabbit. In addition, no previous reports of blood gas analysis results in pet rabbits were found in the contemporary literature.

When comparing two methods the choice of acceptability limits becomes critical. As Jay[33] suggests: "*Limits of acceptability are contentious and there are no hard and fast rules - at least ones that you can get two clinical chemists to agree on*." Thus, acceptability of results is subjective and depends on the analytical performance needed by the operator. Nevertheless, agreement is an objective parameter that can be easily measured.[28] Limits of agreement presented for each variable in this study permit clinicians and researchers to evaluate if the PCA is reliable enough for their scopes.

In the present study the pH presented an acceptable agreement between instruments. Although pH measured by the PCA presented proportional and systematic differences with results obtained by the RA, such analytical fluctuations lead to clinical insignificant differences being the limits of agreement between -0.05 and 0.07. The interpretation of the clinical status of the rabbit would be adequate even at extreme pH values, ie rabbit 16 (RA: 7.16; PCA: 7.11) and rabbit 8 (RA: 7.59; PCA: 7.65).

The PCO_2 analysis presented proportional bias, systematic bias and wide limits of agreement (-13.63 to -4.12 mmHg). Although reference ranges for venous PCO_2 in pet rabbits are lacking, the bias influenced clinical interpretation of PCO_2 each at high and low pressures. Rabbits presenting PCO_2 values determined by the RA between 43.1 and 34.8 mmHg (ie, values in the normal range for unanesthetized dogs and cats),[34] yielded results higher than 45 mmHg by the PCA, which is considered outside the normal ranges for venous sample of dogs and cats. By contrast, most RA values below 30 mmHg, indicating

decreased PCO_2 in dogs and cats,[34] resulted in PCA values inside the normal range for dogs and cats.

Although in rabbits both hyponatremia and hypernatremia have been experimentally induced and corrected,[35,36] sodium disorders are rarely observed in pet rabbits' clinical practice. In the present study the agreement was measured on values of Na^+ included in the normal range reported for the rabbit (ie, 131-155 mmol/L).[37] Although no constant or proportional bias were found, the agreement between instruments was poor (-5.40 to 5.79 mmol/L). This result is not unexpected considering that in venous blood samples from cattle the limits of agreement for Na^+ of the PCA compared to a RA was found to be around -8 to 1 mmol/L.[20] Nevertheless, the poor agreement would have never changed clinical decision basing on the current reference range for Na^+ in rabbits.

Potassium concentration in rabbits is evaluated for several purposes, among others it is suggested to relate to kidney disease.[38] Nevertheless, true hyperkalemia seems to be a rare condition in pet rabbits.[38] One case report described a pet rabbit presenting generalized weakness, tetraparesis, ventroflexion of the neck, and decreased postural reactions, proprioception and spinal reflexes in the four limb, concurrently to hyperkalemia.[i] In that case correction of potassium imbalance led to resolution of the symptoms. Rabbits in which acute hyperkalemia was experimentally induced developed weakness, increase in respiration, tremors and occasionally urination. Untreated animals developed severe convulsions and ventricular fibrillation.[39] Therefore potassium disorders in rabbits, as in other animals, need a prompt diagnosis and adequate treatment.

In fact, all the results obtained by the RA were inside the upper reference range limit for healthy rabbits (6.9 mmol/L).[37] Differently, the PCA gave two samples, rabbit 16 (PCA: 7.9

mmol/L; RA: 5.77 mmol/L) and rabbit 22 (PCA: 7.1 mmol/L; RA: 5.42 mmol/L), exceeding the upper limit of the reference range. Therefore the use of the PCA would have overdiagnosed hyperkalemia. Hypokalemia was not diagnosed in any rabbit of the present study, with the exception of rabbit 13 in which K^+ was below the lower reference range limit (3.6 mmol/L)[37] by both analyzers. According to the Bland-Altman procedure the difference between the PCA and the RA for K^+ concentrations was proportional, i.e. varied with the magnitude of measurement. According with the Passing-Bablok analysis the potassium assay did not show a proportional difference. This analytical difference may be secondary to the presence of two extreme values for which the Passing-Bablok non-parametric procedure is not affected.

The Cl^- was slightly overestimated by the PCA (mean difference: 1.17; 95% CI: 0.44-1.91). The overestimation led to potential clinical misinterpretation of Cl^- values at the upper limit of the normal range. Therefore rabbit 23 (RA: 111 mmol/L; PCA: 113 mmol/L) and rabbit 7 (RA: 111.6 mmol/L; PCA: 114 mmol/L) exceeded the reference range (92-112 mmol/L)[37] with the PCA, but were inside the range according to the RA.

Strong evidence of the clinical value of BUN measurement during kidney or liver disorders in pet rabbits is still lacking. Nevertheless, BUN is often measured in clinical practice as an aid in the evaluation of renal and hepatic function.[38] In the present study no proportional or systematic bias were found with the Bland-Altman plot of the BUN, even if the limits of agreement were wide (-6.13 to 6.99 mg/dL). Most BUN values obtained in the present study were inside the normal range for rabbits (ie, between 13-29 mg/dL).[37] The two instruments agreed in detecting two rabbits outside the upper reference range limits, although an important difference was present in one of those (PCA: 34 mg/dL; RA: 41

mg/dL). The PCA found five rabbits to present BUN values outside the lower reference range limits. Of those five rabbits only two rabbits were below the BUN limits according the results of the RA.

Similarly, glucose was mainly underestimated by the PCA. In some cases reliance on results of the PCA would have resulted in alteration of clinical decisions: there is some recent evidence[41] that extremely high glucose values (ie, around 390 mg/dL) in pet rabbits presenting gut stasis could be indicative of intestinal obstruction, thus necessitating surgical intervention. In such cases glucose underestimation can influence decision-making and patient outcome. Again, hypoglycemia could be overdiagnosed, leading to erroneous clinical suspicion and stressful, time-consuming tests, e.g. pancreas ultrasound for suspected insulin-secreting tumors or pancreatitis.[40] Nevertheless, in the present study minimum glucose value was of 117 mg/dL, so response of PCA to low glucose values in blood samples can only be inferred. It should be noticed that standards in human medicine recommend that the accuracy of a PCA be within 15% of the reference value.[41]

Evaluation of hematologic parameter (Hct and Hb) in pet rabbits provides useful clinical information in rabbits,[42] as in other species. In our study only one rabbit presented anemia (ie, Hb concentration <10.0 g/dL)[37] according to the RA, whereas three rabbits would be diagnosed to have anemia according to PCA results. Similarly, the Hct was mainly underestimated by the PCA, provoking overdiagnosis of low hematocrit (ie, Hct <33%):[37] low Hct would be diagnosed in eight rabbits attending PCA results, but only one rabbit was confirmed to have a Hct inferior than 33% via the RA.

In the present study rabbits suffering several disease conditions were sampled. Some previous studies comparing the performances of a PCA with a RA focused on healthy or

presumed healthy individuals.[20,25] Others were centered on exercising individuals[43,44] or on animals in undetermined health conditions.[19] Ideally, the specimens to be tested should cover a wide range of analyte concentrations in order to make valid use of linear regression analysis from which the best estimate of constant and proportional errors can be obtained.[45] Therefore inclusion of diseased animals permitted a wider range of the variables to be evaluated.

The statistical analysis in research papers reporting comparisons of methods of measuring the same quantity is often inappropriate, with the correlation coefficient *r* often used in studies of method comparison.[46] The deficiencies of *r* in method comparison have been thoroughly described in medical literature.[46,47] Although correlation coefficient is proved to not respond satisfactorily to the agreement question,[29,48] it is still used in instrument comparison.[49] In the present study the authors calculated the Bland-Altman limits of agreement using the mean of the measurements by the two methods as the best estimate.[29] A regression analysis was also used to interpret the data, because it is widely known, thus its interpretation is often more accessible. In previous comparative studies between point-of-care analyzers and bench-top analyzers[20,25,50] Deming regression was used to assess the degree of agreement. Whereas the ordinary linear regression method assumes that only the Y measurements are associated with random measurement errors, the Deming regression takes measurement errors for both methods into accounts.[51] The non-parametric procedure of Passing-Bablok was selected over the Deming regression because shows reliable results in all of most situations.[31] Because of the robustness of this procedure the problem of including or excluding extreme data points (outliers) does not arise.[31] It should be considered that outliers in comparison method studies are not

necessarily gross measurement errors; they may be caused by different properties of the methods with respect to specificity or susceptibility to interferences. Therefore they should not be removed from the calculation without experimental reason.[31]

Conclusion

In conclusion, the present study provides sufficient evidence that the PCA is acceptable for clinical pH measurement in venous blood samples of pet rabbits. Nevertheless, considering the bias between the PCA and the RA for the other variables, instrument-specific reference intervals are needed and a single analyzer should be used if the intent is to evaluate serial results in a patient over time. Furthermore, researchers aware of the limits of agreement for each variable of the cartridge can decide if it is reliable enough for their scopes.

Footnotes

a. i-STAT 1 Analyzer, SN-703112; Abbott Point of Care Inc., Abaxis Inc., Union City, CA

b. EC8+ Testing cartridge, 03P79-25; Abbott, Birmingham, UK

c. Stat Profile Critical Care Xpress 12; Nova Biomedical Corporation, Waltham, MA

d. i-STAT 1 Level 1,2,3 Control; Abbott Point of Care Inc., Abbott Park, IL

e. i-STAT 1 System manual; Abbott Point of Care Inc., East Windsor, NJ

f. Stat Profile Critical Care Xpress Reference manual; Nova Biomedical Corporation, Waltham, MA

g. Westmed 1cc 25 U Balanced Heparin, 3100-25; Westmed Inc., Tucson, AZ

h. MedCalc 12.2.1; MedCalc Software bvba, Mariakerke, BE

i. Papa V, Selleri P. Proceed Italian Soc Exot Anim Veterin (SIVAE) POA Congress, Cremona, 2009

Table 1. Summary statistics of the variables measured by means of the reference analyzer.

	U	Methodology	Mean	95% CI	SD	Percentiles (2.5 - 97.5)
pH		Direct ISE	7.378	7.341 – 7.415	0.098	7.169 – 7.572
PCO_2	mmHg	Severinghaus electrode	29.4*	28.6 – 34.8*	6.6	22.9 – 51.2
Hb	g/dL	Multi-wavelength reflectance/conductivity connection	12.0*	11.6 – 12.6*	1.2	8.7 – 14.5
Na^+	mmol/L	Direct ISE	142.2	141.2 – 143.3	2.9	136.7 – 148.3
K^+	mmol/L	Direct ISE	4.5*	4.3 – 4.6*	0.63	3.4 – 6.4
Cl^-	mmol/L	Direct ISE	106.2	105.0 – 107.4	3.3	98.6 – 111.4
BUN	mg/dL	Enzyme/Direct ISE	16.5*	14.1 – 19.0*	8	11.2 – 47
Glu	mg/dL	Enzyme/Amperometric	164.0*	151.8 – 193.3*	45	117 – 297.5
Hct	%	Conductivity/Na^+ correction	37	35.7 – 38.6	3	27 – 45

U: Measurement units

N: Number of blood samples

* Median and 95% CI for the median were reported due to non-normality of this variable.

Table 2. Summary statistics of the variables measured by means of the portable clinical analyser.

