ABSTRACT

RUIZ-JIMENEZ, FERNANDO. Application of Blood Biochemistry Portable Analyzers in Meat-Type Birds (Under the direction of Dr. Rocio Crespo).

Clinical biochemistry is routinely used in human and veterinary medicine to monitor the health status of patients and aid in the diagnosis of several conditions. Traditionally, blood analyzers have been complex and expensive instruments that are found in specialized clinical laboratories where samples need to be sent to be tested. This makes blood testing for commercial poultry very challenging, since sample transportation and conservation are troublesome, and testing is usually not cost-effective. Nonetheless, the introduction of portable blood analyzers has shifted the paradigm of traditional clinical pathology, making blood testing more accessible and convenient in any clinical setting, and potentially in field conditions. Therefore, the objective of the present thesis was to evaluate the performance of two portable blood analyzers, the i-STAT and the Vetscan VS2, in broiler chickens reared under field conditions, to determine if clinical pathology could be used as a reliable tool to diagnose and better understand the different metabolic conditions that have an impact in the poultry industry. In the first study conducted, the time effect on the blood analytes was evaluated, and the portable instruments were validated by assessing their agreement with reference analyzers. Samples were taken from 60 broiler chickens and tested at three different times in the i-STAT and the VS2, each 20 minutes apart. The last testing was performed in reference analyzers as well. Significant differences in the concentration of the blood gases were noted, but they were caused by exposure of the samples to the environmental air. None of the other analytes showed clinically relevant differences in concentration through time. Only some analytes on each of the portable devices showed agreement with the reference analyzers, indicating that they could not be used interchangeably

and that reference intervals needed to be generated for the portable analyzers. In the second study performed, a total of 134 blood samples from birds of different ages (7d, 21d, and 35d) reared in two different flocks were analyzed using the i-STAT and the Vetscan VS2. Significant differences in concentration among ages were observed in all analytes except for bicarbonate, total carbon dioxide, and glucose. Differences in pH, partial pressure of oxygen, oxygen saturation, partial pressure of carbon dioxide, aspartate aminotransferase, sodium, and albumin, were observed between the flocks. Preliminary reference intervals were obtained for all analytes at each of the age groups using the non-parametric ranking method. These studies demonstrated that the i-STAT and VS2 portable analyzers are a feasible option to perform blood testing of commercial broilers in the field and provided a basis for interpretation of results. It is necessary to generate more data to get a more robust knowledge of the normal values in chickens, but this study shed light on the value that clinical pathology could add to the poultry veterinary field to ultimately improve the well-being of commercial chickens.

Application of Blood Biochemistry Portable Technologies in Meat-Type Birds

by
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DEDICATION

To my mom, who through unmeasurable love and effort made me who I am today. I am so grateful to have you as my mom and I will never finish thanking you for everything that you have done for me. I love you with all my heart.

To Quetzal, who has been a light in my life for more than 5 years and will certainly continue to be so for the years that we have left. I am stronger and better with you, and you make me the happiest man on this planet. I thank the universe every day that it allowed us to coincide and share our lives together. I will forever love you.

To my family and friends, for all your love and support through all this time that I have been far from you. You are always in my heart and mind.

BIOGRAPHY

Fernando Ruiz Jimenez was born in Mexico City on September 24th, 1993. At the age of 9, he and his family moved to a small city named Cordoba in the state of Veracruz, Mexico, which he considers his hometown. As a child, he would dream of becoming a veterinarian, and he used to spend hours playing with his mom, pretending to diagnose and treat every stuffed animal in the house. As he grew older, he was always interested in the scientific field, and the desire of becoming a veterinarian was still deep inside his mind.

At the age of 18, he was accepted into the College of Veterinary Medicine at the National Autonomous University of Mexico (UNAM) in Mexico City. That was a dream come true, as his grandparents, two of the most important persons in his life, were students and professors at that university back in the day. It was also a lifetime opportunity, as UNAM's veterinary school is the best in the country. After his first year, he started getting interested in poultry medicine and decided to learn and get more involved in it. While he was a student, he started working as a volunteer at the UNAM Poultry Teaching Farm, the school's pigeon loft, and the UNAM Poultry Diagnostic Laboratory, where he met and started a good relationship with professors and researchers of avian medicine. After vet school, he decided to do his social service, which is a non-remunerated six-month work that all Mexican students are required to do to retribute the expenses for their education, at the aforementioned laboratory, where he ended up developing the thesis project that led him to get his DVM degree with an honorable mention.

A year later, he was accepted into the combined Master/Residency program in Poultry Health Medicine at North Carolina State University in the city of Raleigh. This thesis materializes his master's research project, and he is soon to finish his residency program and take the exam to become a board-certified poultry veterinarian.

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LIST OF ABBREVIATIONS

Alb	Albumin
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
AP	Alkaline phosphatase
BA	Bile acids
BE	Base excess
CK	Creatine kinase
CLIA	Clinical Laboratory Improvement Amendments
CLSI	Clinical and Laboratory Standards Institute
CSF	Cerebrospinal fluid
CVM-NCSU	College of Veterinary Medicine, North Carolina State University
GGT	Gamma-glutamyl transferase
GLDH	Glutamate dehydrogenase
Glu	Glucose
HCO_3	Bicarbonate
LDH	Lactate dehydrogenase
pCO_2	Partial pressure of carbon dioxide

pO_2	Partial pressure of oxygen
POC	Point-of-care
RI	Reference Intervals
sO_2	Oxygen saturation
TAU	Teaching Animal Unit
tCO_2	Total carbon dioxide
TP	Total protein
UA	Uric acid
VS2	Vetscan VS2

CHAPTER 1. Literature Review

Conventional and Portable Biochemistry Analyzers. Clinical biochemistry refers to the analysis of samples of body fluids, mainly plasma, but can also include feces, urine, CSF, and pleural or peritoneal fluids, for a wide variety of substances and their use in diagnosis and monitoring of disease in human and veterinary patients (1). Since clinical biochemistry is a multidisciplinary field that involves chemistry, biochemistry, immunochemistry, endocrinology, toxicology, engineering, and informatics, the analytical methods to test the samples have always been a challenge. Early on, the tests needed to be performed manually, by medical technologists, and with a variety of analyzers that had limited capabilities and that could only measure a few analytes (2). These factors made the use of clinical biochemistry a cumbersome and expensive task, yet necessary to facilitate the diagnosis of certain conditions in human medicine. It was not until the 1950s that the first automated instrument, the AutoAnalyzer was developed. It was able to measure urea, glucose, and calcium without manual intervention and in a continuous flow. The AutoAnalyzer was a "batch analyzer" which means that it performed the same array of tests on every sample, whether they were requested or not. Many other batch analyzers were developed in the following years, making clinical biochemistry more common and available (3). Parallel to the development of the batch analyzers, "ready-to-use" reagent kits were introduced by the Sigma Chemical Company, boosting the benefits of the automated analyzers, and contributing significantly to the clinical laboratory field (4). Later, centrifugal analysis emerged as an alternative for batch analyzers, with the advantage that the devices were able to perform sequential, discrete, and parallel tests by using a spinning rotor that mixed the sample with the reagents and made them flow into a reaction chamber. The centrifugal analysis was so successful that it remained the preferred option at clinical laboratories for about 20 years (5). In the 1990s,

discrete or random-access analyzers displaced the batch and centrifugal analyzers. Discrete analyzers allowed to test each sample in a separate reaction chamber with individual reagents and used positive displacement pipettes with different volumes, washing steps in-between processes, automatic mixing, and controlled temperature throughout the test (5).

Modern analyzers still use automated discrete systems, but they now count with an array of analytical techniques such as electrophoresis, liquid chromatography-mass spectrometry, colorimetry/ spectrophotometry, potentiometry, and immunoassay (6). They usually have an ion-specific electrode (ISE) for electrolyte analysis and employ spectrophotometry for colorimetric and/or immunoassay tests for the rest of the analytes (2). Modern analyzers can be categorized in two types according to the reagents they use, if they only support reagents produced by the instrument's manufacturer, they are considered a "closed system", while, if reagents from different companies are suitable, they are considered an "open system". The advantage of a closed system is that the reagents are validated and verified by the manufacturer, but they are usually more expensive, and they may not be widely available. On the other side, open systems allow each laboratory to choose the reagent manufacturer of their preference, but each laboratory needs to do a method validation for every assay they want to adopt (2,3). Historically, clinical chemistry analyzers developed for human medicine have been used successfully in veterinary medicine, but evaluating their performance with non-human blood is critical due to the many physiological and biochemical differences among species, resulting in some analyzers that cannot meet the veterinary needs (7). Due to these downsides, in the decade of the 1990s, some companies started developing veterinary-specific chemistry analyzers like the CDC Technologies CHEMVET, and the Idexx VetTest/VetLyte, which quickly gained popularity in the veterinary field (8).

In the same decade, point-of-care (POC) devices were created and represented a breakthrough for human medicine by allowing to measure biochemical markers accurately within minutes in various settings (9). Those analyzers are smaller and more specialized than benchtop instruments, so they are cheaper but can perform fewer tests. They commonly work with single-use consumables that allow the systems to be cleaner and need less maintenance, since the samples are not in direct contact with the analyzer. They also indirectly reduce sample mislabeling and mishandling, and the results are obtained faster, allowing for rapid and effective treatment in critical situations (10). These POC devices were so successful and convenient that in 2009, 25% of the tests done in human medicine were performed at the site of care, and their usage has increased significantly since then (11).

Just as what happened previously with the traditional analyzers, the POC devices were rapidly adopted in veterinary medicine mainly due to their portability, which allowed immediate analysis of the samples even in field conditions (12). Portable analyzers permitted the use of clinical chemistry in species where it was not possible before and enabled the obtention of invaluable information about the physiology and certain metabolic conditions of several domestic and wild animals (13). The major challenge for the routine utilization of portable analyzers in field conditions is the fluctuating environmental conditions in the outdoor settings where they are used, and the devices must be able to compensate for those variations (14). Several POC analyzers have been validated and successfully used in the clinical and field setting for dogs, cats (15–17), cattle, horses, sheep (18), and some species of wild birds such as harlequin ducks (19), amazon parrots (20), and owls (21). Conversely, portable analyzers are seldomly used for diagnosis in commercial poultry species and there are only few validation studies performed in laying hens (22) and broiler chickens (23).

Validation of Portable Blood Analyzers. Method validation in clinical chemistry refers to the process of demonstrating that a device can consistently fulfill the requirements for its intended use (24). It is necessary to thoroughly validate any benchtop and point-of-care analyzer before using it for clinical applications. There are usually two phases in the validation of an analyzer. The first aims to verify the manufacturer performance specifications, which are obtained with in-vitro techniques and all manufacturers are legally required to provide. This phase must be done independently by all laboratories aiming to use a device so they can verify that its characteristics can be reproduced locally, and it is a requirement for laboratories to obtain full CLIA accreditation.

In the past, the first phase was considered enough to validate a new device, but it was questionable whether proof of only meeting pre-specified criteria was sufficient, so a second phase of evaluation to assess the clinical performance of the analyzer in a specific situation, such as a relevant group of patients or location, was implemented. This second phase is accomplished by comparing the results of the new device with those obtained with a "reference" analyzer (25). Validation is critical to assess and monitor POC analyzers since they are more prone to poor performance due to several reasons, such as inadequate and inconsistent quality maintenance, usage in less controlled environments than a laboratory, and handling by a variety of users with different backgrounds and experience. There is a variety of guidelines to perform method validation and requirements vary between countries. In the U.S., chemistry analyzers must meet the Clinical Laboratory Improvement Amendments (CLIA) guidelines (26,27). Before the start of the validation process, it is important to choose the POC device that better adapts to the clinical application and the environmental settings in which it will be used. Once the device is selected, it is critical to choose the samples that will be used throughout the process, since

validation parameters can vary according to that. Equally important, is to use samples from the target patient group in whom the test is to be used, as many parameters can vary even within the same species (25).