	U	Methodology	Mean	95% CI	SD	Minimum	Maximum	Percentiles (2.5 - 97.5)
pH		Direct potentiometry	7.370	7.328 – 7.413	0.114	7.116	7.652	7.124 – 7.623
PCO_2	mmHg	Direct potentiometry	39.1*	37.1 – 44.7*	8.4	28.0	67.7	28.5 – 64.7
Hb	g/dL	Calculated	12.0	11.2 – 12.7	2	6.5	15.6	7.3 – 15.6
Na^+	mmol/L	Direct ISE	142	141 – 143.1	2.8	135	149	135.5 – 148.5
K^+	mmol/L	Direct ISE	4.3*	4.1 – 4.5*	0.9	3.2	7.9	3.3 – 7.7
Cl^-	mmol/L	Direct ISE	105	103.5 – 106.9	4	94	114	94.7 – 113.8
BUN	mg/dL	Enzime/Direct ISE	17*	14 – 18.8*	7.9	10	48	10 – 44.5
Glu	mg/dL	Enzyme/Amperometric	162	149.1 – 176.2	36	105	255	108 – 249.2
Hct	%	Conductivity/Electrolyte correction	35	33.15 – 37.64	6	19	46	21.5 – 46

See table 1 for the remainder key

* Median and 95% CI for the median were reported due to non-normality of this variable.

Table 3**.** Results of PCA and RA comparison in healthy and diseased pet rabbits. RSD: Residual Standard Deviation.

Analyte	**Mean difference**	**Limits of Agreement**	**% Log Transformation**	**Regression Equation**	**95% CI Intercept A**	**95% CI Slope B**
pH	0.007	-0.03 to 0.05	-0.4 to 0.7*	y = -1.05 + 1.14 x	-1.59 to -0.45	1.06 to 1.21
***P*CO$_2$**	-8.876	-13.63 to -4.12	-28 to -13*	y = -0.31 + 1.28 x	-5.17 to 4.11	1.15 to 1.44
Hb	0.060	-1.75 to 1.87	-13 to 18*	y = -9.31 + 1.76 x	-13.15 to -6.22	1.50 to 2.07
Na$^+$	0.193	-5.40 to 5.79	---	y = 23.91 + 0.83 x	-24.64 to 53.46	0.62 to 1.17
K$^+$	0.015	-1.06 to 1.09	-14 to 20*	y = -0.57 + 1.08 x	-2.10 to -0.17	0.99 to 1.42
Cl$^-$	1.177	-2.45 to 4.80	-2 to 4*	y = -30.50 + 1.27 x	-58.38 to -5.68	1.04 to 1.53
BUN	0.433	-6.13 to 6.99	---	y = -3.00 + 1.16 x	-8.00 to -0.50	1.00 to 1.50
Glu	13.466	-12.28 to 39.21	-4 to 20*	y = 13.80 + 0.84 x	1.29 to 29.60	0.75 to 0.91
Hct	1.800	-3.27 to 6.87	-9 to 24*	y = -22.97 + 1.56 x	-39.00 to -14.50	1.33 to 2.00

* Variables presenting differences proportional to the mean underwent logarithmic transformation. Antilogs of the limits of agreement calculated on the log-transformed data are presented as a percentage.[29]

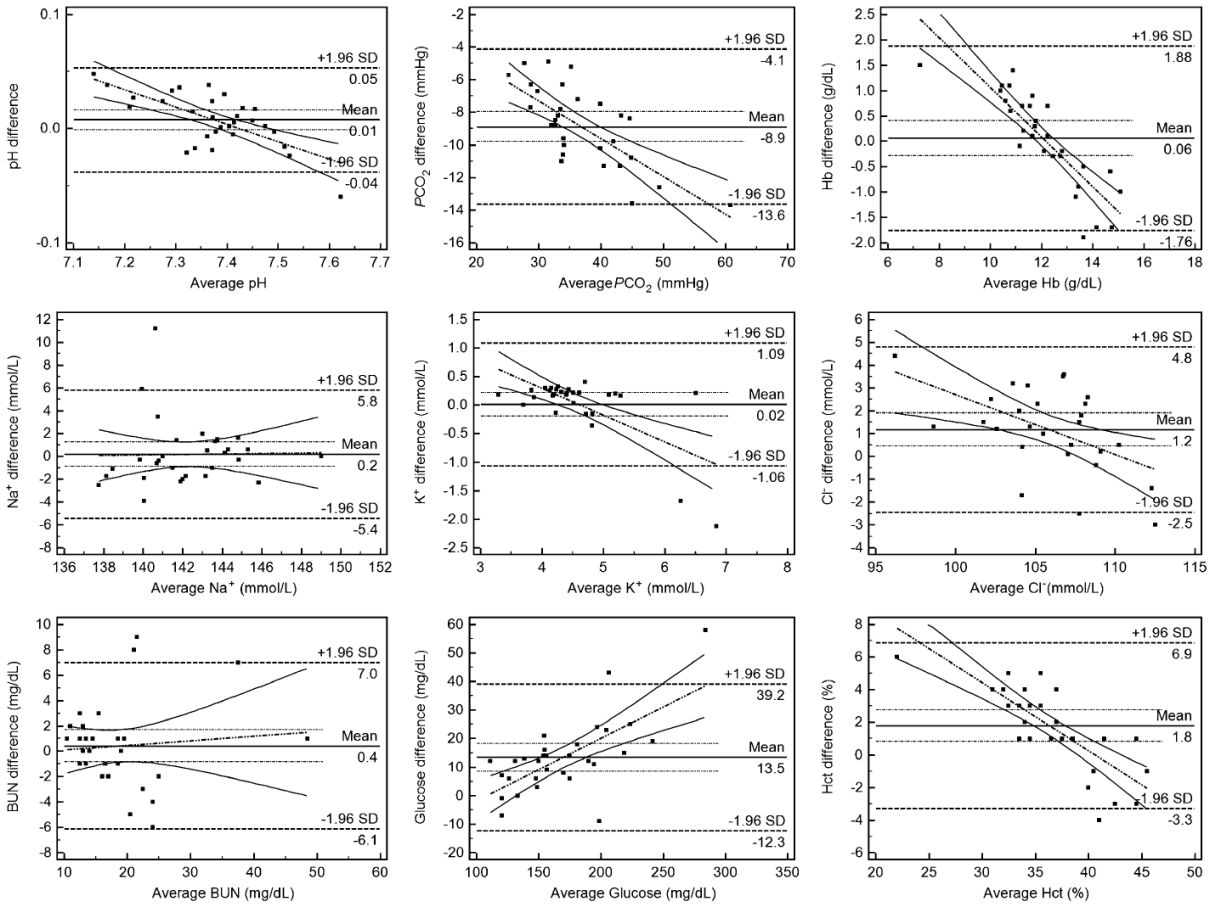

Figure 1**.** Bland-Altman agreement plots for venous blood samples collected from pet rabbits and analyzed with a PCA and a RA. The middle solid horizontal line represents the mean difference between the pairs of measurements, essentially representing the mean 'measurement error'. The upper and lower horizontal dashed lines represent the 95% limits of agreement, i.e. the range of 'measurement errors' that would occur on 95% of occasions. The 95% confidence intervals of mean of differences are depicted as dashed and dotted lines. The dotted line on the '0 difference' is the line of equality. If the line of equality is not included in the 95% confidence interval of mean of difference, there is a significant systematic difference. Regression line with its 95% confidence intervals is depicted to assist the detection of a proportional difference. Bland-Altman plot of Hb is not shown.

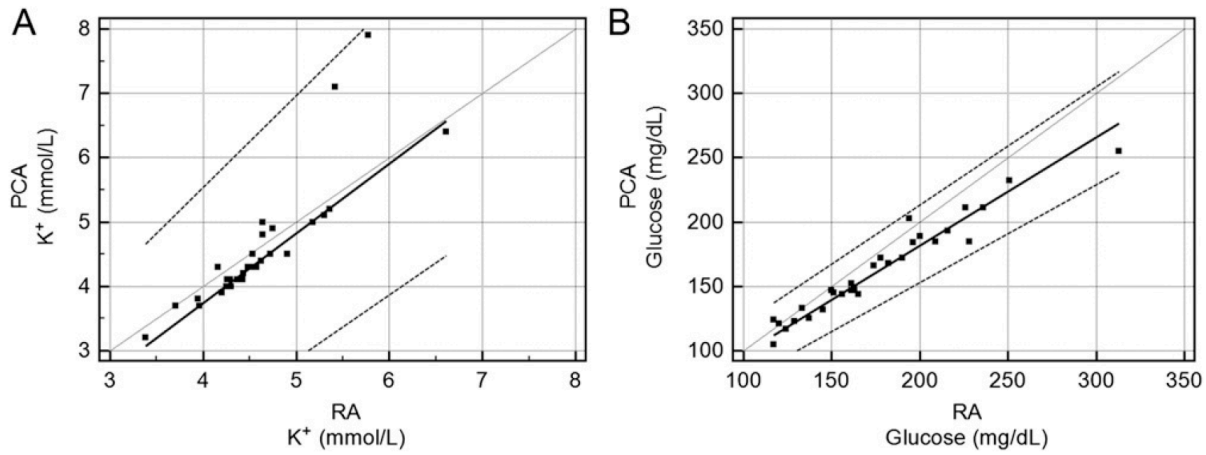

Figure 2**.** Passing-Bablok regression for two selected analytes. The regression line is indicated by the solid line, with the confidence intervals marked as dashed lines. The identity line (x = y) is indicated as the dotted line. **A.** K^+; Presence of proportional bias. Notice the two values (rabbit 16 [PCA: 7.9 mmol/L; RA: 5.77 mmol/L], rabbit 22 [PCA: 7.1 mmol/L; RA: 5.42 mmol/L]) that exceeded the upper limit of the normal range with the PCA, but were inside the reference range according to the RA. **B.** Glucose; Presence of constant and proportional bias.

Performance of two portable blood glucose meters and one bench-top analyzer for blood glucose measurement in pet rabbits

Background

Rabbits are among the most common pets in the households of Europe and United States[1-3] and represent a significant part of most small animal practices' patients in Great Britain.[4] Since blood glucose alterations in rabbits are frequent,[5] recurrent and accurate glucose measurement may be useful. In order to obtain more frequent determination of blood glucose, continuous glucose monitoring and point-of-care (POC) glucose testing may be performed. Although continuous glucose monitoring has being increasingly used in human medicine,[6] its use in veterinary medicine is still limited due to financial and practical concerns.[7] Besides standard laboratory methods, POC testing permits instantaneous reporting of blood glucose concentrations and has become invaluable in human and veterinary medicine.[8,9] Point-of-care testing broadly refers to any laboratory testing performed outside the conventional reference laboratory and implies close proximity to patients.[10] A variety of POC testing methods for glucose are available, including non-instrumental systems (ie, reagent test strips), portable analyzers (ie, portable blood glucose meters [PBGM]), and bench-top analyzers (ie, automated chemistry analyzers).[10-12]

Portable blood glucose meters are handheld instruments that use reagent test strips to provide immediate glucose results. Since the first blood glucose test was

developed in 1965, performance of PBGMs has been a constant concern in human medicine.[9,13] Most commercially available PBGMs were designed for human use. Recently, PBGMs designed for animal use, which should account for species-specific differences in distribution of glucose between plasma and erythrocytes, have been developed. To assess performance of PBGMs, glucose values obtained with PBGMs are typically compared to values obtained by use of a laboratory analyzer via the hexokinase reference method.[14] In veterinary medicine, performance of PBGMs has been assessed in dogs,[14] cats,[15] horses,[16] seabirds,[17] Hispaniolan Amazon parrots,[18] deer,[19] sheep,[20] cattle,[21] alpacas[22] and ferrets.[23] Results of previous studies indicated that discrepancies between PBGMs and reference methods may be clinically relevant, emphasizing the importance of assessing individual meter performance in the target species. Depending on the species, sample characteristics may differently affect accuracy of PBGMs: for instance, hematocrit affected accuracy of PBGMs in dogs,[24] while in cats its effect has not been proved.[25]Although rapid testing and blood volume required makes PBGMs particularly attractive in rabbits' critical care, no data evaluating the performance of POC analyzers for blood glucose measurement in rabbits are currently available.

Experience suggests that hypoglycemia and hyperglycemia are common in young, anorexic rabbits and in critically ill rabbits, respectively.[26] A recent study[5] provides the first evidence that clinical monitoring of blood glucose in pet rabbits should be recommended: blood glucose concentration was determined in a large population of pet rabbits (n=922), demonstrating an eventual application of glucose measurement to distinguish between gut stasis and intestinal

humans was used. Unfortunately, accuracy and precision of that instrument have never been evaluated in rabbits.

Therefore, the objective of this study was to compare the performance and the potential clinical impact of 3 POC analyzers with a laboratory-based analyzer in the measurement of glucose concentration in rabbits. Furthermore we wanted to evaluate the relationship among performance of POC analyzers and sample characteristics, i.e. hematocrit and glucose concentration. The specific hypotheses were that the human PBGM underestimated blood glucose concentration as in other species, and that hematocrit influenced the performance of the instruments.

Materials and Methods

Study design and population- An observational prospective cross-sectional study was planned. Sequentially admitted pet rabbits undergoing blood sampling in the Clinica per Animali Esotici, Roma, Italy for unrelated diagnostic reasons were included in the study. The predefined exclusion criterion was that the rabbit did not need blood collection. All the rabbits blood sampled in the clinic in the study period (February/March, 2013) were included in the study. In order to cover a wide range of analyte concentrations were included both healthy and diseased rabbits. The 'healthy' rabbits were rabbits undergoing elective surgery (castration/spaying) and underwent blood analysis for routine preliminary assessment of their clinical status (ie, hematology and serum chemistry). All the other rabbits included in the present study, in which blood collection was preliminary to semi-elective or urgent surgeries or necessary to confirm the

underwent blood sampling for several primary purposes, including hematology and/or biochemistry and/or protein electrophoresis and/or serologic testing. The study was performed in compliance with the directive 2010/63/EU of the European parliament and of the European council. The owners gave written informed consent to the inclusion of samples of their animals in the study.

Procedures-The rabbits were restrained in lateral recumbency by an experienced operator. Venipuncture site over the left saphenous vein was cleaned with a 70% alcohol swab. Blood samples were collected with a 25-Gauge needle into 1-mL plastic syringes and a drop of fresh whole blood was placed on a glass slide and immediately analyzed by the 4 PBGMs (2 identical human PBGMs[a]; 2 identical veterinary PBGMs[b]). Venipuncture and sample processing were performed by the same operator in order to minimize procedure time. The order in which PBGMs were used was randomized by use of a random sequence generator to avoid bias caused by the effect of time[27] and drop size[14] on glucose concentration. In case of strip failures, another strip from the same vial was immediately used. Blood was not scraped onto the test strips and it was only applied on one side of the veterinary test strips.[d]

The remaining whole blood was immediately transferred from the syringe into tubes containing lithium-heparin. If the sample needed to be analyzed with the bench-top analyzer, 0.1 mL of whole blood from the lithium-heparin tube was inserted in a reagent rotor. Hematocrit was determined in duplicate with 32 x 0.8 mm heparinized capillaries.[e] The lithium-heparin tubes were centrifuged at 3300 rpm for 10 minutes and plasma was harvested within 12 minutes after collection. Plasma glucose concentration was measured in all the samples with a

delay in the procedures, the PBGMs, the bench-top analyzer and the centrifuge were located immediately adjacent to each other and immediately adjacent to the table where blood collection was performed.

Analyzers and quality control-Three commercially available POC instruments, namely 2 PBGMs[a,b] and 1 bench-top analyzer,[c] were evaluated in the present study. Duplicate analysis of the samples[28] via PBGMs was performed using two identical human PBGMs[a] and two identical veterinary PBGMs[b] as in a previous study.[29] The meters and test strips were maintained and operated within a temperature range of 20–24°C degrees. Test strips were stored in their original vial in a cool, dry place between 20-25°C degrees, away from sunlight and heat. The vial was immediately closed after removing two test strips. Strips belonging to 2 different lots were used in the veterinary PBGM and strips belonging to 4 lots were used in the human PBGM to account for lot-to-lot variability.[30] Control solutions[g,h] were used once per week in order to check the performance of the PBGMs. Control solutions were also employed before a new box of test strips was used. The system was considered to perform correctly if the control solution test result felt within the specific control solution ranges listed on the strip vial.

Human amperometric PBGM-Single-use test strips[i] were used in the human PBGM. The human PBGM determined blood glucose concentration amperometrically by means of a pyrroloquinolinequinone-glucose dehydrogenase (GDH) catalyzed reaction. Results were provided in approximately 5 seconds, and the manufacturer's reported range for whole blood glucose concentration measurements was 10 to 600 mg/dL.[j]

Veterinary colorimetric PBGM-Single-use test strips[k] were used in the veterinary

means of a flavin-adenine dinucleotide-GDH catalyzed reaction.[h] The veterinary PBGM provided results in approximately 8 seconds, and the manufacturer's reported range for whole blood glucose concentration measurements was 20 to 750 mg/dL.[d] The 2 veterinary PBGMs were set on "canine" setting for the first 40 samples and on "feline" setting for the remaining samples. Glucose values obtained with the "canine" code were converted to the "feline" code and vice versa by use of an algorithm property of the manufacturer.[l]

Bench-top analyzer-The bench-top POC analyzer[c] with reagent rotors specific for mammalian biochemical analyses[m] was used in the cases in which a general biochemical profile was needed. The bench-top analyzer employed a modified version of the hexokinase method to measure glucose concentration.[n] Manufacturer's reported spectrum for glucose concentration measurements was 10 to 700 mg/dL.[n] Rotors of the bench-top analyzer were stored at 4°C degrees. The device was serviced by the local provider few days before the beginning of the study.

Laboratory analyzer-A laboratory-based automated chemistry analyzer[f] that measures glucose concentration via an enzymatic hexokinase oxidase reaction was used as reference method. Linear calibration[o] of the laboratory analyzer was performed weekly during study period.

Statistical analysis

Accuracy-accuracy of POC analyzers was assessed with Bland-Altman bias plots and regression techniques. Deming regression analysis was used for PBGMs because duplicate measurements were obtained, while Passing-Bablok regression analysis was used for the bench-top analyzer as only single

The limits of agreement (LoA) were determined from Bland-Altman plots by ±1.96 SD centered on the mean difference. The association between the difference and the analyte concentration was examined by standard regression analysis of the difference between the two methods on their average. If a significant slope of the regression line was detected, logarithmic transformation of both measurements before analysis was performed.[31] The LoA derived from log transformed data were back-transformed to give limits for the ratio of the actual measurements.[32]

By use of Deming regression the slope B and intercept A were calculated with their standard error and 95% confidence interval.[33] Passing-Bablok regression was performed as described elsewhere.[34-36] Constant bias were present if the 95% confidence interval for the y-intercept did not include the value 0. Proportional bias were present if the 95% confidence interval for the slope did not include the value 1.[35]

To assess relationship between accuracy, blood glucose concentration and hematocrit, the difference among laboratory results and POC analyzer results (ie, hexokinase results - POC results) were included as dependent variable in a multiple regression stepwise model with hematocrit and blood glucose concentration as independent variables. If the two independent variables concurred in the prediction of the dependent variable the multiple correlation coefficient (MCC) was provided. In case that only one variable fitted the model the Pearson correlation coefficient was provided for the significant variable.

Precision-repeatability of PBGMs was measured calculating the CV from duplicate measurement.[37] As no data were found in the current literature

for the hexokinase method was calculated. Four plasma samples were divided in 4 vials each, and were sent to the laboratory as different samples. In this way the technician was not aware that multiple aliquots of the same samples were measured.

To assess relationship between repeatability, blood glucose concentration and hematocrit, in PBGMs the difference (absolute value) among duplicate measurement were included as dependent variable in a multiple regression stepwise model with hematocrit and blood glucose concentration as independent variables. If the two independent variables concurred in the prediction of the dependent variable the MCC was provided. In case that only one variable fitted the model the Pearson correlation coefficient was provided for the significant variable.

Total error-Total analytic error reflects the sum of random error (imprecision) and systematic error (bias). No clear consensus exists as to best method to calculate total analytic error for instrument validation.[28,38,39] In the present study total error observed (TE_{obs}) has been calculated as described elsewhere:[40]

$TE_{obs} = 2\ CV + |Bias\ (\%)|$

Bias (%) were calculated as:

$Bias = (Mean_{hexokinase} - Mean_{PBGM}) / Mean_{hexokinase} \times 100$

Basing on the guidelines of the American Society for Veterinary Clinical Pathology[40] allowable total error for glucose measurement was 10% in hypoglycemic samples, 20% in normoglycemic samples, and 20% in hyperglycemic samples.

Alteration in diagnostic and treatment decisions-Error grid plots[41] were developed to evaluate clinical impact if the POC analyzers were used rather than the laboratory. The Clarke error grid analysis method and acceptance criteria was modified because veterinary critical limits are different than those used in humans.[16,42,43] The grid system assigns predicted glucose values (POC analyzers, y-axis) versus actual glucose values (hexokinase, x-axis) to 4 zones (A through D). Zone A included POC readings that deviate from the laboratory result by no more than 20%, or POC readings that are in the hyper or hypoglycemic range when the laboratory result is also in the hyper or hypoglycemic range, respectively. Twenty percent limits were plotted around the line of equality and hypoglycemic limit was considered below 75 mg/dL while hyperglycemic limit over 150 mg/dL, according to a recent survey[5] and to reference ranges.[44,45] Zone B was defined as values outside the normal range basing on the POC analyzers but in the normal range basing on the reference analyzer, ie included POC readings that would lead to over-diagnosis and treatment of either hypo- or hyperglycemia. Zone C was defined as values inside the normal range basing on the POC analyzers but outside the normal range basing on the reference analyzer, ie included POC readings that would lead to under-diagnosis and treatment of either hypo- or hyperglycemia. Zone D was defined as POC readings that were opposite of the hexokinase glucose readings, leading to treatment of hypoglycemia rather than hyperglycemia or vice versa. The POC analyzer would be considered clinically acceptable if at least 95% of its readings were within zone A.[16,42,46]

All data obtained, including those gathered after a previous strip failed, were included in the statistical analysis. Data were analyzed using commercial software.[p] Two tailed p-values of less than 0.05 were considered significant.

Results

Population summary-Overall 356 test strips were employed on 89 rabbits that underwent measurement of blood glucose by use of human PBGMs, veterinary PBGMs and laboratory analyzer (Table 1). The bench-top analyzer was used to measure blood glucose concentration on 32 samples. Hematocrit was measured in 84 rabbits. Rabbits ranged from 1 to 132 months of age (median ± SD, 24 ± 32.3), 46 were female (of which 23 neutered) and 43 were males (of which 11 neutered). Thirty-five rabbits were healthy, of which 11 were presented for neutering. Veterinary PBGMs did not yield results for 7 times. One of the veterinary PBGM for two times did not read the samples twice (for a totality of 4 errors), the other veterinary PBGM on three samples did not read the sample once (for a totality of 3 errors).