The first step towards the verification process is to assess the imprecision or random error of the POC device. This parameter measures the analytical variability of the analyzer and it is usually provided by the manufacturer but if not, it can be estimated by testing 20 or more samples in duplicate in a single run, and calculate the coefficient of variation of the results (28,29). Subsequently, the systematic error needs to be calculated since it estimates how different the POC analyzer results are from the values obtained with the reference analyzer. Systematic error is subdivided into constant and proportional bias. Constant bias means that one analyzer measures consistently higher or lower values in comparison with the other, while a proportional bias indicates that the differences between the two analyzers are proportionally related to the number of the measurements (30,31). Systematic error can be analyzed using the Passing-Bablok regression or the Deming regression; constant bias exists if the intercept differs from 0, and a proportional bias is present if the slope differs from 1 (32,33).

Finally, the accuracy needs to be determined by measuring the agreement between the results of the POC device and the reference analyzer, which is fully validated and quality controlled (34). It is recommended to test at least 40 samples across the working range of the analyzers, which should cover the spectrum of conditions expected in their routine usage. Also, it is better to perform a duplicate analysis of each sample so obvious outliers can be identified (35,36). The most common way to determine accuracy is to plot the data obtained with the POC device and the reference analyzer in a difference plot (also known as Bland-Altman plot). This method plots the difference between the results on the y-axis (A-B) against the mean of the

results on the x-axis ([A+B]/2). Then, limits of agreement are calculated and added to the Bland-Altman plot. The limits of agreement can be estimated using the inherent imprecision (coefficient of variation) of each analyzer (37), or by calculating two standard deviations above and below from the mean difference of the results (38–40). If the differences are symmetrically distributed around 0 and more than 95% of the differences lie within the limits of agreement, the POC analyzer is accurate and in agreement with the laboratory standard, so the analyzers can be used interchangeably (37,41). If the POC device is deemed inaccurate, it cannot be considered identical to the already established laboratory standards and new reference intervals for the POC analyzer must be calculated (37).

Determination of Blood Reference Intervals. A reference interval (RI) refers to the range of values that are considered normal for a physiological measurement in healthy individuals of a specific population. RIs are necessary for clinicians to interpret laboratory test results and they usually consist of the central 95% of the values obtained from reference individuals, with the limits being the 0.025 and 0.0975 fractiles (42–45). The Clinical and Laboratory Standards Institute (CLSI) recommends two general approaches to determine reference intervals for any analyte in a certain population; the first is to verify an already existing RI, either from the analyzer's manufacturer or from another clinical laboratory, and the second is to establish new RI from tests made in reference individuals (46). The RI verification is a relatively simple process that consists of collecting and testing samples from 20 apparently healthy patients in the new analyzer, and if fewer than 2 samples fall outside the RI being verified, it is statistically valid to adopt them (46,47). Nonetheless, the verification is only valid if the RI were obtained properly and in a well-documented process, and if the analyzers and the patient populations are comparable (48). The generation of new RI on the other hand, is a long

process that can be hard for some laboratories to conduct, but necessary if the validation of a RI fails or if there are no prior RI to verify (46,48). Any laboratory conducting a RI determination must fulfill the following requirements before the samples obtention: a) minimize all factors of variation for each analyte; b) establish inclusion and exclusion criteria for the selection of reference individuals according to the population to be tested; c) standardize and document the whole process; and d) keep up-to-date and reliable quality control on the analyzer to be used (48–50). It is recommended to test a minimum of 120 samples to obtain reliable values using a non-parametric technique, but if it is too difficult to achieve that number, the laboratory should run as many samples as possible and/or consider using parametric techniques (46). Once the data is obtained, it needs to be evaluated to identify any errors, tested for outliers using the Dixon-Reed or Tukey's tests (51), and subsequently calculate the RI and their confidence intervals. The CLSI recommends calculating the RI using the non-parametric ranking method since it makes no assumptions about the data distribution, but parametric techniques can be considered as well if the data shows a normal distribution or can be transformed into it (51–55). Fewer samples are required (~40 samples) when using parametric methods, but the data usually need to be transformed and there is a higher potential for bias when calculating the upper RI in highly right-skewed distributions (56). Separation of RI into subcategories according to sex or age may be considered if it is clinically relevant to the species studied (46). Each laboratory should select the method that fits better to the type of data they collect, and the calculated RI values must not vary if the samples are collected and analyzed properly (46,47).

Avian Blood Biochemistry. Widespread utilization and research of clinical biochemistry in avian species started later than in mammals, since older analyzers required large blood sample volumes that were impossible to obtain from most birds. Its development was very important

since it provided avian clinicians with an alternative diagnostic tool to necropsy, which was, and still is very common in commercial poultry but was not feasible for routine diagnosis in pet or wild birds (57). Nowadays, clinical biochemistry is used extensively in pet and wild bird medicine, and lately, due to the increased availability and accessibility of portable analyzers, it is drawing some attention to be used for diagnostic purposes in commercial flocks of chickens and turkeys (57–60).

Many factors must be considered when using clinical biochemistry in avian species, from the handling of the birds and the sample collection to the interpretation of the results according to their species, physiology, and purpose. Birds should be allowed 10 to 15 minutes after the physical exam to perform the sample collection, and it must be completed as fast as possible, since handling and restraining of the birds induce significant stress that could be associated with changes in certain biochemical parameters. For instance, significant elevations in glucose concentrations and decreased uric acid levels have been observed in songbirds subjected to acute capture and restraint (57). It has been demonstrated that in general, birds withstand blood loss better than mammals, with certain species tolerating losses of up to 2% of their body weight (61). Nonetheless, it is recommended that a sample volume should never exceed 1% of the body weight of the bird unless it is strictly necessary. The preferred site for blood collection in most avian species is the right jugular vein since it is readily available, and it is less prone to hematomas due to its thick wall (62,63). The radial vein, which is located under the wing of all birds, is a good alternative and it can be easier to locate than the right jugular vein in some species; the downside though is that it is more fragile and the development of hematomas after the procedure is very likely (61). Another anatomic site used for collection, primarily in

waterfowl (i.e. ducks and geese), is the metatarsal vein, located on the medial side of the leg, next to the hock joint (61).

Heparin is the preferred anticoagulant for avian blood since EDTA disrupts the red blood cells of many avian species (64). Nearly all the analytes that are measured routinely in mammalian species are useful for avian species as well. Measurement of **plasma proteins** is critical since, when interpreted along with other analytes, it can aid in the diagnosis of several conditions. By determining the Total protein (TP) value, and the Albumin/Globulin (A/G) ratio, clinicians can evaluate different processes in birds such as dehydration, acute and chronic inflammation, malnutrition, and gastrointestinal diseases (57,65). It is important to consider that female birds that are in the onset of, and during laying, will show significantly higher levels of plasma total protein because the yolk precursors are being transported from the liver to the ovary (65,66).

Renal function and damage are primarily assessed with the uric acid (UA) concentrations. Because birds are considered uricotelic animals, which means they excrete uric acid instead of urea as nitrogenous waste (67), creatinine is not produced and blood urea nitrogen (BUN) is produced in minimum amounts. Uric acid is effectively excreted by the kidneys in a process independent of the hydration status, so UA levels are not a good indicator of prerenal azotemia conditions, such as dehydration (68). Nonetheless, it is useful to detect direct renal damage and its concentration will increase after the kidneys function falls below 30% of their original capacity. Elevated UA concentrations may also indicate excessive protein intake in the diet or postrenal obstructions, which can result in visceral and/or articular gout in the short to medium term, as the uric acid precipitates in the abdominal viscera and the joints of affected birds (57,69).

Several enzymes are available to evaluate **liver function** and their diagnostic value depends on their half-life time and the cell part where they occur (e.g., cytoplasm [aspartate aminotransferase, AST; alanine aminotransferase, ALT; lactate dehydrogenase, LDH], cell membranes [alkaline phosphatase, AP; gamma-glutamyl transferase, GGT], mitochondria [glutamate dehydrogenase, GLDH; AST]). In general, an increase in these enzymes indicate organ damage rather than decreased function. Cytoplasmic enzymes will be released early after cell damage, while mitochondrial enzymes are released after sustained and severe damage (70). AST and bile acids (BA) are the most sensitive indicators of liver damage, followed by ALT, GGT, and LDH. GLDH elevations are observed only after moderate liver necrosis is present (57). It is important to consider that AST concentrations can also increase as a result of muscle damage; so, plasma levels of GLDH (liver-specific) and CK (muscle-specific) need to be reviewed to be able to tell where the elevation might be originating. If the differentiating enzymes are not available, BA concentrations can be used instead of AST (57,60,65). GGT is a reliable indicator of cholestatic diseases in birds, whereas AP is rarely associated with liver conditions and is more indicative of increased osteoblastic activity and impending ovulation (61,65).

Creatine kinase (CK) is the most significant and specific enzyme to **evaluate muscle** damage in avian species; other less specific enzymes that can be useful are AST, ALT, and LDH (60,65). It is usual to find increases in the concentration of muscle enzymes caused by other factors rather than disease, such as capture, handling, stress, exercise, or recent intramuscular injections (71). Some common causes of direct muscle damage are ionophore toxicity (72), exertional rhabdomyolysis (73,74), and vitamin E or selenium deficiency (69).

Physiological values of **glucose** vary among species of birds and increases can be observed in periods of stress. Diabetes is uncommon in avian species, but a spontaneous form of the disease has been reported sporadically in several wild birds (60,65). The disease known as "starve-out" is often observed in commercial chicks, and although the etiology is unclear, it is suspected to be related to a sustained hypoglycemic condition (69).

Electrolytes are commonly evaluated in avian species and their function is essentially the same as in mammals; regulation of osmotic pressure, water distribution, electroneutrality, and regulation of nervous, cardiac, and muscular function. The most assessed analytes are sodium (Na^+), potassium (K^+), and chloride (Cl^-), and any abnormalities in their concentration are indicative of a disturbance in the water distribution or in the acid-base status (65). Calcium is another important electrolyte that can be assessed in birds since it has a critical role in many physiological processes such as ossification, excitability of nervous tissues, and oviposition. Between 50 to 60% of the plasma calcium present in the organism is bound to blood proteins and non-protein anions and it is biologically inactive (67). There are two different ways to determine calcium concentration; the total calcium (tCa) which measures free and protein-bound calcium, and the ionized calcium (iCa) that only measures the free and active calcium. In the past, it was only possible to measure total calcium, so correction formulas based on total protein and albumin levels were necessary to obtain an estimated amount of the free calcium present (75,76). Nowadays, analyzers can determine the levels of iCa directly, providing a more accurate assessment of the calcium status. Some common conditions that cause hypercalcemia in birds are over-supplementation of calcium or vitamin D_3 in the diet, osteolytic skeletal tumors, and estrogen-induced hypercalcemia, which is associated with oviposition (57). Hypocalcemia is frequently observed in laying hens with insufficient levels of calcium in their diet; a condition

that has been denominated “cage layer fatigue” (69). Levels of phosphorus (P) can also be assessed, particularly in conjunction with calcium to obtain the Ca:P ratio. In healthy laying birds, this ratio should always stay above 1 and any decrease should be of concern as it may indicate a renal issue (60). A comprehensive review of all the analytes that can be assessed in avian species, along with their measurement methods, their physiology, and their diagnostic value can be found in (65).

Reference Intervals in Domestic and Wild Birds. Reference intervals are the cornerstone for the interpretation of clinical biochemistry results as they serve as a guideline for the normal values expected in healthy patients. As avian medicine gained popularity, many research groups and clinical laboratories started establishing reference intervals for wild bird species using a variety of analyzers (65). Reference intervals exist for many species of psittacines (57,65,77–79), owls (21,79), ostriches (79,80), ducks (79), pigeons (57,65,79), wild turkeys (81), white storks (82), eagles (79), and hawks and falcons (57,65,79). Reference intervals for commercial poultry are scarcer due to difficulties with sample handling and transportation to clinical laboratories, but with the increased accessibility to portable analyzers in the latest years, some RI have been determined for backyard hens (83), broiler breeders (84), laying hens (85), and replacement pullets (86). Data about normal biochemical values is still lacking on broiler chickens and commercial turkeys. More information on clinical chemistry and reference intervals for commercial poultry is necessary to provide clinicians with a reliable and solid tool to diagnose and better understand some of the metabolic conditions that currently affect broiler chickens.

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CHAPTER 2. Comparison of Biochemical Test Results between Portable and Conventional Laboratory Analyzers in Chickens

Ruiz-Jimenez F., Gruber E., Correa M., Crespo R. Comparison of Portable and Conventional Laboratory Analyzers for Biochemical Tests in Chickens. Poult. Sci. 2021;100(2):746-754.