Accuracy-The degree of agreement between POC analyzers and laboratory method varied among instruments (Table 2). Bland-Altman plots demonstrated proportional and constant bias for the veterinary PBGM (both canine and feline setting) and at least constant bias for the human PBGM. No significant bias was demonstrated for the bench-top analyzer (Figure 1). Results of regression analyses (Figure 2) confirmed at least proportional bias for the veterinary PBGM (either setting) (Table 2). On the basis of these findings, the veterinary PBGM significantly overestimated blood glucose concentrations and the human PBGM

Increase in hematocrit was related with a decrease in accuracy (r=0.46, p<0.001) in the human PBGM (Figure 3), while blood glucose concentration had no effect on accuracy. In the veterinary PBGM accuracy decreased with increasing blood glucose concentration and decreasing hematocrit (Canine: MCC=0.77, p<0.001; Feline: MCC=0.68, p<0.001). Accuracy of the bench-top analyzer was not related to blood glucose concentration or to hematocrit.

Precision-The human PBGM presented an overall better repeatability (CV: 2.99%) than the veterinary PBGMs, either canine or feline settings (4.13% and 4.16%). Coefficient of variation of the hexokinase-based laboratory analyzer was 0.8%.

In all the PBGMs repeatability decreased with increasing glucose concentration (Human: r=0.38, p=0.0002; Canine: r=0.34, p=0.001) and decreased with decreasing hematocrit in the feline setting (MCC=0.38, p=0.002)(Figure 3).

Total error-The TE_{obs} for the human PBGM was 11.4%. The canine setting presented a TE_{obs} of 29.8% and the feline setting of 15.5%. The TE_{obs} at different glucose concentration are described in Table 3.

Alteration in diagnostic and treatment decisions-Basing on results of the error grid analysis (Figure 4) all the glucose values obtained with the human PBGM and the bench-top analyzer deviate from the reference by no more than 20%, or were correctly identified as hypoglycemic, normoglycemic or hyperglycemic. The use of the canine setting of the veterinary PBGM would have provoked in 9% of the cases misdiagnosis of hyperglycemia in normoglycemic pet rabbits. The use of the feline setting would have provoked in 5.6% of the cases misdiagnosis of hyperglycemia in normoglycemic pet rabbits, and in 1.1% of the cases

Discussion

Tight glucose control is increasingly being recognized as a priority in the management of critical human and animal patients.[47-49] For instance hypoglycemia and hyperglycemia are both associated with increased morbidity and mortality in pediatric intensive care unit.[47] Furthermore, blood glucose levels may assist in the diagnosis of pathological status in most animals,[50-54] including rabbits.[5] Nevertheless, PBGMs should be always evaluated in the target species, as their results may have significant divergence from true glucose value.[17,18,20,42] In the present study the bench-top analyzer presented the better agreement with the laboratory analyzer for measurement of blood glucose in rabbits' whole blood. This is not unexpected as the bench-top analyzer has a hexokinase-based methodology for glucose determination. Among PBGMs, the one intended for human use was more accurate and precise in measurement of rabbit blood glucose concentrations. Both canine and feline settings of the PBGM designed for veterinary use tended to proportionally overestimate blood glucose concentrations. The overestimation was statistically significant (mean difference was 48 mg/dL [LoA: -20 to 116] and 25 mg/dL [LoA: -27 to 78] for canine and feline settings, respectively) and clinically significant, as showed by the modified error grid analysis.

Accuracy of the veterinary PBGM

The veterinary PBGM employed in the current study has been already evaluated in dogs,[55] cats,[56] horses,[16] alpacas[22] and ferrets.[23] The accuracy of the veterinary PBGM in rabbits was similar to the accuracy reported in alpacas.[22] In whole blood from alpacas the veterinary PBGM tended to overestimate glucose

57.1 mg/dL) when compared to a laboratory analyzer.[22] In contrast, accuracy of the veterinary PBGM in dogs, cats, horses and ferrets was different that in rabbits. In dogs the veterinary PBGM did not provide results that were consistently lower or higher than the reference glucose concentration, although 43% of the results yielded blood glucose concentrations higher than the reference analyzer.[55] As in the present report, the veterinary PBGM in dogs decreased its accuracy with increasing blood glucose concentration (ie, presented proportional bias).[55] Reporting of mean difference with his 95% CI would have assisted in the evaluation of a significant constant overestimation of blood glucose concentration.[31,57] In cats the results obtained with the veterinary PBGM did not differ significantly from results obtained with the laboratory analyzer.[56] Unfortunately no data are available regarding mean difference among the veterinary PBGM and the reference analyzer in cats. In horses constant over/underestimation of blood glucose by use of the veterinary PBGM was not reported.[16] The veterinary PBGM was considered clinically acceptable, with nearly 97% of the readings that would have resulted in appropriate clinical decisions.[16] In ferrets the canine setting of the PBGM presented a negligible mean difference (1.9 mg/dL) compared to results of the laboratory analyzer, but wide LoA (-29 to 34 mg/dL; values extrapolated from the difference plot)[23] that may have an impact in clinical decision-making, as also stated by the authors.[23] Differently, the feline setting significantly underestimated the blood glucose concentration.[23] These interspecific differences emphasize the importance of assessing the performance of veterinary meters in their target species. Furthermore, the disparate statistical analyses performed in each study makes

results difficult to be compared. This stress the need for a common design and statistical protocol in method comparison studies.[31,40]

Accuracy of the human PBGM

The human PBGM constantly underestimated blood glucose concentration in rabbits of an average of 9.5 mg/dL. Identical human PBGMs have been already evaluated in horses,[58] in parrots,[18] in alpacas[22,42] and in ferrets.[23] In all those species the same human PBGM consistently underestimated blood glucose concentrations. A possible explanation for this finding is that such instruments are designed for self-monitoring of blood glucose concentration by humans with diabetes, who adjust their dosage of insulin according to the PBGM reading. With PBGM readings that are slightly less than actual blood glucose values, the diabetic would avoid hypoglycemia by injecting less insulin or by treating potential hypoglycemia earlier.[15] This explanation seems unlikely, as in humans the identical PBGM presents some proportional bias (mean difference among the PBGM and the reference analyzer: -2.7 mg/dL [-0.15 mmol/L]; LoA: 26.1 to -31.3 mg/dL [1.45 to -1.74 mmol/L])[59] but not a constant underestimation of blood glucose.[9,59]

Furthermore, it has been proposed that filters used in test strips to separate erythrocytes from plasma may cause inaccuracy in PBGMs[58] accounting for the differences between results for the PBGM and the reference hexokinase method. That hypothesis is challenged by the finding that dogs, humans and rabbits have erythrocytes of similar size (approximately 7 µm, 6 to 8 µm, and 6.5 µm respectively).[60-62]

Another proposed explanation is that values obtained with the reference

values obtained with the PBGM, for which whole blood was used, due to the quantity of water in plasma and whole blood. In fact, glucose equilibrates into the aqueous portion of a blood sample. The concentration of water in serum/plasma is higher than the concentration of water in the cellular portion of blood. Therefore serum/plasma has higher water content, and higher glucose concentration, than whole blood.[63] In humans the average volume of plasma consisting of water is 93%, the average volume of packed red blood cells consisting of water is 71%.[64] Considering an average hematocrit of 43%, the constant factor to convert whole blood molality to the equivalent plasma glucose molarity in humans is 1.11.[65] For instance, a fixed volume of plasma has higher water content and therefore has higher glucose concentrations of approximately 11–12% compared with whole blood already at a normal hematocrit of approximately 45%.[27] Some PBGMs have calibrations to correct this incongruity, assuming the patient has hematocrit within a given reference interval.[63] Difference among whole blood glucose molality and equivalent plasma glucose molarity is clearly more pronounced in the case of high hematocrit values.

A further explanation could be the different distribution of glucose in plasma and erythrocytes among species.[16] In dogs and cats, approximately 12% and 7% of glucose is within erythrocytes, respectively.[66] In humans, differently, glucose is distributed around 60% within erythrocytes and 40% within plasma.[67] Interestingly in rabbits approximately 15% of the glucose is distributed in the erythrocytes[67] with some fluctuations among studies.[66-68] A distribution of glucose in the blood of rabbits more similar to humans could justify the performance of the human PBGM observed in this study. However, it should be

considered that results obtained with the PBGM used in this study may not apply to different PBGMs intended for human use.

Impact of PBGM use in clinical practice

In human medicine, although modern glucose meters still show variable results with respect to analytical measures of accuracy, error grid analysis often demonstrates that measurements are clinically acceptable.[69] Poor clinical agreement of the veterinary PBGM with the reference analyzer was unexpected, as in other studies the veterinary PBGM proved to be more or equally accurate than human PBGMs in animal patients.[23,55] The canine and feline settings of the veterinary PBGM were not clinically acceptable in rabbits because inaccurately indicated normoglycemic blood samples as hyperglycemic; this occurred in testing 9% samples with the canine setting and 5.6% with the feline setting. Although the human PBGM used in this study constantly underestimated blood glucose in rabbits, basing on the modified error grid analysis it was acceptable for clinical use in rabbits. Our findings differ from what previously found in ferrets, in which 31% of the samples were incorrectly labeled as hypoglycemic with the same human PBGM.[23] Rabbits and ferrets together represent the more significant part of small exotic mammals.[1] The opportunity to use a single PBGM to measure blood glucose in both species would be advisable. Unfortunately, basing on our results and on the previous study conducted on ferrets,[23] veterinarians caring for exotic mammals should have available both the veterinary PBGM (for ferrets) and the human PBGM used in this study (for rabbits).

To employ the veterinary PBGM in rabbits, it may be possible to mathematically

would be to validate independent reference intervals and critical decision limits for that particular PBGM, but the lower repeatability would likely penalize such instrument. In this study, we observed that repeatability of the veterinary PBGM was similar to that reported in dogs (approximately 4%),[55] and that repeatability was influenced by blood glucose concentration and hematocrit. To our knowledge the effect of hematocrit on precision of PBGMs has not been previously described on animal patients.

Total analytic error can be used as an assessment of an individual instrument's analytical performance for instrument selection or for assessment of in clinic instrument performance.[39] Analytical imprecision (repeatability of the result) and bias (constant error) were combined into a single measure of the uncertainty of a test result. The ideal situation is to have highly accurate and precise measurement, i.e. low bias and low CV, respectively. Although total error when first introduced was proposed with a CV multiplication factor of 1.[65,70] several multiplication factors for the CV has been used by different authors for method validation.[39] In the present study a factor of 2 CV was used, as suggested by the guidelines for total allowable error of the American Society for Veterinary Clinical Pathology,[40] which is consistent with the Clinical Laboratory Improvement Amendments of 1988.[71] Allowable total error for glucose measurement should be 10% in hypoglycemic samples, 20% in normoglycemic samples, and 20% in hyperglycemic samples.[40] Both the human and the veterinary PBGM evaluated in this study exceeded the allowable total error. Nevertheless, no single standard exists to assess acceptable accuracy of a PBGM, so the determination of accuracy will vary by country and recommendation

utilized for the judgment.[63] Therefore, in the present study we calculated the TE_{obs} to compare the PBGMs, more than for describing absolute acceptability.