ABSTRACT

Antemortem blood biochemical and blood gas analyses are routinely used in health screening and diagnosis of disease in domestic veterinary species. These testing modalities are not routinely performed in poultry, in part, due to the distance from the diagnostic laboratory. Portable blood analyzers such as the i-STAT and VetScan (VS2) can be used to obtain results on the farm without delay, potentially offering a more practical option for poultry practitioners. We investigated the time effect on blood chemistry values and compared the results obtained using the i-STAT and VS2 with those obtained using conventional laboratory analyzers (GEM Premier 3000 and Cobas c501, respectively).We tested blood from 60 healthy chickens. Each sample was tested in triplicate using each of the portable analyzers and once using conventional analyzers. All samples were analyzed within 60 minutes of collection. The concentrations of some analytes were outside the limit of detection of the portable analyzers (i.e., bile acids). Although statistically significant differences were found for some biochemical analytes over time, the actual mean or median differences were too small to be considered of clinical importance. As observed in mammals, significant time-dependent changes in blood gas analytes were observed in whole blood samples exposed to ambient air. Correlation coefficients between portable and conventional analyzers were moderate to high for most of the analytes. For the most part, there was an agreement between the portable and conventional analyzers. We identified constant and

proportional biases in the measurement of multiple analytes by both the i-STAT and VS2. Future studies are warranted to establish analyzer-specific reference intervals for poultry.

INTRODUCTION

Antemortem blood tests, including the complete blood count and biochemical analysis, are routinely used to evaluate health status, diagnose disease, guide medical decisions, and assess the progression of disease in a variety of veterinary species (1). The lack of quality of biochemical data in poultry may impair our ability to properly diagnose diseases and monitor flock health status (2,3). Although other routine poultry diagnostic methods including necropsy, serology, and microbiologic testing are excellent for diagnosis of infectious diseases (4,5), these testing modalities neither are sufficient to diagnose metabolic conditions (6), nor can be used as part of flock health management to determine nutritional deficiencies before the development of clinical signs. Therefore, the antemortem biochemical analysis may be an important diagnostic modality in poultry medicine that deserves further investigation.

A delay between sample collection and testing can cause significant changes in the concentration of some blood analytes owing to factors including hemolysis, continued cellular metabolism, evaporation, and altered enzyme activities (7). In human and small animal medicine, this may not represent a significant challenge, given that in most cases, clinics and hospitals have an on-site laboratory and blood samples can be tested with minimal delay. In production animals, prompt testing may be hindered by the physical distance and time needed to reach a laboratory. In addition, avian red blood cells are nucleated, and their metabolism could be faster than their mammalian counterparts (7), potentially accelerating storage-related changes to analyte concentrations.

Several studies have reported reference intervals for hematologic, biochemistry, and blood gas analytes in production poultry (2,8–13). However, owing to lack of prompt access to laboratory services, requirements for sample preservation and handling, and cost, these tests are rarely used in routine poultry medicine (2). Portable analyzers, such as the i-STAT (Abbott Laboratories, Chicago, IL) or VetScan VS2 (VS2; Abaxis, Inc., Union City, CA), can be used to measure biochemical analytes directly on the farm, thus mitigating sample handling and transportation challenges. These analyzers have been used in clinical settings for other avian species, including Strigiformes and Psittacines (3,14,15).

Before the i-STAT and VS2 portable analyzers can be used in commercial poultry with confidence, it is important to determine whether the results obtained using these instruments are comparable with those obtained using conventional laboratory analyzers. Hence, the aims of this study were to 1) assess the comparability of the i-STAT using the CG81 cartridge and VS2 using the Avian/Reptile rotor with their respective conventional laboratory analyzers: the GEM 3000 and Cobas c501, and 2) evaluate the effects that time has on blood analytes.

MATERIALS AND METHODS

Animal and Housing. Five thousand 1-day-old Ross 708 chickens provided by a commercial broiler integrator were housed at the poultry barn of the Teaching Animal Unit at the College of Veterinary Medicine, North Carolina State University (CVM-NCSU). The chickens were vaccinated in ovo for Marek's Disease and Infectious Bursal Disease. They were vaccinated for Coccidiosis and Infectious Bronchitis Virus after hatching and before delivery. All birds were managed under the same environmental conditions. A commercial feed was provided by the integrator. All animal handling and blood collection protocols were reviewed

and approved by the NCSU Institutional Animal Care and Use Committee (IACUC protocol number: 19-001).

Sample Collection and Handling. Sixty chickens were sampled in 3 groups of 20 chickens on 3 different days. At each sampling time, birds were marked with food coloring dye to ensure that no bird was tested more than once. Venipuncture was performed via the jugular vein in non-anesthetized chickens using disposable 1-mL or 3-mL syringes with 21-gauge heparin flashed needles. As recommended by *Owen et al.* (16), to prevent hemolysis, the needle was removed before the immediate transfer of 1 to 2 mL of blood into a lithium heparin collection tube (BD Biosciences, Franklin Lakes, NJ). The blood was gently mixed and stored at 4°C until analysis.

Blood Chemistry and Gas Analysis. The following analytes were measured in whole blood using CG8+ cartridges on the i-STAT: sodium (Na^+; mmol/L), potassium (K^+; mmol/L), ionized calcium (iCa; mmol/L), glucose (Glu; mg/dl), hematocrit (Hct), pH, oxygen partial pressure (pO_2; mm Hg), carbon dioxide partial pressure (pCO_2; mm Hg), bicarbonate (HCO_3; mmol/L), total carbon dioxide (TCO_2; mmol/L), base excess (BE; mmol/L), and oxygen saturation (sO_2; %). The samples were measured according to the manufacturer's instructions, and instrumentation was kept within the recommended operating temperature parameters. The results obtained using the i-STAT were compared with those obtained using the GEM Premier 3000 blood gas system (Instrumentation Laboratories, Bedford, MA), which was calibrated and maintained by the CVM-NCSU Clinical Pathology laboratory.

The following analytes were measured in whole blood using avian/reptilian specific reagent rotors on the VS2: aspartate aminotransferase (AST; U/L), bile acids (BA; μmol/L), creatine kinase (CK; U/L), uric acid (UA; mg/dL), glucose (Glu; mg/dL), total calcium (Ca;

mg/dL), phosphorus (P; mg/dL), total protein (TP; g/dL), albumin (Alb; g/dL), potassium (K^+; mmol/L), and sodium (Na^+; mmol/L). The samples were measured according to the manufacturer's instructions, and instrumentation was kept within the recommended operating temperature parameters. The results obtained using the VS2 were compared with those obtained using the Roche Cobas c501 chemistry analyzer (Roche Diagnostics, Basel, Switzerland), which is calibrated and maintained by the CVM-NCSU Clinical Pathology laboratory.

Figure 2.1 is a schematic of the experimental workflow. In brief, each blood sample was analyzed in 3 serial replicates in each instrument, approximately 20 minutes apart. Blood samples were kept at 4°C between testing times, and all analyses were completed within 60 minutes. The first 2 replicates (time 1 [T1] and time 2 [T2]) were completed at the Teaching Animal Unit poultry barn. The third replicate (T3) was completed in the Clinical Pathology laboratory at CVM-NCSU to allow simultaneous measurement by the portable analyzers and the GEM Premier 3000 and Cobas c501. Analysis using the Cobas c501 required an additional step to separate the plasma from the sample by centrifugation at 800 *x g* for 10 minutes. The values obtained using each portable analyzer at T3 were compared with results obtained simultaneously using either the GEM Premier 3000 or Cobas c501.

Statistical analysis. Statistical analyses and figures were obtained using MedCalc software (Medcalc version 19.1.5; Ostend, Belgium; https://www.medcalc.org; 2020). Descriptive statistics were estimated for each analyte using the portable analyzers at each time of measurement and the conventional instruments. The normality of the data was determined using the D'Agostino-Pearson normality test (17). If data were not normally distributed, nonparametric tests were considered.

Comparison of analytes measured over time with the portable analyzers. Repeated measures ANOVA or Friedman's tests were considered for comparisons of analytes through time for the i-STAT and VS2 analyzers. The α-value was set at ≤0.05. Mountain plots were used for visualization of possible differences between times for the same analyte (18). These plots compare the median of tests for T2 and T3 against T1 (given that T1 was in the immediacy of blood collection). In this plot, the closer the median difference value is to 0 (median of T2 and T3 values to T1), the closer the test results are to T1.

Comparison of portable and conventional analyzers. Pearson correlation coefficients were obtained to measure the linear association of portable and conventional analyzers. Bland–Altman plots were used for visualization and quantification of the agreement of the results obtained by the i-STAT and VS2 analyzers at T3 with the results obtained using the respective conventional analyzers. Passing–Bablok regression analysis was used to estimate constant and proportional bias between analytical methods. Constant bias indicates that the test analyzer consistently measures an analyte concentration to be higher or lower in comparison with the reference analyzer. Proportional bias indicates that the differences in measurements of the test and reference analyzer are proportional to the level of measurement. This is a nonparametric regression, and nonlinear samples are not suitable for Passing–Bablok analysis (19–21).

RESULTS

Descriptive statistics of all the analytes are summarized in Tables 2.1 and 2.2. Data results outside the detection range of the instruments were excluded. More than 90% of the bile acid measurements were lower than the detection limits of the VS2; thus, this analyte was excluded from further analysis. The low concentrations of this enzyme may be due to a normal

lower production in commercial broilers. For CK, only 41 samples (68%) were included. The other 19 samples had a CK value higher than the limit of detection for the VS2 and were excluded.

All the blood gas analytes (i.e., HCO_3, pCO_2, pO_2, sO_2, tCO_2, BE, and pH) measured using the i-STAT analyzer showed statistically significant differences between measurements taken at T1, T2, and T3 (Table 2.3). Ionized calcium and K^+ showed statistically significant differences between times as well. Glucose, Hct, and Na^+ did not show a statistically significant difference between measurement times (Table 2.3). The mountain plot for pO_2 (Figure 2.2) is a representative plot illustrating the time-dependent differences in blood gas concentrations.

On the VS2, statistically significant differences in replicate measurements were observed for AST, CK, UA, Ca, P, K1, and Na^+ (Table 2.4). Notably, the numeric differences were small for each of these analytes. Glucose, TP, and ALB did not show any statistically significant differences between time points (Table 2.4). The mountain plot for TP (Figure 2.3) is a representative example illustrating an analyte with no statistically significant time-dependent differences in concentration.

The i-STAT showed a constant negative bias for Hct and a proportional bias for pCO_2. Base excess, pH, and sO_2 had both constant and proportional bias. The Bland–Altman plots showed a high agreement (>95%) between the i-STAT and GEM 3000 for iCa, K^+, Na^+, HCO_3, pCO_2, pH, and tCO_2. Agreement was moderate (50–95%) for Glu, Hct, BE, pO_2, and sO_2 (Table 2.5, Figure 2.4). None of the analytes showed less than 50% of agreement.

The statistical results for the comparison between VS2 and Cobas c501 are summarized in Table 2.6. The VS2 had a constant positive bias for Ca and TP and a negative constant bias for Na^+. The VS2 had a negative proportional bias for Glu. The VS2 had combined constant and

proportional biases for AST, UA, and K^+. The Bland–Altman plots showed a high agreement (>95%) between the VS2 and the Cobas c501 for Ca, TP, Alb, K^+, and Na^+ (Table 2.6 and Figure 2.5). Moderate agreement (50–94%) was observed for AST, CK, UA, Glu, and P. None of the analytes had a poor agreement (<50%).

DISCUSSION

Because the i-STAT and the VS2 cartridges measured different analytes, we keep the discussion for these analyzers separate. We first discuss the finding for the i-STAT over time, followed by the VS2. Then, we discuss differences between the portable and the conventional analyzers.

Significant time-dependent differences in blood gas concentrations (e.g., pO_2, sO_2, pCO_2, tCO_2) were observed as measured using the i-STAT. The blood collection container was opened 3 different times for analysis, exposing the sample to air. Ambient air oxygen and carbon dioxide concentrations are higher and lower than in blood, respectively. In mammalian species, blood gases diffuse readily, and it has been shown that the presence of even 1% of air bubbles in a blood sample can result in significant modifications in the blood gases concentration (e.g., pO_2, sO_2, pCO_2, or tCO_2) (22). In addition, there is evidence that modifications in blood pH are inversely related to changes in pCO_2 concentrations (7). In our studies, we demonstrate that similar rapid changes in blood gas concentrations are observed in avian blood exposed to ambient air. The decreases in iCa and K^+ concentration over time are most likely due to the increased pH. Increased pH, or alkalosis, promotes calcium binding to blood proteins, thus decreasing measured iCa (23). Alkalosis also induces transcellular shifting of K^+. Extracellular K^+ is driven into the cells in exchange for intracellular hydrogen, thus reducing measured

serum/plasma K^+ (24). Glucose content was relatively stable across time points, which was expected given the short duration of the experiment and that the samples were stored in ice, thus decreasing cellular metabolism (25).

For analytes measured using the VS2, we observed statistically significant differences in concentrations of AST, CK, UA, Ca, P, K^+, and Na^+ over time. When interpreting these findings, it is critical to consider the degree of change in each analyte because a statistically significant change may not correspond to a clinically relevant change (26). Over the short time course of these experiments, we interpreted the time-dependent changes in all analytes measured using the VS2 to likely be negligible and unlikely to change clinical interpretation. Still, it is important to note that there are no studies of stability on avian or reptilian blood; thus, further studies on this subject are advised.

In our study, the i-STAT analyzer with the CG8+ cartridge showed moderate to high agreement for measurements in comparison with the GEM 3000 across the entire range of values measured. *Steinmetz et al.* (27), evaluated the performance of the i-STAT CG7+ cartridge, which measures the same analytes as the CG8+ except for Glu, to the Siemens Rapidlab 800 analyzer in anesthetized chickens and found moderate to high correlation between all the analytes, except for K^+ and BE. The agreement between the portable and the conventional analyzer used in this study was not reported.

The agreement between VS2 and Cobas c501 was high for Ca, TP, Alb, K^+, and Na^+ and moderate (90–95%) for AST, CK, UA, Glu, and P. In contrast, other studies on Psittacines (15) and Strigiformes (3) found high to moderate agreement in fewer analytes. Nevertheless, the results from both studies coincided with ours for AST, CK, Glu, and TP.

It is important to consider the method that each of the instruments uses to report the concentration of the analytes because differences could contribute to the degree of agreement (21). All 4 analyzers used in this study use ion-selective electrode potentiometry to measure the concentration of Na^+, K^+, and pH. Still, the exact composition of the electrodes may differ, contributing to minor differences in the measurement of these analytes. The blood gas analytes, HCO_3 and BE, are calculated values derived from the directly measured pH and pCO_2. The GEM 3000 and i-STAT use the same calculations for HCO_3 and BE: $HCO3 = 10^{(pH+\log(pCO2)-7.608)}$ and $BE = HCO3 - 24.8 + 16.2 * (pH - 7.4)$ (28–31). Therefore, differences in the calculation do not explain the differences in the reported values for these analytes. Temperature is another important factor that could affect the results, particularly for pH. Because pH is temperature dependent, it is typically measured at 37°C, and a correction is needed whenever the temperature of the sample varies (32). In this study, the blood was at chickens' temperature only for the T1 analysis, and it was kept at 4°C afterward. The GEM 3000 preheats the samples at 37°C before analysis (28), but the i-STAT does not (30); thus, it is possible that the reported i-STAT pH measurements at T2 and T3 are higher than if they had been measured in blood at 37°C. The effect of temperature on the measurement of pH in avian blood is beyond the scope of the present study, but would warrant further investigation and consideration, particularly for studies using blood pH as a critical indicator of health or metabolic status.

Passing–Bablok regression analysis (21) was used to determine constant and proportional bias of each analyte. The analysis showed either constant and/or proportional bias for some analytes (Tables 2.5 and 2.6). It is important to take biases into account because they can lead to erroneous interpretations of the results (33). With a constant bias, the new instrument values stay higher or lower than the reference instrument values by a certain amount as the level of the

analyte is increased. If the exact amount of modification is calculated, then this error can be fixed by just adjusting the results given by the new instrument. On the other hand, in a proportional bias, the new analyzer values are a fixed percentage of the reference analyzer values at all concentrations tested. We demonstrated constant and proportional biases for BE, pH, and sO_2 when using the CG8+ cartridge in the i-STAT analyzer, and for AST, UA, and K^+ using the avian and reptilian rotor in the VS2 analyzer. The presence of both constant and proportional biases complicates the interpretation of these analytes when using published reference intervals. Together, these results underscore the need to establish reference intervals for each analytic device.

Intriguingly, 19 of the 60 samples were above the limits of detection for CK, an enzyme found primarily in myocytes and commonly used as an indicator of muscular disease in poultry and many other species (34). Increased CK activity has been associated with muscle damage owing to either a normal physiological response to exercise (35–37) or pathologic conditions such as rhabdomyolysis, infections, metabolic disorders, prolonged inactivity, or temperature-induced states as malignant hyperthermia (38,39). Increases in this enzyme content have also been noted in poultry when the birds are subjected to acute heat stress (40,41) and ionophore toxicity (42). Further studies will need to be conducted to determine if the marked CK elevations observed in this study were due to the rapid but physiological growth of the modern chicken's muscles or if there is any chronic muscular damage or environmental condition involved.

Although clinically relevant differences in concentration of analytes are negligible within 1 hour of collection, time-dependent changes in multiple analytes were still observed, indicating the importance of testing blood samples as soon as possible after collection. The changes in blood gas concentrations reported here demonstrate the need to minimize exposure to air. Future

studies to determine the effect of time on blood gas analytes in whole blood collected anaerobically may be warranted.

Although the overall agreement was moderate to high for most analytes, constant, proportional, and mixed constant and proportional biases were observed for both analyzers. Thus, interpretation of results reported by the i-STAT or VS2 using published poultry reference intervals from conventional analyzers may lead to an erroneous diagnosis. Analyzer-specific reference intervals must be established for these analyzers to have confidence in the results.

DISCLOSURES

The authors declare that there are no conflicts of interest.

TABLES

Table 2.1. Descriptive statistics for the biochemical analytes measured using the i-STAT with CG8+ cartridges and the GEM 3000 analyzers.

Analyte	i-STAT (T1)			i-STAT (T2)			i-STAT (T3)			GEM 3000		
	Median	**Mean**	**SD**	**Median**	**Mean**	**SD**	**Median**	**Mean**	**SD**	**Median**	**Mean**	**SD**
Glu	228	227.86	22.14	228	228.29	22.13	229.5	228.57	21.74	227	226.22	22.1
Hct	19	20.54	5.25	19	20.84	5.17	19	20.65	5.25	25	27.2	5.98
iCa	1.16	1.17	0.15	1.17	1.16	0.15	1.16	1.15	0.14	1.28	1.27	0.13
K^+	5	5.13	0.69	5	5.1	0.68	5.1	5.08	0.62	5	5.2	0.7
Na^+	142	142.68	8.35	142	142.89	8.33	142	143.05	8.23	143	145.63	7.46
BE	-0.5	0.63	5.22	0	1.1	5.66	1	1.58	5.96	1.5	2.14	4.28
HCO_3	24.15	24.52	4.01	23.6	24.18	4.16	23.1	23.9	4.25	24.1	24.78	4.32
pCO_2	34.65	34.96	8.2	32.1	31.7	8.44	29.9	28.28	8.62	31	30.33	9.52
pH	7.44	7.46	0.12	7.5	7.5	0.14	7.52	7.55	0.16	7.54	7.54	0.12
pO_2	52	59.9	20.88	56	67.53	28.96	93	99.55	41.7	127	123.86	45.5
sO_2	88	88.39	6.89	91	91.18	5.84	98	95.81	4.94	99	97.58	3.45
tCO_2	25.5	25.65	4.11	25	25.19	4.22	24	24.9	4.69	25.15	25.71	4.43

Abbreviations: BE, base excess; Glu, glucose; Hct, hematocrit; iCa, ionized calcium; K^+, potassium; Na^+, sodium; pO_2, partial pressure of oxygen; sO_2, oxygen saturation; tCO_2, total carbon dioxide.

Table 2.2. Descriptive statistics for the biochemical analytes measured by the Vetscan VS2 (VS2) with Avian/Exotic rotors and the Cobas c501 analyzers.

Analyte	VS2 (T1)			VS2 (T2)			VS2 (T3)			Cobas c501		
	Median	**Mean**	**SD**	**Median**	**Mean**	**SD**	**Median**	**Mean**	**SD**	**Median**	**Mean**	**SD**
AST	173	236.8	157.5	173	240.6	158	178	236.7	150.8	172.0	239.6	168.6
CK	874	1123.2	641.5	887	1130.5	651.1	884	1111.3	639.6	905.0	1190.6	693.1
UA	4.9	5.1	2.0	4.9	5.2	2.0	4.6	5.2	2.1	5.6	5.9	1.9
Glu	235	235.3	16.4	236.0	235.5	15.8	233.5	235.3	16.2	250.5	252.2	17.8
Ca	11.3	11.4	0.7	11.5	11.5	0.7	11.4	11.4	0.6	10.9	10.9	0.9
P	6.5	6.6	0.8	6.8	6.8	0.8	6.8	7.0	0.9	6.9	7.0	0.8
TP	2.9	2.9	0.6	2.8	2.9	0.6	2.8	2.9	0.5	2.6	2.7	0.6
Alb	2.2	2.2	0.3	2.2	2.2	0.3	2.2	2.2	0.3	1.0	1.1	0.3
K^+	5.9	6.6	1.7	5.9	6.8	1.8	5.8	6.7	1.9	5.0	5.1	0.5
Na^+	150	151.3	4.0	150	150.7	4.1	149	150.0	3.7	150.5	151.3	3.6

Abbreviations: Alb, albumin; AST, aspartate aminotransferase; CK, creatine kinase; Glu, glucose; K^+, potassium; Na^+, sodium; TP, total protein; UA, uric acid.

Table 2.3. Friedman test results for the difference in the results over time for the i-STAT analyzer.

Analytes	P-value	Difference	Clinical relevance of the difference [1]
Glu	0.42350	---	---
Hct	0.24938	---	---
iCa	0.01813	T1 vs T3	Negligible
K^+	0.00016	T3 vs T1 and T2	Negligible
Na^+	0.32261	---	---
BE	0.00055	T3 vs T1 and T2	Negligible
HCO_3	<0.00001	T1 vs T2 and T3	Negligible
pCO_2	<0.00001	T1 vs T2 and T3, T2 vs T3	Negligible
pH	<0.00001	T1 vs T2 and T3	Negligible
pO_2	<0.00001	T1 vs T2 and T3	Effect of opening tubes
sO_2	<0.00001	T1 vs T2 and T3	Effect of opening tubes
tCO_2	<0.00001	T1 vs T2 and T3	Negligible

Abbreviations: BE, base excess; Glu, glucose; Hct, hematocrit; iCa, ionized calcium; K^+, potassium; Na^+, sodium; pCO_2, partial pressure of carbon dioxide; pO_2, partial pressure of oxygen; sO_2, oxygen saturation; tCO_2, total carbon dioxide.

[1] Compared with means or medians from Table 2.1

Table 2.4. Friedman test results for the difference in the results over time for the Vetscan VS2 analyzer

Analytes	P-value	Difference	Clinical relevance of the difference [1]
AST	<0.00001	T2 vs T1 and T3	Negligible
CK	0.00270	T1 vs T2 and T3	Negligible
UA	0.00008	T2 vs T1 and T3	Negligible
Glu	0.16396	---	---
Ca	<0.00001	T2 vs T1 and T3	Negligible
P	0.03695	T2 vs T3	Negligible
TP	0.09216	---	---
Alb	0.51123	---	---
K^+	0.03695	T2 vs T3	Negligible
Na^+	0.03695	T2 vs T3	Negligible

Abbreviations: Alb, albumin; AST, aspartate aminotransferase; CK, creatine kinase; Glu, glucose; K^+, potassium; Na^+, sodium; TP, total protein; UA, uric acid.

[1] Compared to means or medians from Table 2.4.

Table 2.5. Correlation, Passing-Bablok regression with constant and proportional bias, and Bland-Altman results for agreement between the i-STAT and the GEM 3000 analyzers.