Effect of sample characteristics on glucose measurement

There are several factors that can adversely affect blood glucose concentrations reported by PBGMs.[65]Hematocrit has long been known to affect the accuracy of PBGMs.[72,73] Several hypotheses have been proposed to explain the impact of abnormal hematocrit values on blood glucose testing, such as altered viscosity of blood, prevention of plasma from reaching the reaction surface of the test strip, change in diffusion kinetics, and/or increased packed red cell volume and displacement of plasma volume leading to insufficient plasma volume for accurate testing.[74] In rabbits, we observed that hematocrit influenced accuracy of all the PBGMs, but on an instrument-dependent basis: the human PBGM increased the underestimation of blood glucose with increasing hematocrit values, while the veterinary PBGM increased the overestimation of blood glucose with decreasing hematocrit values. Therefore, the PBGM for use in humans is likely to be less accurate in polycythemic rabbits and more accurate in anemic ones, whereas the PBGM for veterinary use is more likely to be more accurate when used in polycythemic rabbits but less accurate in anemic ones. Interestingly, similar results have been observed when human and veterinary PBGMs were used in dogs:[24] in a study the effect of hematocrit on accuracy of PBGMs has been assessed in greyhounds (ie, presenting naturally high hematocrit) and in anemic dogs. As observed in rabbits, in dogs the veterinary PBGM decreased his accuracy at low hematocrit values, while the human PBGM decreased his accuracy at high hematocrit values.[24] Considering that the effect of

secondary to the different technology employed by the veterinary and the human PBGMs. To diminish hematocrit interference mathematical algorithms based on the differences in the kinetics of the electrochemical reactions of glucose and confounding substances have been developed. This method is referred to as "dynamic electrochemistry" and has been incorporated into commercially available PBGMs[75] with promising results.[76]

Apart from hematocrit, other analytes in the blood may alter the accuracy of PBGMs. One study investigated the quantity of total error contributed by variations in hematocrit, maltose, ascorbate in PBGMs for use in humans.[65] Interestingly, all the PBGMs performed well so long as there was near-normal hematocrit and no maltose or ascorbate present. But in case of 20% hematocrit and 0.29 mmol/liter ascorbate, the estimated total analytical error approached 30% for the meters evaluated.[65] Future studies should take in account also these sources of analytical error when PBGMs are evaluated in animal patients.

In the present study 1 drop of whole blood was immediately analyzed with the 4 glucose meters and the rest of the sample was placed in lithium-heparin tubes. Although the anticoagulant could be a source of analytical variation, in dogs and cats significant differences were not detected among glucose concentrations of fresh blood without anticoagulant, EDTA-anticoagulated blood, and lithium-heparinized blood.[14,15] It should be also considered that glucose is unstable in whole blood; erythrocytes metabolize glucose at a rate of 6 to 10 mg/dL/h at 25°C in human blood.[27] Therefore, to prevent artifactual hypoglycemia caused by glycolysis, separation of the erythrocytes should be performed soon after sample collection. All samples in the study reported here were evaluated on each PBGM

separate the plasma 2 minutes after collection. Considering the strict procedure timing, glycolysis in the present study should have been minimal. This assumption is supported by the good agreement observed between the bench-top analyzer (which employed whole blood) and the laboratory analyzer. Therefore the overestimation observed in the readings of the veterinary PBGM was not a consequence of falsely low concentrations detected by the laboratory analyzer.

Portable blood glucose meters are designed to measure glucose concentration in capillary blood. The present study was performed on pet rabbits that need venous blood collection for other purposes. Although, we cannot be certain that repeating the study with capillary blood samples would yield identical results, in cats no clinical important differences were found among capillary samples (obtained from the marginal ear vein) and venous samples.[77] Considering that all samples evaluated in this study were of venous origin, all the instruments should have been affected similarly.

Future studies evaluating PBGMs in rabbits should take in account other relevant factors in POC measurement of blood glucose, such as difference between plasma and whole blood measurement, difference between capillary and venous blood samples and interference of other analytes, i.e. maltose and ascorbate, with glucose determination.

Collectively, these data indicate that the human PBGM and the bench-top analyzer employed in the present study can be safely employed for glucose measurement of pet rabbits among a wide range of hematocrit and blood glucose concentration. The feline setting of the veterinary PBGM provided more accurate

presence of constant and proportional bias, high TE_{obs}, and less than 95% results in zone A of the error grid analysis, results obtained with both settings of the veterinary PBGM were not deemed acceptable in pet rabbits. Further work is required for calibrating and validating the veterinary PBGM for use in pet rabbits.

Footnotes

a. Accu-Chek Aviva (SN: 52811780506; SN: 53839834016), Roche Diagnostics, Indianapolis, IN

b. AlphaTrak 2 Blood Glucose Monitoring System (Lot: 03S213D, SN: CMFS201-M0240; Lot: 03S213D; SN: CMFS201-M0153), Abbott laboratories, Abbott Park, Ill

c. VetScan VS2 (SN: 0000000V01071), Abaxis, Inc., Union City, CA

d. AlphaTrak, User Guide, Abbott Laboratories, Ill

e. Capillary hematocrit, Drummond Scientific Co., Broomall, PA

f. Dimension EXL, Siemens Healthcare Diagnostics Inc., Tarrytown, NY

g. AccuChek Aviva Control Solution, Roche Diagnostics GmbH, Mannheim, Germany

h. AlphaTrak 2 Control Solution, Abbott laboratories, Abbott Park, Ill

i. AccuChek Aviva Test Strips, Roche Diagnostics GmbH, Mannheim, Germany

j. AccuChek Aviva user guide, Roche Diagnostics GmbH, Mannheim, Germany

k. AlphaTrak 2 Blood Glucose Test Strips, Abbott laboratories, Abbott Park, Ill

l. Algorithm property of Abbott, Abbott laboratories, Abbott Park, Ill

m. VetScan Comprehensive Diagnostic Profile, Abaxis Europe GmbH, Darmstadt, Germany

n. VetScan Comprehensive Diagnostic Profile, manual, Abaxis, Inc., Union City, CA

o. CHEM I Calibrator, Siemens Healthcare Diagnostics Inc., Tarrytown, NY

p. MedCalc 12.2.1, MedCalc Software bvba, Mariakerke, BE

Table 1. Glucose values obtained with a reference laboratory analyzer, a human PBGM, a veterinary canine PBGM, a veterinary feline PBGM and a bench-top analyzer in blood samples from healthy and diseased pet rabbits.

	Reference method	**Human PBGM**	**Canine PBGM**	**Feline PBGM**	**Bench-top analyzer**
Minimum (mg/dL)	31	29	30	28	80
Maximum (mg/dL)	405	417.5	550.5	494.5	360
Mean ± SD (mg/dL)	175.2 ± 70.1	165.6 ± 70.2	223.3 ± 96.6	200.6 ± 86.9	172.9 ± 53.3
Sample size (n)	89	89	89	89	32
Euglycemic values (n)	25	37	10	19	12
Hypoglycemic values (n)	5	6	5	5	0
Hyperglycemic values (n)	59	46	74	65	20

SD: Standard deviation. n: Number of samples.

Table 2. Accuracy of human PBGM, veterinary canine PBGM, veterinary feline PBGM and a bench-top analyzer to measure blood glucose concentration in whole blood samples from healthy and diseased pet rabbits.

		Bland-Altman analysis		
Instrument	**Sample size (n)**	**Mean difference mg/dL**	**Limits of Agreement mg/dL**	**Log Transformation %**
Human PBGM	89	-9.56	-33.80 to 14.68	---
Canine PBGM	89	48.17	-20.04 to 116.39*	-1.2 to 59.8
Feline PBGM	89	25.43	-27.78 to 78.66*	-11.3 to 43.4
Bench-top analyzer	32	1.25	-7.03 to 9.53	---

* Variables presenting differences proportional to the mean underwent logarithmic transformation. Antilogs of the limits of agreement calculated on the log transformed data are presented as a percentage.[31,32]

†All *P* values for *r* were less than 0.0001.

‡RSD was 5.2 and there was no significant deviation from linearity (Cusum test: P = 0.49).

Table 3. Accuracy, precision and total error of a human PBGM, a veterinary canine PBGM and a veterinary feline PBGM for determination of glucose in blood samples from pet rabbits at different range of glucose concentrations tested.

Glucose range	Human PBGM			Canine PBGM			Feline PBGM		
	Mean difference (LoA) mg/dL	**CV %**	**TE_{obs} %**	**Mean difference (LoA) mg/dL**	**CV %**	**TE_{obs} %**	**Mean difference (LoA) mg/dL**	**CV %**	**TE_{obs} %**
Euglycemic (n=25)†	-8.3 (-31.1 to 14.3)	2.1	10.7	32.2 (-16.2 to 80.7)	3.2	24.8	15.9 (-27.1 to 59.1)	3.2	7.3
Hypoglycemic (n=5)	-2.6 (-10.2 to 5.0)	4.6	15.0	7.3 (-5.6 to 20.2)	4.2	31.6	1.4 (-9.8 to 12.6)	2.1	18.9
Hyperglycemic (n=59)	-10.6 (-36.1 to 14.8)	2.9	10.9	58.3 (-10.1 to 126.9)	4.1	36.4	31.4(-23.6 to 86.5)	4.0	23.2

†Values between 75-150 mg/dL as determined by use of the laboratory analyzer.

LoA: Limits of Agreement. CV: Coefficient of Variation. TE_{obs}: Total error observed

Table 4. Results (%) of modified Clarke error grid analysis used to determine the percentage of results that would have potentially led to inappropriate diagnostic/therapeutic decisions with POC analyzers. Zone A included POC readings that deviate from the laboratory result by no more than 20%, or POC readings that are in the hyper or hypoglycemic range when the laboratory result is also in the hyper or hypoglycemic range, respectively. Zone B included readings that would lead to over-diagnosis of either hypo- or hyperglycemia. Zone C are included readings that would lead to under-diagnosis of either hypo- or hyperglycemia. Zone D included readings that were opposite of the hexokinase glucose readings, leading to treatment of hypoglycemia rather than hyperglycemia or vice versa. A POC analyzer would be considered clinically acceptable if at least 95% of its readings were within zone A.

Error grid zones	**Human PBGM *n* = 89**	**Canine PBGM *n* = 89**	**Feline PBGM *n* = 89**	**Bench-top analyzer *n* = 32**
A	100	91	93.3	100
B	0	9	5.6	0
C	0	0	1.1	0
D	0	0	0	0

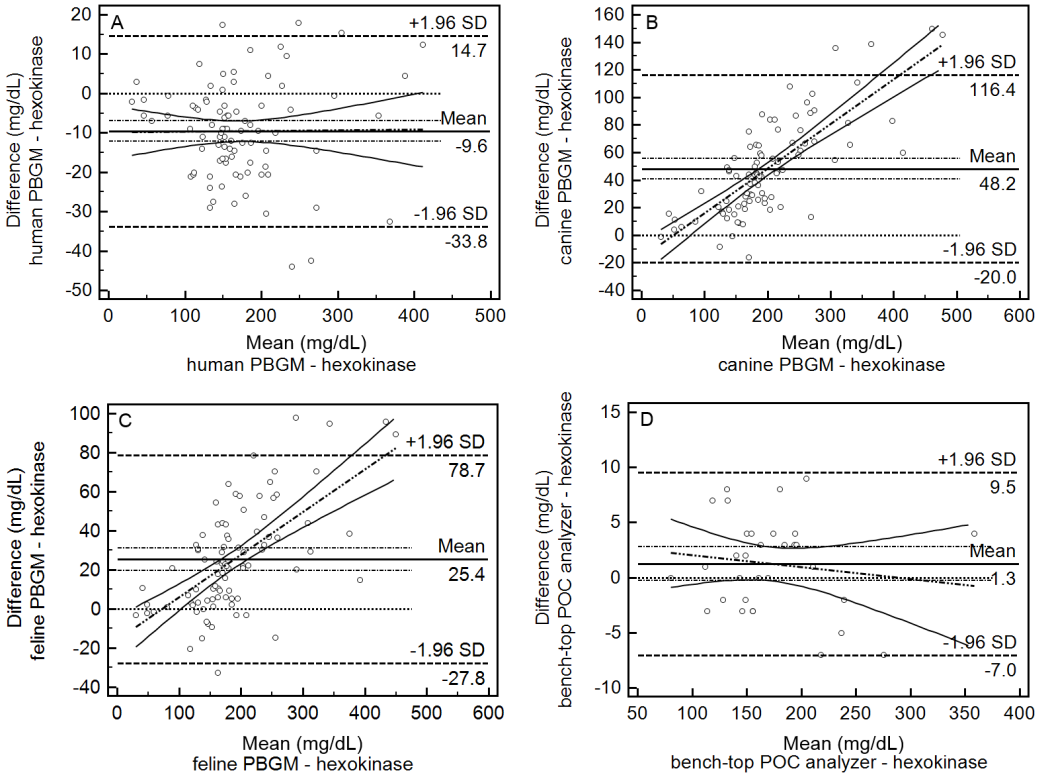

Figure 1. Bland-Altman agreement plots for venous blood samples collected from pet rabbits and analyzed with POC analyzers and a laboratory analyzer (hexokinase). A-Human PBGM. B-Veterinary canine PBGM. C-Veterinary feline PBGM. D-Bench-top analyzer. The middle solid horizontal line represents the mean difference between the pairs of measurements. The upper and lower horizontal dashed lines represent the 95% LoA. The 95% confidence intervals of mean of differences are depicted as dashed and dotted lines. If the 95% confidence intervals of mean of differences do not included the 0 value, there is a significant constant bias. Regression line with its 95% confidence intervals is depicted to assist the detection of a proportional bias.