		Passing-Bablok Linear Regression Analysis						**Bland-Altman Plot**	
Analytes	Correlation[1]	y-intercept	95% CI	Constant bias[2]	Slope	95% CI	Proportional bias[3]	WL[4]	%
Glu	0.81	21.53	-5.16 to 48.06	No	1.17	1 to 1.33	No	50/54	92.59
Hct	0.93	-3.22	-6 to -1.68	Yes	0.89	0.383 to 1.00	No	40/44	93.02
iCa	0.83	-0.27	-0.56 to 0.046	No	1.11	0.94 to 1.35	No	43/42	97.72
K^+	0.94	0.32	0 to 0.76	No	0.94	0.86 to 1.00	No	55/57	96.49
Na^+	0.69	-6.89	-37.50 to 1.00	No	1.04	1 to 1.25	No	55/57	96.49
BE	0.91	-1.54	-1.59 to -1.55	Yes	1.15	1.02 to 1.28	Yes	53/56	94.64
HCO_3	0.90	1.08	-1.64 to 3.75	No	0.91	0.79 to 1.02	No	55/56	98.21
pCO_2	0.91	1.90	-0.70 to 4.59	No	0.90	0.81 to 0.98	Yes	55/57	96.49
pH	0.95	-1.08	-1.69 to -0.44	Yes	1.14	1.06 to 1.22	Yes	55/57	96.49
pO_2	0.76	-6.77	-22 to 4.75	No	0.91	0.81 to 1.06	No	54/57	94.74
sO_2	0.80	-66.66	-100 to -37.69	Yes	1.67	1.38 to 2.00	Yes	54/56	94.73
tCO_2	0.90	1.05	-2.53 to 3.85	No	0.91	0.80 to 1.05	No	54/56	96.43

Abbreviations: BE, base excess; Glu, glucose; Hct, hematocrit; iCa, ionized calcium; K+, potassium; Na+, sodium; pCO_2, partial pressure of carbon dioxide; pO_2, partial pressure of oxygen; sO_2, oxygen saturation; tCO_2, total carbon dioxide.

[1] Pearson correlation coefficient

[2] Constant bias: the confidence interval for the y-intercept must include 0, otherwise, there is evidence of bias.

[3] Proportional bias: the confidence interval for the slope must include 1, otherwise, there is evidence of bias.

[4] WL indicates values that were within the limits of agreement based on the Bland-Altman plot. Denominators per analyte vary.

Table 2.6. Correlation, Passing-Bablok regression with constant and proportional bias, and Bland-Altman results for agreement between the VS2 and the Cobas c501 analyzers.

		Passing-Bablok Linear Regression Analysis						**Bland-Altman Plot**	
Analytes	Correlation[1]	y-intercept	95% CI	Constant bias[2]	Slope	95% CI	Proportional bias[3]	WL[4]	%
AST	0.98	19.60	15.42 to 22.17	Yes	0.90	0.89 to 0.93	Yes	51/54	94.44
CK	0.90	-65.69	-543. To 42.57	No	1.05	0.93 to 1.60	No	18/19	94.74
UA	0.88	-1.38	-1.83 to -0.91	Yes	1.11	1.03 to 1.18	Yes	55/59	93.22
Glu	0.87	21.53	-5.16 to 48.06	No	0.24	0.74 to 0.95	Yes	56/60	93.33
Ca	0.84	1.27	0.50 to 2.66	Yes	0.93	0.80 to 1.00	No	58/60	96.67
P	0.55	-0.10	-1.69 to 0.77	No	1.00	0.88 to 1.23	No	51/58	87.93
TP	0.97	0.20	0.20 to 0.20	Yes	1.00	1.00 to 1.00	No	57/60	95.00
Alb	0.37	0.60	0.00 to 1.15	No	1.5	1.00 to 2.00	No	59/60	98.33
K^+	0.50	-12.86	-19.71 to -8.13	Yes	3.85	2.91 to 5.23	Yes	55/57	96.50
Na^+	0.77	-12.73	-51.50 to -1.00	Yes	1.08	1.00 to 1.33	No	57/60	95.00

Abbreviations: Alb, albumin; AST, aspartate aminotransferase; CK, creatine kinase; Glu, glucose; K^+, potassium; Na^+, sodium; TP, total protein; UA, uric acid.

[a] Pearson correlation coefficient

[b] Constant bias: the confidence interval for the y-intercept must include 0, otherwise, there is evidence of bias.

[c] Proportional bias: the confidence interval for the slope must include 1, otherwise, there is evidence of bias.

[d] WL indicates the number of values that were within the limits of agreement based on the Bland-Altman plot. Denominators per analyte vary.

FIGURES

Figure 2.1. Experimental design and flow of the blood samples.

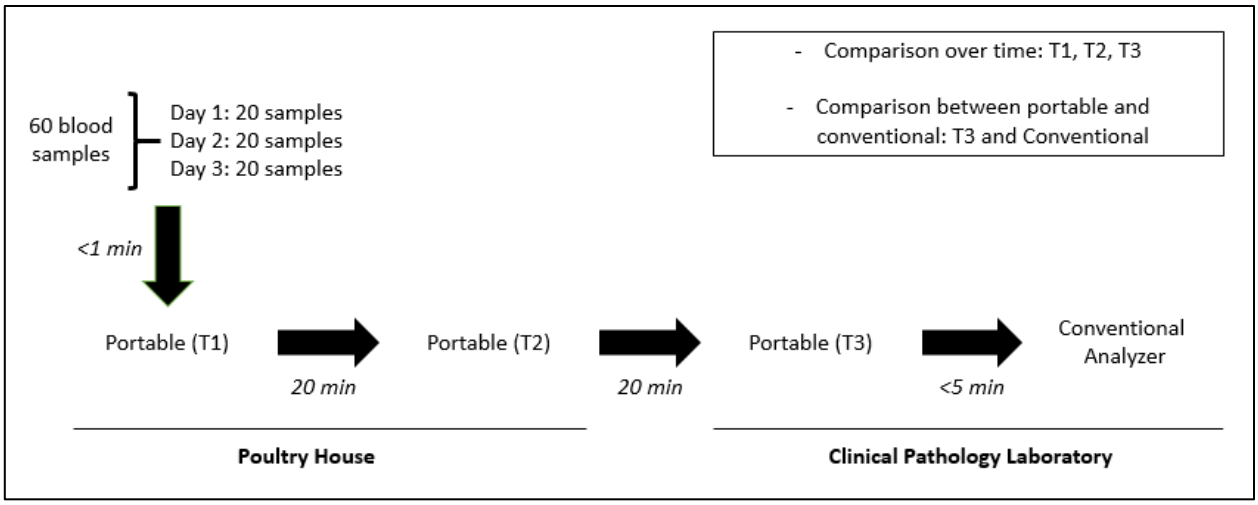


Figure 2.2. Mountain Plot showing an increase in the partial pressure of oxygen (pO_2) in whole blood at T2 and T3 compared with T1, as measured using the i-STAT.

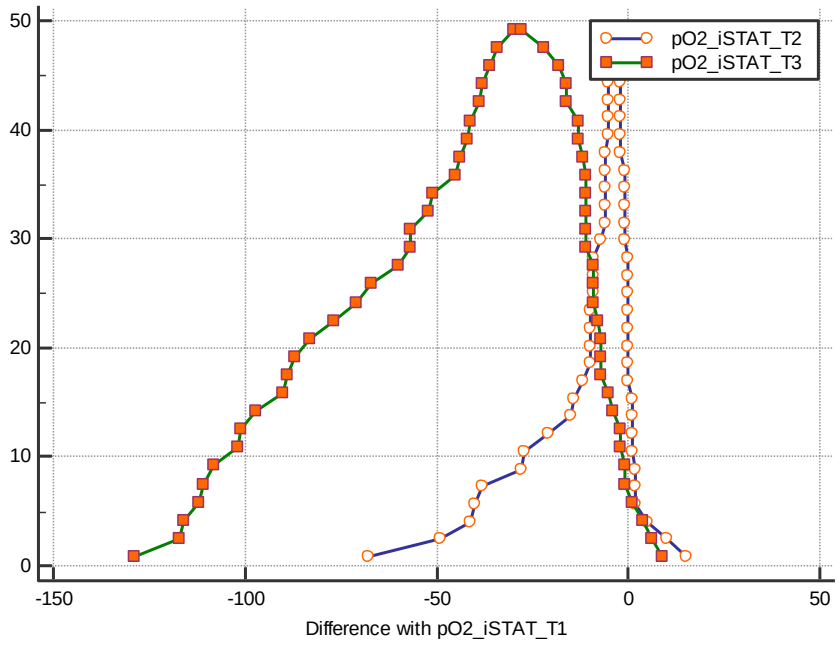


Figure 2.3. Mountain Plot showing no significant differences in total protein (TP) in whole blood at T2 or T3 compared with T1, as measured using the Vetscan VS2.

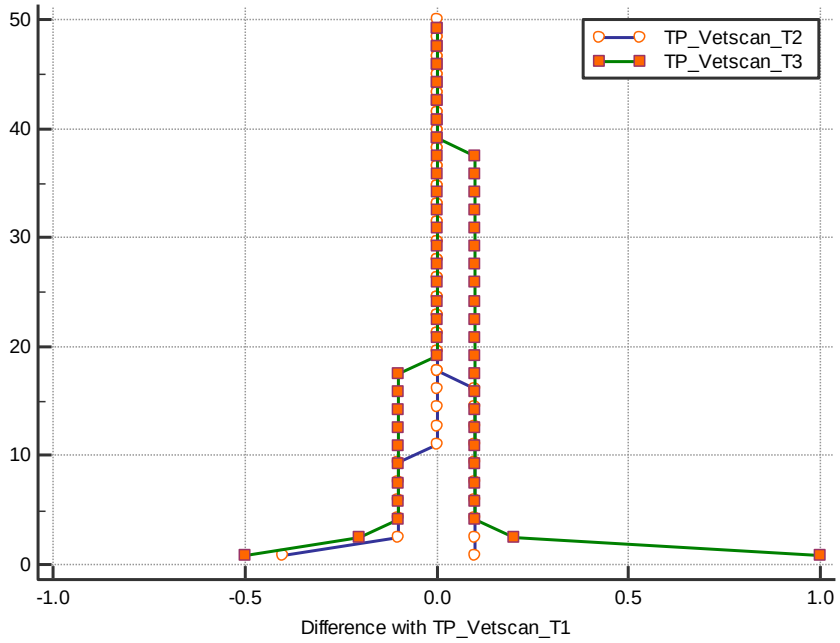
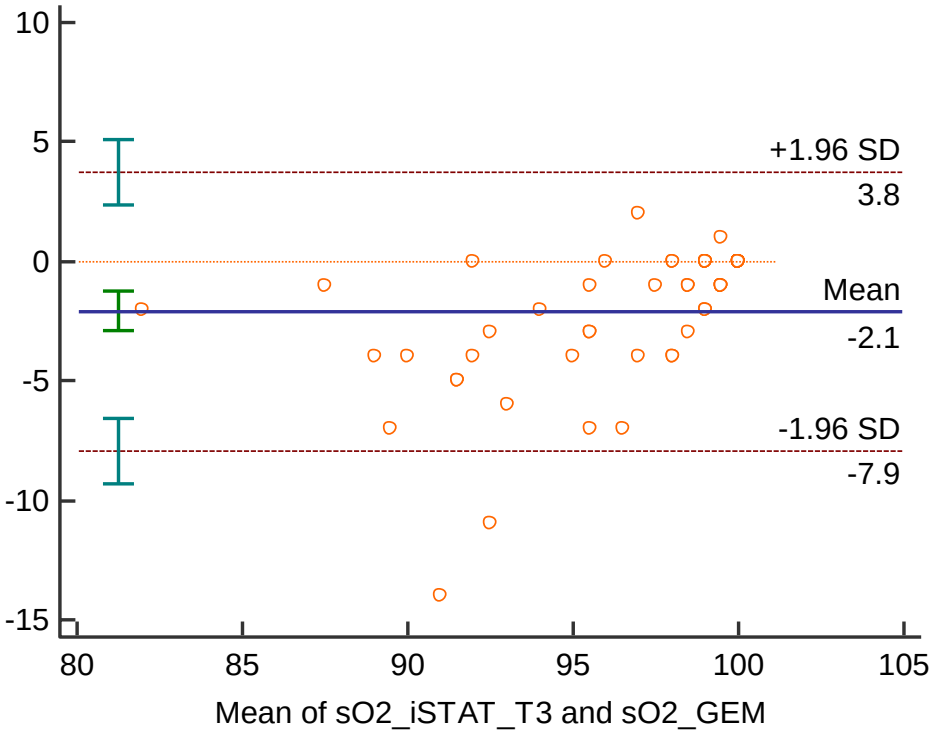
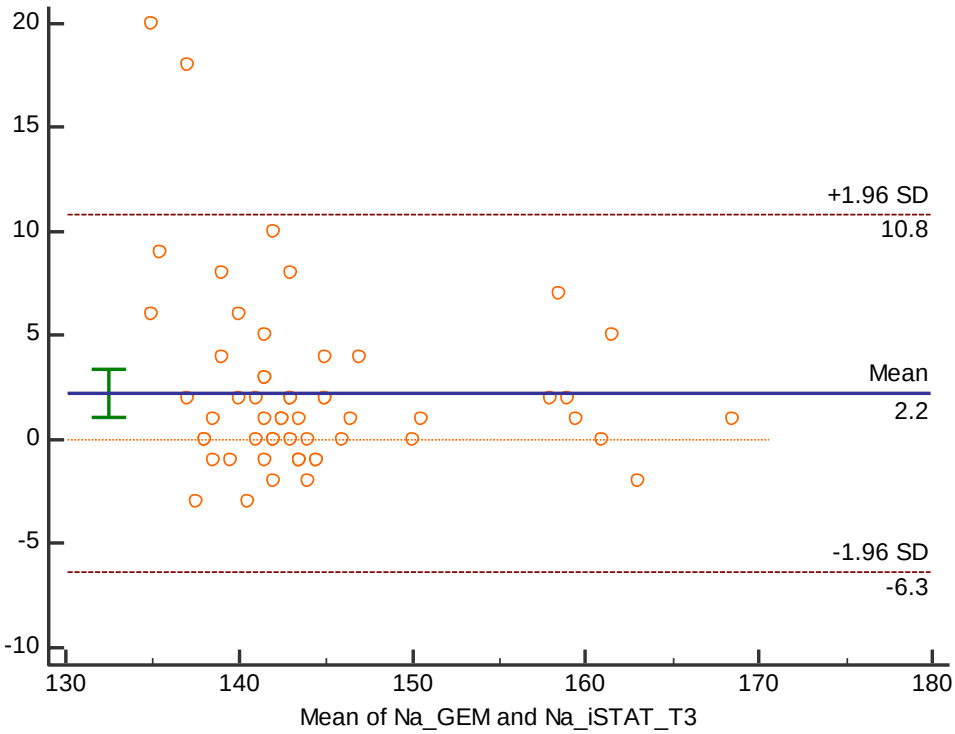