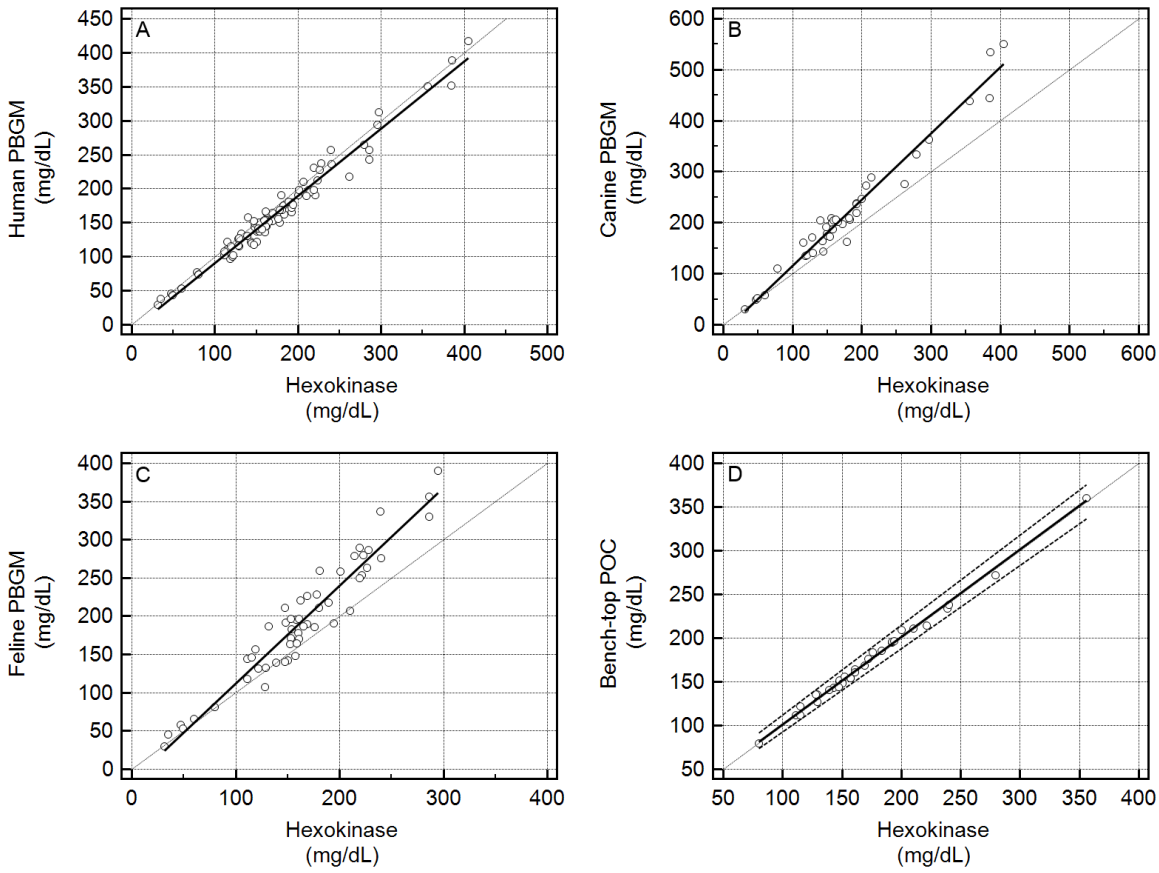

Figure 2. Regression analyses between POC analyzers and a laboratory analyzer (hexokinase) for measurement of blood glucose concentration in pet rabbits. A-Deming regression, human PBGM versus hexokinase. B-Deming regression, veterinary canine PBGM versus hexokinase. C-Deming regression, veterinary feline PBGM versus hexokinase. D-Passing-Bablok regression, bench-top analyzer versus hexokinase. The identity line (x = y) is indicated as the dotted line. The regression line is indicated by the solid line. In the Passing-Bablok regression the confidence intervals of the regression line are marked as dashed lines.

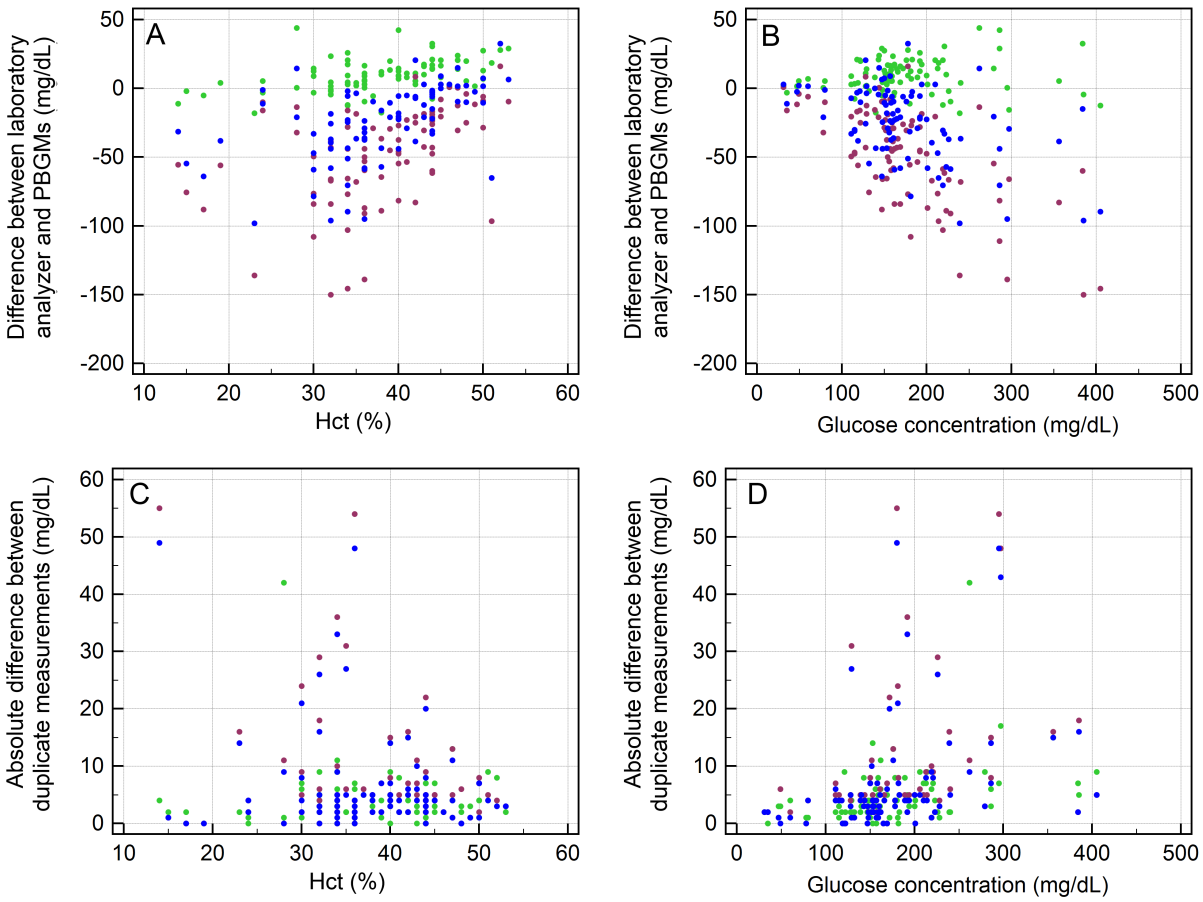

Figure 3. Effect of hematocrit and blood glucose concentration on accuracy and precision of PBGMs used in pet rabbits. A-Accuracy decreased with increasing hematocrit in the human PBGM; Accuracy decreased with decreasing hematocrit in the veterinary PBGM (either setting). B-Blood glucose concentration did not affect accuracy of the human PBGM. The veterinary PBGM presented a decrease in accuracy with increasing blood glucose concentrations. C-Precision decreased with decreasing hematocrit in the feline veterinary PBGM. D-Precision decreased with increasing blood glucose concentration in the three PBGMs. Green dots: human PBGM; Purple dots: veterinary canine PBGM; Blue dots: veterinary feline PBGM.

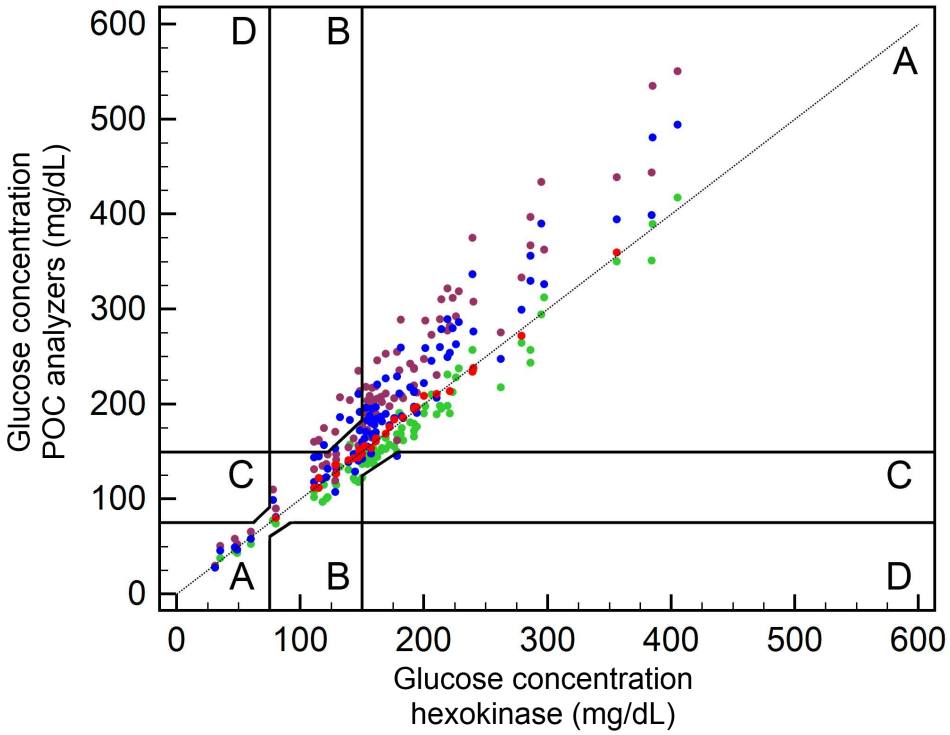

Figure 4. Modified error grid analysis. Whole blood glucose results from 4 point-of-care glucometers (1 human PBGM, 1 veterinary PBGM [canine and feline settings], 1 bench-top analyzer) and serum glucose results obtained from a laboratory hexokinase-based analyzer are plotted. The solid diagonal line is x = y, and lines representing the 10% limits of the x = y line are present (Zone A). Hypoglycemic limit was considered below 75 mg/dL, hyperglycemic limit over 150 mg/dL. Zone B was defined as POC readings that would lead to overdiagnosis of either hypo or hyperglycemia and overtreatment for hypo or hyperglycemia. Zone C was defined as POC readings that would lead to inadequate diagnosis and treatment for either hypo- or hyperglycemia. Zone D was defined as POC readings that were opposite of the hexokinase glucose readings, leading to treatment of hypoglycemia rather than hyperglycemia or vice versa. A POC analyzer would be considered clinically acceptable if at least 95% of its readings were within zone A. Green dots: human PBGM; Purple dots: veterinary canine PBGM; Blue dots: veterinary feline PBGM; Red dots: bench-top analyzer.

Variability of serum aldosterone concentrations in ferrets

Background

Elevation of serum aldosterone concentration is a well-described occurrence in human and veterinary medicine, classified in primary and secondary hyperaldosteronism. Two ferrets with suspect hyperaldosteronism have been described so far (Desmarchelier et al. 2008; Di Girolamo et al. 2013), however no published data regarding serum aldosterone concentration in ferrets are currently available. Our aims were (1) to explore the source of variability of the concentration of aldosterone in a population of healthy and diseased ferrets, (2) to determine preliminary reference intervals for serum aldosterone in healthy ferrets, (3) to provide a decision limit to discriminate healthy and diseased ferrets by their serum aldosterone concentration.

Materials and Methods

Consecutively admitted ferrets at the Clinica per Animali Esotici (Rome, Italy) from March 2013 to October 2013 were included in the study. Ferrets were not included if: (1) the owner did not give consent to inclusion; (2) the ferret was uncooperative and it was not possible to obtain the amount of blood required for the analysis without chemical restraint; (3) the ferret was in a clinical condition (e.g., severe anemia) that did not allow a safe sampling of the minimum quantity of blood required for the analysis (0.8 ml). The owners signed an informed consent for inclusion of their animals in the study.

Analytical methods

The samples were shipped to the Clinical Endocrinology Service, University of Tennessee for aldosterone analysis. Serum aldosterone concentrations were measured by use of a commercially available ^{125}I solid-phase competitive radioimmunoassay kit (Siemens Healthcare Diagnostics, Los Angeles, CA). Performance characteristics for the aldosterone assay were determined with pooled sera from ferret samples submitted to the Clinical Endocrinology Service for analysis. Intra-assay and inter-assay coefficient of variation for the pooled sera was 11.6% and 10.9%, respectively. Mean recovery of known amounts of aldosterone standards added to the pooled sera was 96.0%. When pooled ferret sera was serially diluted, results were 89.4%, 106.8%, 107.3%, and 106.5% of the expected values. Analytical sensitivity of the assay was 11 pg/ml on the basis of information provided by the manufacturer.

Statistical analysis

Summary statistics, including Shapiro-Wilk normality test, were compiled. Correlation between age and aldosterone was assessed with Spearmen rho coefficient. Differences in aldosterone concentrations due to sex, sexual activity and clinical status were assessed with the Mann-Whitney U test. Reference intervals for healthy ferrets were calculated with robust method as indicated by ASVCP guidelines (Friedrichs et al. 2012). Sensitivity and specificity of different aldosterone cut-offs to discriminate healthy and diseased ferrets were estimated with ROC curve analysis. Analyses were performed with commercial software (SPSS v22.0, IBM, Chicago, IL; MedCalc Software bvba, Mariakerke, BE) at a significance level of $P < 0.05$. Values are reported as interquartile range (IQR) and

Results

In the recruiting period, 86 samples were obtained. Eight of those were not analyzed due to technical problems (five, quantity not sufficient; three, not received). Of the 78 samples analyzed, 43 (55%) were from female and 35 (45%) were from male ferrets. Thirty-two ferrets (41%) were sexually inactive at the time of sampling (i.e., surgically or chemically neutered). Twenty-eight of the female ferrets were sexually active (65%), versus 18 (51%) of the male ferrets. Fifty-six (72%) of the animals were clinically healthy, while 22 had one or more disorders. Median age of the ferret was 24 months (IQR: 35; Range: 5-72) and the variable was not normally distributed (Shapiro-Wilk: $p<0.001$).

Aldosterone concentrations in the overall population showed wide variability and had a non-normal, positive-skewed distribution with right tail extremes (skewness 2.9; Shapiro-Wilk: $p<0.001$)(Figure 1), with a median of 4.75 pg/ml (17.9; 0.02-283.9) and 75% of the samples lower than 18 pg/ml. Healthy ferrets had a median aldosterone concentration of 1.9 pg/ml (8.4; 0.02-156.7) while diseased ferrets of 43.9 pg/ml (86.6; 0.05-283.9)($P<0.001$).

Older age and neutering were associated with significantly lower aldosterone concentrations (Spearmen rho: -0.24; $P=0.033$), with sexually active ferrets having median aldosterone concentrations of 8.1 pg/ml (32.5; 0.14-239.5) and sexually inactive ferrets of 0.9 pg/ml (5.5; 0.02-283.9)($P=0.003$). No significant differences in aldosterone were observed by sex.

Upper limit of the reference interval for healthy ferrets was 13.3 pg/ml (90%CI: 9.9 to 16.9). Subgroup-specific reference intervals were not calculated due to the limited sample size.

ROC curve analysis revealed that the cut-off value of aldosterone at 7.6 pg/ml could differentiate healthy from diseased ferrets with a sensitivity of 72.7% and a specificity of 73.2% (AUC: 0.79; 95% CI 0.67 to 0.91)(Figure 2). To maximize sensitivity, values of aldosterone of 0.9 pg/ml have a sensitivity of 90.9% and a specificity of 44.6%. To maximize specificity, values of aldosterone of 28.1 pg/ml have a sensitivity of 54.5% and a specificity of 91.9%.

Discussion

In the present study, a wide range of aldosterone concentrations was observed in ferrets, especially in unhealthy ferrets. Based on these results, high aldosterone concentrations are common in client-owned ferrets. Like in other mammals, a high aldosterone concentration should not be considered diagnostic of primary hyperaldosteronism.

Aldosterone concentrations in the overall population of ferrets had a positive skewed distribution, similar to the one observed in cats (Yu and Morris, 1998), with three quarters of the concentrations below 18 pg/ml. These distributions influence the determination of reference intervals possibly resulting in biased estimations (Boyd, 2010). For clinical purposes, rather than the upper limit of the reference interval calculated in the healthy population (13.3 pg/ml), we suggest to employ the decision limits obtained with the ROC curve analysis (7.6 pg/mL). Currently, this is a standard approach also in other medical specialties (Morrow and Cook, 2011).

In cats, a suspicion of primary hyperaldosteronism is based on clinical signs (e.g., weakness, hypertension), laboratory abnormalities (e.g., hypokalemia) and

et al. 2013; Daniel et al. 2015). In ferrets, adrenal enlargement is extremely common due to its association with gonadectomy and usually results in increased sexual hormones (Rosenthal and Peterson, 1996; Li et al. 1998; Shoemaker et al. 2000). This fact limits the utility of diagnostic imaging and makes the clinical suspicion of primary hyperaldosteronism more difficult than in other species.

Conclusions

Due to the number of ferrets with high aldosterone concentrations, there is the need to develop further testing to confirm primary hyperaldosteronism. In cats, an elevated plasma aldosterone-to-renin ratio, and lack of decrease of aldosterone during a fludrocortisone suppression test are currently considered the reference standard for diagnosis of hyperaldosteronism (Djajadiningrat-Laanen et al. 2013; Matsuda et al. 2015). In the future, these tests should be validated in ferrets for discrimination of primary hyperaldosteronism.

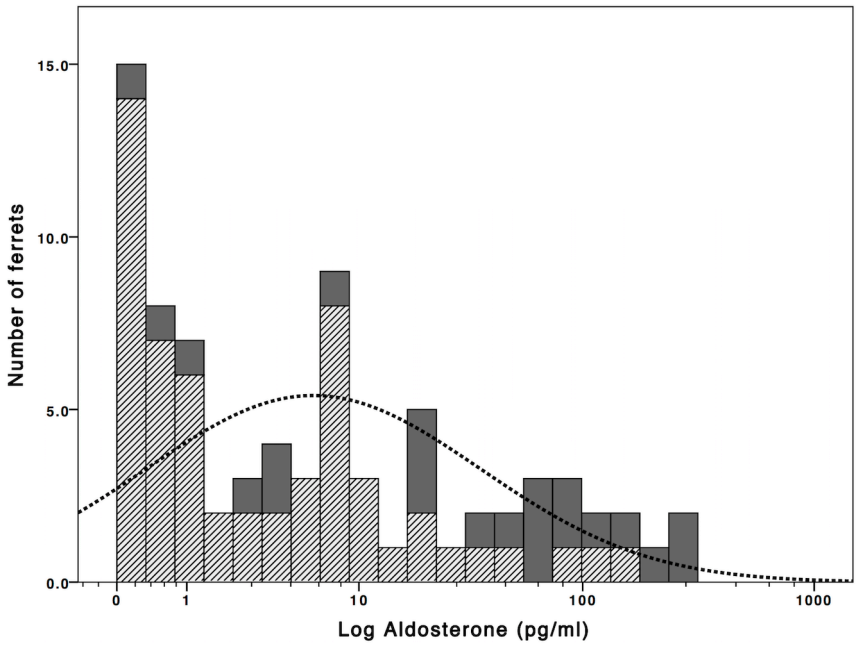

Figure 1. Distribution of aldosterone concentrations in healthy (diagonal pattern white) and diseased (solid grey) ferrets (n=78). Notice the non-normal, positive-skewed distribution with right tail extremes. The aldosterone concentrations are plotted on a logarithmic scale.