Figure 2.4. Bland-Altman plot comparing oxygen saturation (sO_2) results measured using the i-STAT and GEM 3000; difference between both tests plotted against the mean difference between the tests.



The mean line indicates the difference between both tests expected to be = 0; dotted lines are the upper and lower 95% confidence interval for the mean; and the 95% confidence of limits of agreement represented by the vertical bars. The closer the data points are to the mean line or are within the 95% confidence interval for the mean represent the agreement between tests.

Figure 2.5. Bland-Altman plot comparing sodium (Na^+) results measured using the Vetscan VS2 and Cobas c501; difference between both tests plotted against the mean difference between the tests.



The mean line indicates the difference between both tests expected to be = 0; dotted lines are the upper and lower 95% confidence interval for the mean; and the 95% confidence of limits of agreement represented by the vertical bars. The closer the data points are to the mean line or are within the 95% confidence interval for the mean represent the agreement between tests.

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CHAPTER 3. Establishment of Age-Specific Whole Blood Biochemistry and Gas Reference Intervals in Ross 708 Broiler Chickens using the i-STAT and the Vetscan VS2 Portable Analyzers

ABSTRACT

Whole blood biochemistry and blood gas analysis are uncommonly used in poultry but are powerful tools that could improve diagnosis of certain diseases or aid in monitoring flock health or changes in management. To create preliminary reference intervals for selected blood analytes in broilers using the i-STAT and Vetscan VS2 portable analyzers, we tested broilers at 7, 21, and 35 days of age. A total of 134 venous blood samples from healthy chickens of two different flocks were analyzed. There were significant age-related increases in concentration for glucose, hematocrit, ionized calcium, sodium, and carbon dioxide partial pressure on the i-STAT; and for AST, CK, total calcium, phosphorus, and total protein on the Vetscan VS2. Conversely, significant decreases in concentration were observed for pH, oxygen partial pressure, oxygen saturation on the i-STAT, and uric acid and albumin on the Vetscan VS2. Additionally, significant differences were found for blood parameters among the two flocks, indicating that different management procedures can impact blood analytes' concentration. Extremely high CK values were found on broilers after 21 days of age, indicating a possible degree of muscle injury during the grow-out. Reference intervals for all the analytes at each of the age groups were obtained. This study's data provide a starting point for the interpretation of blood analysis in broiler chickens at different ages and offers a new approach to investigate certain metabolic diseases that affect commercial poultry.

INTRODUCTION

Standard techniques for diagnosing disease in commercial poultry include gross and histopathologic examinations and detection of infectious agents by isolation or polymerase chain reaction (PCR) assays (1). However, some metabolic and nutritional diseases cannot be diagnosed using these techniques or detected at an advanced stage, such as hypoglycemia, calcium tetany, hypernatremia, and osteoporosis. Other methodologies, such as blood biochemistry and blood gases analysis, may help diagnose certain metabolic diseases before death or narrow the list of possible problems. Additionally, blood biochemistry and or blood gas analysis could help monitor flocks' health status and assess the impact of different management practices.

Blood biochemistry analysis includes analytes including electrolytes, minerals, enzymes, proteins, and glucose. In contrast, blood gas analysis includes partial pressure of oxygen and carbon dioxide, oxygen saturation, total carbon dioxide, and pH. Interpretation of these results in individual animals requires reference intervals generated from healthy individuals from that population. While several studies report reference intervals on long-lived poultry, including broiler breeders (2), layers (3,4), and backyard hens (5), there are no reference intervals for broilers. Meat-type chickens differ from other poultry in several significant ways, including genetics, growth rate, and market-age. Broilers' rapid growth rate is associated with drastic changes in their metabolism (6). Additionally, variability among birds' genetic lines and the different management procedures may influence the biochemistry and blood gas values. Therefore, published reference intervals in other poultry may be unsuitable for broilers.

Portable biochemistry analyzers, such as the i-STAT (Abbott Laboratories; *Chicago, IL*) and the Vetscan VS2 (VS2, Abaxis, Inc; *Union City, CA*), might be useful for on-farm testing.

Each assay requires 0.1 mL of whole blood, and results are available within minutes, thus minimizing the impact on the bird and eliminating the risk of storage-related artifacts during transport to a diagnostic laboratory. Portable analyzers are widely used in small animal and equine practice (7–10). Results are comparable to the benchtop analyzers, making them preferable over the traditional analyzers when a short turn-around time is needed, such as in the emergency room (11). Portable analyzers have been evaluated in psittacines (12), strigiformes (13), and poultry (2,4,5). We recently showed that results from conventional and portable analyzers are comparable in broiler chickens, but we identified biases that warrant the establishment of analyzer-specific reference intervals (14). Notably, there are no reference ranges reported for broiler chickens using portable analyzers.

This study's objectives were; a) to determine whether age-specific differences are present in chickens at three different ages, and b) to provide preliminary reference intervals for selected blood analytes measured by the i-STAT and VS2 portable blood analyzers in Ross 708 broiler chickens. The values obtained in this study will provide a basis to interpret blood parameters from broilers at various stages of development, potentially giving a new approach for the diagnosis of metabolic disorders in meat-type chickens.

MATERIALS AND METHODS

Animals and Housing. Two different flocks of five thousand Ross 708 broiler chickens each were used for this study. The flocks were raised between January and March in 2019 and 2020. A vertically integrated company provided the birds, and they were housed at the Teaching Animal Unit (TAU) at the College of Veterinary Medicine, North Carolina State University (CVM-NCSU). All the chickens were vaccinated *in-ovo* against Marek's Disease and Infectious

Bursal Disease, and at one day-of-age against coccidiosis and Infectious Bronchitis. The integrator provided standard commercial feed. Water acidified with acetic acid and copper sulfate was used on one of the flocks (Flock 2), starting when the chickens were three weeks and up to the end of their productive cycle. The study was approved by the NCSU Institutional Animal Care and Use Committee (IACUC Protocol Number: 19-001-A).

A total of 134 samples were evaluated on the i-STAT and 123 samples on the VS2 (Table 3.1). All the chickens sampled were clinically healthy based on observation by the veterinarians in charge. Each sampled bird was marked with food coloring dye to avoid multiple testing on the same subject. Approximately 1.5 - 2 mL of blood was collected from each non-anesthetized bird's jugular vein, using 3 mL syringes with 21-gauge needles flushed with heparin. The needle was immediately removed after collection, and the blood was transferred into lithium-heparin collection tubes (BD Biosciences, Franklin Lakes, NJ) and gently mixed.

Blood Analysis. The i-STAT and VS2 analyzers were placed in the poultry barn anteroom to minimize dust contamination. The analyzers were kept under the recommended operating temperature (15 – 32º C), and the i-STAT cartridges and the VS2 rotors were kept at 4°C until used. Around 1 mL of blood from the collection tubes was taken using disposable plastic Pasteur pipettes to fill the i-STAT cartridge and the VS2 rotor and processed afterward. Samples were processed within 15 minutes of collection.

i-STAT analysis was done using CG8+ cartridges, which measure the following analytes: sodium (Na^+; mmol/L), potassium (K^+; mmol/L), ionized calcium (iCa; mmol/L), glucose (Glu; mg/dl), hematocrit (Hct), pH, oxygen partial pressure (pO_2; mm Hg), carbon dioxide partial pressure (pCO_2; mm Hg), bicarbonate (HCO_3; mmol/L), total carbon dioxide (TCO_2; mmol/L), base excess (BE; mmol/L), and oxygen saturation (sO_2; %).

VS2 analysis was done using the Avian/Reptilian Profile Plus reagent rotors, which measure the following analytes: aspartate aminotransferase (AST; U/L), bile acids (BA; μmol/L), creatine kinase (CK; U/L), uric acid (UA; mg/dL), glucose (Glu; mg/dL), total calcium (Ca; mg/dL), phosphorus (P; mg/dL), total protein (TP; g/dL), albumin (Alb; g/dL), potassium (K^+; mmol/L), and sodium (Na^+; mmol/L).

Statistical Analysis. For each analyte, descriptive statistics including mean, median, standard deviation, maximum value, and minimum value were generated using MedCalc software (version 19.1.5, Ostend, Belgium; https://www.medcalc.org; 2020). All the analytes were tested for normal distribution using a D'Agostino-Pearson normality test. Significant differences in the means between flocks and age groups (7, 21, and 35d) were determined using a one-way ANOVA with a Bonferroni correction ($\alpha = 0.05$). Figures were generated using MedCalc software. Reference intervals were calculated by a non-parametric method using the Reference Value Advisor v2.1 (available at http://www.biostat.envt.fr/reference-value-advisor/) (15) free software.

RESULTS

We first evaluated analytes with the i-STAT from 7d-, 21d-, and 35d-old chickens in both flocks (*Table 3.2*). One sample from the 7d and 35d age groups in Flock 1 was discarded due to cartridge error. We observed an age-related difference in multiple analytes. The 35d birds showed significantly higher iCa, Na^+, and pCO_2 than the 7d and 21d groups, while the concentrations of pH, pO_2, and sO_2 were lower in the 21d and 35d groups than in the 7d birds. Significant lower concentrations of K^+ and BE were noted between the 21d birds and the other two age groups. For HCO_3 and tCO_2, there were no statistical differences between ages. To

determine whether there were flock-related differences, we compared each age group between flocks (*Table 3.3*). Flock 1 showed significantly higher pH, pO_2, and sO_2 than Flock 2. pCO_2 concentrations were significantly lower in Flock 1.

We next evaluated the analytes measured with the VS2 from 7d-, 21d-, and 35d-old chickens in both flocks (*Table 3.4*). Six samples were discarded due to cartridge failure. Discarded samples included two samples from the 35d old birds in Flock 1, one sample from the 7d birds, and three samples from the 35d old birds in Flock 2. There was not a specific reason for these failures. No statistical analysis was performed on BA values because more than 95% of the measurements were below the VS2 detection limit. Thirty-five percent (n=41) of the CK measurements were above the detection limit; 16 samples from the 21d birds and 25 samples from the 35d birds. For each case with a CK measurement above the detection limit, we assigned the highest detectable value (14,000 U/L) rather than discard these samples, which would bias the results if only lower CK concentrations were included.

Significantly higher AST concentrations, CK, Ca, P, and TP was detected in blood from the 21d and 35d birds than 7d old birds. In contrast, UA concentration was lower in the 21d and 35d birds compared to the 7d birds. The blood concentration of Alb was significantly lower in 21d birds compared to the other two groups. Measurements of K^+ determined by the VS2 were included but must be interpreted cautiously since elevated CK falsely increases K+ (16). Differences between flocks were observed for the average concentrations of Alb, AST, and Na^+.

Finally, we calculated age-specific reference intervals for each analyte measured by the i-STAT and VS2 (*Tables 3.6, 3.7, and 3.8*).

DISCUSSION

For this study, three ages (7, 21, and 35 days) were selected based on critical events in broiler chickens' life. Their yolk sac is fully absorbed between 4 to 7 days of age and their primary source of energy switches from a lipid-based source to a carbohydrate-based source. Maternal antibodies are depleted at around 21 days of age, and the adult feathers finish growing. The next significant change happens at approximately 35 days of age, as the protein in their feed is reduced and the energy in their diet increased. It is also at 35 days up to 52 days of age when broilers are sent to market (6).

The BE, pCO_2, pO_2, and sO_2 values obtained by the i-STAT were similar to what has been observed in broiler breeders (2) and laying hens (3,4). However, when compared to those studies, the 7d broilers showed substantially lower pCO_2 concentrations, while the BE, pO_2, and sO_2 values were considerably higher in the 21d and 35d groups. Martin et al. (2) found decreased pCO_2 and BE concentrations from 25 to 36 weeks of age, which is similar to the direction they followed in the birds used in this study, though it is important to consider that the broiler chickens were younger. Therefore, to determine if this reduction trend follows the bird's life, research for a more extended period is needed.

In our study, the calculated intervals for most of the blood analytes measured with the VS2 were similar to the intervals reported on backyard hens (5), even though the chickens in our study were fast-growing meat-type birds and the backyard hens were adult laying hens. The exceptions were AST and CK, for which we obtained higher concentrations, and the concentrations increased significantly as the birds aged. AST is an enzyme present in high concentrations in hepatocytes, skeletal muscle, cardiac muscle, and some species' erythrocytes. Increased AST activity is most often due to hepatocellular or skeletal muscle injury. CK is an

enzyme present in skeletal and cardiac muscle that is increased as a secondary response to muscle damage due to injury or disease (17). CK activity exceeded the upper limit of detection for the VS2 rotor (14,000 U/L) in 25 birds from the 21d and 35d age groups. Rather than discarding these samples, we assigned the highest detectable value (14,000 U/L) to the birds with a CK above the detection limit. Therefore, the mean and median are likely higher than reported herein. Dilution studies to determine the actual CK activity were attempted in a few samples; however, the VS2 could not provide measurements of samples diluted to 1:2. Waldock et al. (18) diluted the samples of thick-billed parrots (*Rhynchopsitta pachyrhyncha*) up to 1:3.5 and still obtained readable results. However, since dilution may not result in linear results (19) and the manufacturer recommends avoiding dilution, these samples would better be analyzed by a different test method (16).

The mean CK value found in backyard hens using the VS2 was 703 U/L (5), lower than the mean found in this study for the youngest broilers (1,141 U/L). There are no reported CK values for other types of commercial birds like layers or breeders. Given their lower growth rate, decreased adult weight, and different purposes (meat vs. egg-laying), we anticipate that the values would not be as high as those obtained for broiler chickens; however further studies need to be conducted to confirm or refute this hypothesis. AST was significantly different amongst all age groups, while CK was only significantly different in the 21d group. We do not know whether the elevated CK concentrations, in conjunction with the AST increases, are expected in meat-type chickens due to their rapid growth rate, or if this concentration reflects muscle damage that intensifies with age.

Alterations in blood gas concentrations and CK activity may provide insight into clinical and management practices. For example, birds with clinical signs and lesions of Wooden breast

had decreased pO_2, sO_2, and pH concentrations with increased pCO_2 values (20). In a separate study, birds with and without suspected wooden breast had high CK concentrations (21). The age-related high CK concentration in apparently healthy broiler chickens suggests that these birds have an occult muscle injury, likely related to intense growing conditions. Early diagnosis of myopathy may open new paths for research management, treatment, and prevention.

A consequence of the muscle injury, as reflected by the increased CK, is the effect on accurate measurement of K^+ using the VS2. The VS2 measures K^+ by an enzymatic method based on pyruvate kinase activation with potassium (16). In the organism, pyruvate kinase is present as four different tissue-specific isoenzymes, including muscle. Therefore, muscle damage/injury releases an increased amount of pyruvate kinase into the blood. The increased concentration of pyruvate kinase participates in the enzymatic measurement of K^+ in the VS2, thus artificially increasing reported K^+ (22). Therefore, potassium concentrations in broilers with elevated CK cannot be reliably measured by the VS2. In these cases, accurate measurement of K^+ would require a different methodology, such as ion-selective electrode potentiometry used by other analyzers. For this study, we relied on the i-STAT analyzer results for more accurate K^+.

Hematocrit values were significantly lower in the 7d old birds when compared with the 21d and 35d groups. It is known that hematocrit values increase with age in mammals and wild birds due to the maturation of the hematopoiesis process and the increased RBC lifespan and hemoglobin concentration (23,24). These data suggest a similar maturation of hematopoiesis in young commercial chickens. An additional consideration is the effect of temperature on hematocrit. *Kubena et al.* (25) found that hematocrit values in broilers negatively correlate with the temperature the birds are reared on, which could be an additional factor influencing the results obtained in the present study.

Broiler chickens have a higher metabolic rate than most commercial birds due to their rapid growth rate, causing an increase in their cardiac output, pulmonary hypertension, and chronic respiratory insufficiency (20,26). Here, we report venous blood pCO_2 values were significantly higher as age increased, which may indicate either increased transit time of blood or increased use of oxygen, possibly due to increased body size.

iCa concentration was higher in the 21d and 35d groups, which could be related to the pH decrease with age. The increased amount of hydrogen ions present in blood with lower pH displace calcium from its protein binding sites, resulting in increased ionized calcium (27). Differences in iCa could also be physiologic and related to bone growth.

Although both flocks were raised in the same facility, minor differences in flock management occurred. For example, the drinking water in Flock 2 was acidified from their third week of life until the end of their production cycle, at 52 days of age. Drinking water acidification is a common practice in commercial broiler production. There are different types of acidifiers, and it is not well known if their effect is limited to the intestine or if they have a systemic impact on the chickens. In general, water acidification lowers the intestinal pH and modulates the intestinal microbiota, preventing the proliferation of pathogenic bacteria (28–33). Despite these variations, most of the analytes were not significantly different between the two flocks. However, we observed significant differences in the pCO_2, pH, pO_2, sO_2, AST, Alb, and Na^+ values. pH values from Flock 2 birds were significantly lower than those from Flock 1. Even though the difference is not striking, this could indicate that the acid had a systemic effect on the birds at ages 21d and 35d. The exact mechanism of the systemic effect is uncertain, but there are several possible pathways. First, an increased acid uptake leads directly to higher absorption in the intestine (34). Second, a reduced water intake causing hypovolemia and decreased perfusion

leads to an increased lactic acid production (35). Third, the higher concentration of K^+ from the water acidifier (Clear View Enterprises, AR), might have caused higher removal of Na^+, as happens in other species (36). It is beyond this study's scope to determine if the acidification of the drinking water directly impacted the birds' health. Still, it suggests that the drinking water's acidity and the salts in these acidifiers may need to be considered when interpreting pH values in commercial birds, and further studies are required.

It is important to note that the reference intervals generated in this study are specific to 7d, 21d, and 35d-old Ross 708 broiler chickens as measured by the i-STAT and VS2 portable analyzers. We recently assessed the comparability of the i-STAT and VS2 to their benchtop counterparts (GEM 3000 and Cobas c501, respectively). We observed that 5 out of 12 analytes were not comparable between the i-STAT and the GEM 3000. Simultaneously, 5 out of 10 analytes were not comparable between the VS2 and the Cobas c501 (14). The reference intervals reported here should be considered preliminary. The current recommendation for obtaining robust reference intervals is to use a sample of at least 120 individuals (37). Additionally, management conditions likely affect the biochemistry and or blood gas analyte concentrations. Therefore, more samples and more flocks grown under different management conditions will need to be analyzed to determine whether these intervals can be more broadly applied across different management and environmental conditions.

In conclusion, we demonstrated changes in blood analytes with age in the Ross 708 broiler chickens using the i-STAT and VS2 analyzers. These intervals provide an initial basis to interpret blood tests in this type of birds according to their age. Different broiler strains and other management procedures may impact the values; thus, these data should be used with caution.

Still, these data mark an important starting point for subsequent studies investigating the utility of blood gas and chemistry analysis to detect and diagnose metabolic disease.

TABLES

Table 3.1. Summary of the number of samples collected for each flock.

		7 days	21 days	35 days
Flock 1	i-STAT	21	21	21
	VS2	20	20	20
Flock 2	i-STAT	20	26	25
	VS2	21	21	21

Table 3.2. Summary statistics i-STAT. Blood parameters data from 132 broiler chickens at 7, 21, and 35 days of age.

		Glu	Hct	iCa	K^+	Na^+	BE	HCO_3	pCO_2	pH	pO_2	sO_2	tCO_2
7d n=41	Mean	214.57 ^{A}	16.76 ^{A}	1.12 ^{A}	5.00 ^{A}	134.98 ^{A}	3.51 ^{A}	26.21	31.55 ^{A}	7.53 ^{A}	60.15 ^{A}	89.51 ^{A}	27.15
	SD	24.45	1.98	0.16	0.72	3.80	6.45	5.41	7.20	0.10	25.19	6.23	5.54
	Median	217.00	16.00	1.15	4.85	136.00	4.00	26.40	31.70	7.51	48.00	88.00	27.00
21d n=46	Mean	236.65 ^{B}	21.09 ^{B}	1.22 ^{B}	4.90 ^{B}	145.10 ^{B}	1.02 ^{B}	25.16	36.93 ^{B}	7.44 ^{B}	52.48	86.41 ^{B}	26.33
	SD	21.34	4.90	0.14	0.67	6.95	4.22	3.15	6.38	0.09	10.42	5.92	3.15
	Median	237.50	20.00	1.26	4.90	143.00	1.00	25.00	36.00	7.45	49.50	87.00	26.00
35d n=45	Mean	231.93 ^{B}	20.52 ^{B}	1.42 ^{C}	5.32 ^{A}	145.16 ^{B}	2.13 ^{A}	26.65	41.43 ^{C}	7.4 ^{B}	47.66 ^{B}	82.75 ^{C}	27.89
	SD	19.82	2.00	0.09	0.43	3.42	2.56	2.30	5.03	0.06	5.81	5.09	2.50
	Median	231.00	21.00	1.44	5.20	144.00	3.00	27.10	40.60	7.40	47.00	83.00	28.00

Letter superscripts indicate significant differences in the mean value of the analyte. α=0.05

Table 3.3. Summary statistics i-STAT. Blood parameters data from 132 broiler chickens grouped according to flock number.