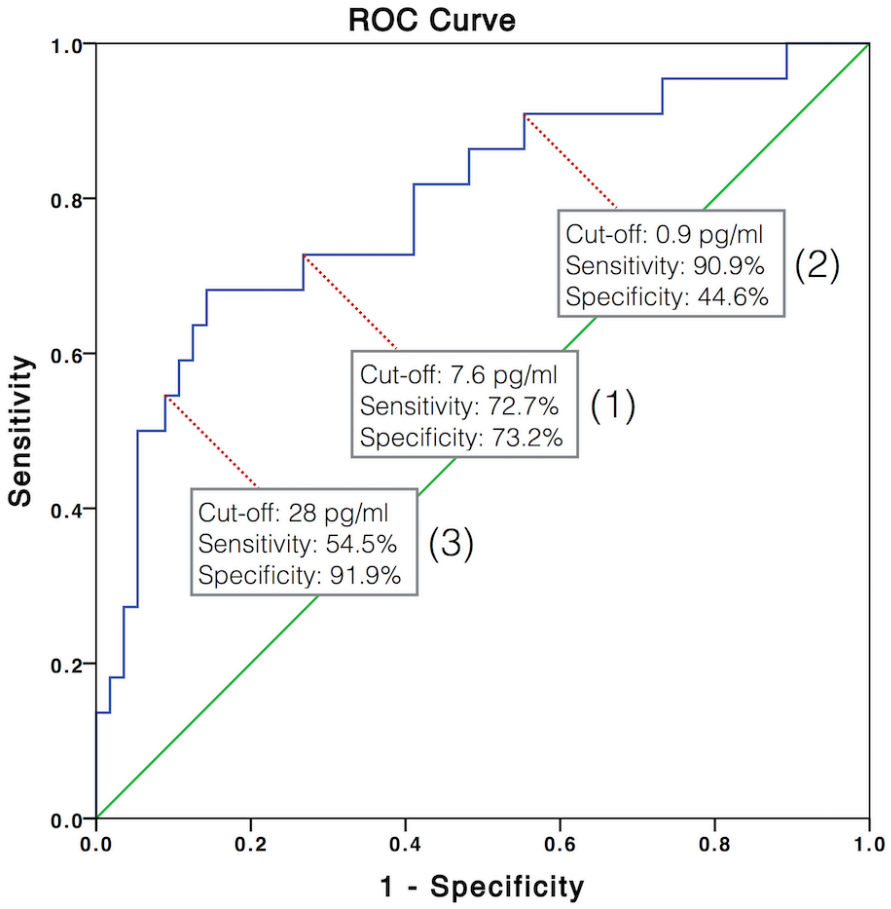

Figure 2. Receiver operating characteristic (ROC) curve of aldosterone concentrations to discriminate diseased ferrets (AUC: 0.79; 95% CI 0.67 to 0.91). Cut-off points represent: (1) optimal compromise between sensitivity and specificity, (2) optimal sensitivity, (3) optimal specificity.

DISCUSSION & CONCLUSIONS

With the increasing diffusion of several animal species in households, there is urgent need of improving the care that veterinarians can provide them. A first step for improvement in medical care is to enhance the diagnostic capabilities of practitioners. For this purpose, reference intervals and validation of clinical instruments is required.

We have found that although the use of point-of-care devices in small animals is intriguing due to the versatility of the instruments and the minimal amount of blood required, results provided by POC analyzers may lack agreement with laboratory-based analyzers. Specifically, bromocresol green dye-binding method for determination of albumin concentration can be inadequate in Hermann's tortoises, as reported for other reptiles. The overestimation of albumin by bromocresol green is a common finding due to nonspecific interactions with other plasma proteins. Therefore, we suggest quantifying plasma albumin by agarose gel electrophoresis in reptiles. We found that portable blood gas analyzer underestimated pH and PO_2, while PCO_2 was overestimated. Therefore, there is the potential risk to over-diagnose some conditions (e.g., respiratory acidosis characterized by decreased pH and increased PCO_2). We have found that among portable glucometer, the one intended for human use was more accurate and precise in measurement of rabbit blood glucose concentrations. The portable glucometer intended for veterinary use tended to proportionally overestimate blood glucose concentrations, resulting in potential clinical misdiagnosis.

As regards reference intervals, we have found that Hermann's tortoises need

environmental and physiological factors should be considered when evaluating hematology, plasma biochemistry and protein electrophoresis of this species. Due to the differences observed between species, the most relevant findings of our work are summarized below.

Hermann's tortoises

In one of the study included in this dissertation we evaluated the use of a PGA and a PCA in tortoises by comparing them to analyzers routinely used in clinical laboratories. Furthermore, we provided preliminary reference intervals for venous blood gas analysis in Hermann's tortoises in summer. Although several benefits are associated with the use of POC analyzers, one of the major concerns remains the validity of their measurements compared to primary, bench-top instruments used in the laboratory (Jacobs et al. 1993; Klein et al. 1999; Sediame et al. 1999; Papadea et al. 2002). Only an adequate agreement between instruments ensures comparability of test results (Papadea et al. 2002).

Method comparison studies testing analytical performances of PGAs have been done in domestic, laboratory and wild animals (Grosenbaugh et al. 1998, Tinkey et al. 2006, Steinmetz et al. 2007, Wenker et al. 2007, Peiró et al. 2010, Burdick et al. 2012, Selleri and Di Girolamo 2014). In reptiles, despite the employment of this PGA in previous studies (Harms et al. 2003, Lewbart et al. 2014), agreement with reference analyzers has been only evaluated for Cl, glucose, K, Na, and Hct measurements (Wolf et al. 2008; McCain et al. 2010). In the present study, the PGA had constant and/or proportional bias for each of the analytes except Na.

Regarding acid-base and blood gases evaluation, the PGA underestimated pH and PO_2, while PCO_2 was overestimated. Therefore, there is the potential risk to over-

pH and increased PCO_2). The poor agreement of PO_2 is unlikely to be clinically significant given the lack of clinical importance of venous PO_2 in tortoises (Khan et al. 2010). Both POC analyzers underestimated blood K concentrations. POC analyzers may be especially useful for evaluation of K in the field, as in chelonians late separation of red cells from serum or plasma after blood collection may interfere with the reliability of K measurements (Abou-Madi and Jacobson 2003). Underestimation of K may potentially lead to an under-diagnosis of conditions that result in hyperkalemia, e.g., acute renal failure (Divers 2000). Sodium did not present constant bias with both POC analyzers, but with the PCA there was increasing bias at increasing Na values. A great advantage of the PGA is the measurement of the iCa that may have an important role in tortoise medicine (Eatwell 2009a; Eatwell 2009b). iCa was significantly overestimated by the PGA. Decreased iCa is typically observed in chelonians suffering nutritional secondary hyperparathyroidism (Stringer et al. 2010) and renal disease (Selleri and Hernandez-Divers 2006). Therefore, the overestimation of iCa by the PGA may potentially result in under-diagnosis of such disorders.

In the other survey on Hermann's tortoises included in this dissertation, we have provided reference ranges on several hematological and biochemical variables. We have confirmed the reliability of Romanowsky-type (May Grünwald-Giemsa and Wright) stains for morphologic analysis of peripheral blood cells in *T.hermanni*. Most of the measured biochemical analytes in *T. hermanni* presented values in accordance with those reported for other healthy tortoises and were used for the calculation of reference intervals. There were significant differences

timepoint. Therefore, physiological and environmental factors should to be taken into account when evaluating tortoise health status including laboratory data analysis. The reference ranges defined in the present study for hematologic and biochemical blood values can be considered a useful tool for clinical pathologists, clinicians and researchers working in tortoise medicine and conservation. However, more interaction among laboratories working with non-conventional species would be useful to minimize inter-laboratory differences and to devise standard criteria and analysis protocols in protein electrophoresis.

In the last survey on Hermann's tortoises included in this dissertation, we described important findings related to hematology of this species. We found range values in our study of manually counted RBC and WBC and HGB are wider than those previously reported in the literature for *T.hermanni*. Possible explanations for the differences found here, can be attributed to the size of sample population, to seasonal variability, to sub-specific differences, to the venipuncture site, to the anticoagulant used or to the counting. In fact, some sampling sites are more prone to lead to lymph contamination in chelonians, causing spurious low values of blood cell count. In our experience, lymph contamination is less likely to occur when sampling from the jugular vein.

We found differences in RBC count between males and females in both the studies. This information is not novel, as such differences are commonly reported in reptiles (Duguy 1970). Since erythrocytic indexes are correlated to RBC, HTC and HGB, the same gender-based differences are likely to be found in MCV, MCH and MCHC. The finding of a gender-based difference in selected parameters in *T. h. boettgeri* may have a clinical relevance and suggest the need

blood cells counter that yielded comparable results with the manual count for RBC, HTC and erythrocytic indexes. The automated count seems to overestimate the RBC count and to underestimate the HTC determination in a way not likely to affect clinical judgment. A possible explanation is that some small WBC or thrombocytes are counted as RBC and that in the PCV are usually comprised other cells (WBC, thrombocytes) other than RBC only. Even though some authors recommend the use of an automated or semiautomated counting machines for reptile RBC and HTC determinations, according to our findings the use of such equipments should be previously validated in order to obtain reliable results.

Rabbits

In one of the study included in this dissertation focusing on the use of glucometers in rabbits we found that the bench-top analyzer presented the better agreement with the laboratory analyzer for measurement of blood glucose in rabbits' whole blood. This is not unexpected as the bench-top analyzer has a hexokinase-based methodology for glucose determination. Among PBGMs, the one intended for human use was more accurate and precise in measurement of rabbit blood glucose concentrations. Both canine and feline settings of the PBGM designed for veterinary use tended to proportionally overestimate blood glucose concentrations. The overestimation was statistically significant and clinically significant. Even if among the PBGMs tested, the human PBGM was superior, it constantly underestimated blood glucose concentration in rabbits of an average of 9.5 mg/dL. A possible reason for this finding is that such instrumentis designed for self-monitoring of blood glucose concentration by humans with

With PBGM readings slightly less than actual blood glucose values, the diabetic would avoid hypoglycemia by injecting less insulin or by treating potential hypoglycemia earlier. This explanation seems unlikely, as in humans the identical PBGM presents some proportional bias (mean difference among the PBGM and the reference analyzer: -2.7 mg/dL [-0.15 mmol/L]; LoA: 26.1 to -31.3 mg/dL [1.45 to -1.74 mmol/L]) but not a constant underestimation of blood glucose. Furthermore, it has been proposed that filters used in test strips to separate erythrocytes from plasma may cause inaccuracy in PBGMs accounting for the differences between results for the PBGM and the reference hexokinase method. That hypothesis is challenged by the finding that dogs, humans and rabbits have erythrocytes of similar size (approximately 7 μm, 6 to 8 μm, and 6.5 μm respectively)(Persons 1929; Meinkoth et al. 2000; Poljičak-Milas et al. 2009). Another proposed explanation is that values obtained with the reference method, for which plasma or serum was used, can be expected to be higher than values obtained with the PBGM, for which whole blood was used, due to the quantity of water in plasma and whole blood. In fact, glucose equilibrates into the aqueous portion of a blood sample. The concentration of water in serum/plasma is higher than the concentration of water in the cellular portion of blood. Therefor, serum/plasma has higher water content, and higher glucose concentration, than whole blood. In humans the average volume of plasma consisting of water is 93%, the average volume of packed red blood cells consisting of water is 71%(Lyon et al. 2010). Considering an average hematocrit of 43%, the constant factor to convert whole blood molality to the equivalent plasma glucose molarity in humans is 1.11 (Coldman 1967). For instance, a fixed

concentrations of approximately 11–12% compared with whole blood already at a normal hematocrit of approximately 45%. Some PBGMs have calibrations to correct this incongruity, assuming the patient has hematocrit within a given reference interval.

In the other study included in this dissertation we found that agreement of blood gas analysis, selected electrolyte values, selected chemistries values, Hct and Hb between the PCA and the RA was poor, the only exception made by pH. The inadequate agreement found in such study it is not completely unexpected. In the veterinary literature a poor agreement between PCAs and RAs for certain assays has already been described (Dechant et al. 2006; Tinkey et al. 2011). Although the need to evaluate point-of-care units in exotic species has already been pointed out, to the best of the authors' knowledge the present was the first report employing a point-of-care analyzer in the rabbit.

Ferrets

In this dissertation we observed a wide range of aldosterone concentrations, especially in unhealthy ferrets. For clinical purposes, rather than the upper limit of the reference interval calculated in the healthy population (13.3 pg/ml), we suggest to employ the decision limits obtained with the ROC curve analysis. Currently, this is a standard approach also in other medical specialties Due to the number of ferrets with high aldosterone concentrations, there is the need to develop and validate further testing for discrimination of tprimary hyperaldosteronism.

Conclusions

In conclusion, animal species requires individual validation of laboratory methods and reference intervals. Lack of consideration of these findings may result in clinical misdiagnosis and improper treatment of animals.

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