Flock #			Glu	Hct	iCa	K^+	Na^+	BE	HCO_3	pCO_2	pH	pO_2	sO_2	tCO_2
1	Total n=61	Mean	228.95	18.84	1.26	5.18	140.87	1.95	25.48	34.54 ^{A}	7.48 ^{A}	58.28 ^{A}	88.03 ^{A}	26.54
		SD	18.75	2.59	0.16	0.64	5.38	4.83	3.93	7.49	0.10	21.42	7.04	4.10
		Median	231	18.00	1.27	5.10	142.00	2	26.00	35.10	7.47	50.50	87.00	27.00
2	Total n=71	Mean	228.75	21.20	1.29	4.99	142.93	2.36	26.44	38.73 ^{B}	7.43 ^{B}	49.01 ^{B}	84.56 ^{B}	27.60
		SD	26.59	4.29	0.19	0.63	7.84	4.61	3.67	6.73	0.10	8.63	5.20	3.71
		Median	228.00	21.00	1.35	5.10	142.00	3.00	26.10	38.80	7.42	47.00	84.00	27.00
1	7d n=21	Mean	219.20	16.00	1.10	5.3 ^{A}	135.38	1.05 ^{A}	23.81 ^{A}	28.99 ^{A}	7.53	75.43 ^{A}	93.80 ^{A}	24.71 ^{A}
		SD	22.47	1.55	0.16	0.83	4.20	6.64	5.19	8.43	0.13	27.49	5.47	5.36
		Median	218.00	15.50	1.13	5.15	137.00	-1.00	22.70	26.30	7.51	73.00	96.00	24.00
2	7d n=20	Mean	211.10	17.20	1.14	4.71 ^{B}	134.55	6.10 ^{B}	28.72 ^{B}	34.25 ^{B}	7.53	44.10 ^{B}	85.00 ^{B}	29.70 ^{B}
		SD	25.85	2.15	0.18	0.42	3.39	5.23	4.49	4.41	0.06	3.37	2.87	4.59
		Median	212.00	16.00	1.16	4.65	135.00	5.50	28.75	35.20	7.51	44.00	85.00	30.00
1	21d n=20	Mean	237.60	18.10 ^{A}	1.27 ^{A}	5.02	142.60 ^{A}	3.15 ^{A}	26.63 ^{A}	35.16	7.49 ^{A}	49.80	86.45	27.75 ^{A}
		SD	17.42	2.10	0.09	0.55	1.98	4.30	3.36	4.88	0.08	9.17	5.20	3.42
		Median	236.50	17.50	1.27	4.90	143.00	4.00	26.55	34.85	7.50	47.50	87.00	28.00
2	21d n=26	Mean	235.92	23.38 ^{B}	1.84 ^{B}	4.82	147.04 ^{B}	-0.62 ^{B}	24.02 ^{B}	38.30	7.41 ^{B}	54.54	86.38	25.23 ^{B}
		SD	24.25	5.22	0.16	0.74	8.66	3.42	2.49	7.12	0.09	11.02	6.52	2.48
		Median	238.00	22.00	1.17	4.65	143.00	-1.00	24.45	37.85	7.41	51.50	87.00	26.00
1	35d n=20	Mean	227.60	20.53	1.37 ^{A}	5.20	144.90	1.70	26.07	39.75 ^{A}	7.43 ^{A}	48.26	83.32	27.25
		SD	12.96	2.20	0.13	0.46	3.92	2.47	2.10	3.97	0.04	6.83	5.98	2.31
		Median	231.00	20.00	1.40	5.20	144.00	2.00	26.15	38.35	7.43	47.00	85.00	27.00
2	35d n=25	Mean	235.40	20.52	1.46 ^{B}	5.40	145.36	2.48	27.12	42.78 ^{B}	7.38 ^{B}	47.20	82.32	28.40
		SD	23.64	1.90	0.04	0.39	3.03	2.63	2.39	5.45	0.07	5.00	4.37	2.57
		Median	229.00	21.00	1.46	5.20	145.00	3.00	27.10	41.40	7.38	46.00	83.00	28.00

Letter superscripts indicate significant differences in the mean value of the analyte between flocks. α=0.05

Table 3.4. Summary statistics Vetscan VS2. Blood parameters data from 117 broiler chickens at 7, 21, and 35 days of age.

		AST	CK	UA	Glu	Ca	P	TP	Alb	K^{+} [1]	Na^{+}
7d n=40	Mean	149.39 ^{A}	1141.37 ^{A}	7.61 ^{A}	225.45	10.97 ^{A}	6.23 ^{A}	2.55 ^{A}	5.66 ^{A}	2.12	149.13 ^{A}
	SD	17.35	628.10	1.64	23.59	0.68	0.68	0.32	0.58	0.27	2.42
	Median	148.50	876.00	7.40	227.00	11.00	6.20	2.50	5.60	2.10	149.00
21d n=41	Mean	280.98 ^{B}	7055.41 ^{B}	4.70 ^{B}	236.88	11.56 ^{B}	6.71 ^{B}	3.03 ^{B}	6.75 ^{B}	2.09 ^{A}	152.43 ^{B}
	SD	185.17	4977.23	1.90	13.44	0.69	0.73	0.66	1.65	0.28	4.22
	Median	198.00	5494.00	4.30	239.00	11.50	6.60	2.90	6.20	2.10	151.00
35d n=36	Mean	384.57 ^{B}	11390.78 ^{C}	3.68 ^{B}	232.05	11.55 ^{B}	6.86 ^{B}	3.17 ^{B}	7.60 ^{B}	2.28 ^{B}	151.61 ^{B}
	SD	218.53	4194.48	1.68	10.08	0.41	0.54	0.29	1.04	0.16	2.97
	Median	279.00	>14000.00	3.40	232.00	11.55	6.80	3.15	7.40	2.30	151.00

Letter superscripts indicate significant differences in the mean value of the analyte. α=0.05

[1] K^{+} values may be artificially elevated due to known interference from muscle damage. They are reported here for completeness.

Table 3.5. Summary statistics Vetscan VS2. Blood parameters data from 132 broiler chickens grouped according to flock number.

Flock #			AST	CK	UA	Glu	Ca	P	TP	Alb	K^{+} [1]	Na^{+}
1	Total n=58	Mean	220.58 [A]	5268.73	5.17	232.95	11.32	6.58	2.85	2.21 [A]	6.45	149.95 [A]
		SD	126.24	4907.76	2.08	14.42	0.56	0.68	0.4	0.24	1.21	2.09
		Median	178.00	3246.50	4.40	234.00	11.35	6.55	2.80	2.30	6.10	150.00
2	Total n=59	Mean	323.42 [B]	7756.15	5.48	230.11	11.41	6.64	2.99	2.11 [B]	6.56	152.31 [B]
		SD	230.00	6066.59	2.70	19.94	0.77	0.74	0.64	0.26	1.64	4.41
		Median	206	8000.00	4.90	232.00	11.50	6.60	3.00	2.10	6.10	151.00
1	7d n=20	Mean	147.65	1103.85	7.34	233.05 [A]	11.08	6.42	2.53	2.24 [A]	5.66	148.40 [A]
		SD	13.35	630.19	0.87	20.11	0.48	0.72	0.16	0.12	0.68	1.35
		Median	143.50	870.50	7.40	227.50	11.10	6.40	2.55	2.20	5.60	148.50
2	7d n=20	Mean	151.33	1183.06	7.89	217.00 [B]	10.85	6.02	2.56	1.99 [B]	5.66	149.94 [B]
		SD	21.18	641.32	2.18	24.81	0.84	0.60	0.44	0.33	0.48	3.06
		Median	156.00	980.00	7.40	216.00	10.75	6.00	2.40	2.00	5.55	149.50
1	21d n=20	Mean	196.75 [A]	4344.50 [A]	4.65	234.30	11.38	6.43 [A]	2.92	2.09	6.32	150.80 [A]
		SD	54.34	2293.94	1.25	11.73	0.69	0.72	0.46	0.34	0.94	1.67
		Median	176.00	4099.50	4.30	239.00	11.50	6.40	2.85	2.10	6.10	151.00
2	21d n=21	Mean	365.20 [B]	9637.24 [B]	4.74	239.33	11.73	6.98 [B]	3.13	2.09	7.21	154.05 [B]
		SD	229.11	5495.32	2.40	14.75	0.66	0.66	0.81	0.22	2.10	5.32
		Median	284.50	14000.00	4.40	238.00	11.70	7.00	3.00	2.10	6.65	153.50
1	35d n=18	Mean	317.35 [A]	10357.85	3.63	231.50	11.50	6.87	3.11	2.30	7.63	150.65 [A]
		SD	174.08	4765.90	1.85	9.97	0.40	0.54	0.27	0.16	1.16	2.25
		Median	250.50	>14000.00	3.20	233.00	11.45	6.80	3.10	2.30	7.40	150.50
2	35d n=18	Mean	474.20 [B]	12681.94	3.74	232.75	11.62	6.85	3.24	2.25	7.48	152.81 [B]
		SD	244.51	3013.35	1.49	10.51	0.43	0.57	0.30	0.15	0.59	3.37
		Median	431.00	>14000.00	3.45	231.50	11.60	6.85	3.30	2.30	7.80	152.00

Letter superscripts indicate significant differences in the mean value of the analyte between flocks. α=0.05.

[1] K^{+} values may be artificially elevated due to known interference from muscle damage. They are reported here for completeness.

Table 3.6. Reference Intervals for broilers at 7 days of age

		7 days of age (n=41)		
	Analyte	Reference Interval	90% Confidence Interval	
			Lower Limit	Upper Limit
i-STAT	Glu (mg/dL)	153.60 - 256.30	132.00 - 175.30	248.90 - 264.20
	Hct (%)	15.00 - 21.00	15.00 - 15.00	19.00 - 21.00
	iCa (mmol/L)	0.81 - 1.55	0.77 - 0.91	1.43 - 1.66
	K^+ (mmol/L)	4.01 - 7.58	4.00 - 4.20	5.80 - 7.60
	Na^+ (mmol/L)	123.2 - 141.00	123.00 - 129.00	139.90 - 141.00
	BE (mmol/L)	-8.00 - 19.8	-8.00 - (-5.00)	12.00 - 20.00
	HCO_3 (mmol/L)	16.83 - 40.32	16.80 - 17.79	32.97 - 40.70
	pCO_2 (mm Hg)	18.55 - 53.10	18.50 - 22.21	39.58 - 53.60
	pH	7.35 - 7.85	7.35 - 7.40	7.68 - 7.86
	pO_2 (mm Hg)	38.1 - 154.00	38.00 - 41.00	110.60 - 156.00
	sO_2 (%)	80.00 - 100.00	80.00 - 81.10	99.00 - 100.00
	tCO_2 (mmol/L)	18.00 - 41.70	18.00 - 18.10	34.00 - 42.00
Vetscan VS2	AST (U/L)	117.70 - 189.40	111.90 - 124.10	178.70 - 201.30
	CK (U/L)	525.30 - 2717.00	505.60 - 563.60	1982.10 - 3878.10
	UA (mg/dL)	4.38 - 10.86	3.67 - 5.21	9.86 - 11.85
	Glu (mg/dL)	177.1 - 274.00	165.20 - 190.00	261.10 - 287.40
	Ca (mg/dL)	9.42 - 12.19	8.79 - 9.90	11.89 - 12.47
	P (mg/dL)	4.93 - 7.75	4.71 - 5.20	7.34 - 8.17
	TP (g/dL)	2.04 - 3.37	1.96 - 2.13	3.08 - 3.77
	Alb (g/dL)	1.48 - 2.60	1.25 - 1.66	2.51 - 2.69
	K^+ (mmol/L)	4.87 - 7.22	4.76 - 5.01	6.64 - 8.03
	Na^+ (mmol/L)	144.20 - 154.10	142.60 - 146.00	151.60 - 156.80

K^+ values must be interpreted with caution in animals with evidence of muscle injury.

Table 3.7. Reference Intervals for broilers at 21 days of age

		21 days of age (n=46)		
	Analyte	Reference Interval	90% Confidence Interval	
			Lower Limit	Upper Limit
i-STAT	Glu (mg/dL)	195.90 - 289.30	194.00 - 206.00	274.00 - 290.00
	Hct (%)	16.00 - 38.00	16.00 - 16.00	31.10 - 39.00
	iCa (mmol/L)	0.92 - 1.44	0.91 - 1.01	1.39 - 1.44
	K^+ (mmol/L)	3.82 - 6.80	3.80 - 4.02	5.89 - 6.90
	Na^+ (mmol/L)	139.00 - 167.00	139.00 - 140.00	160.70 - 168.00
	BE (mmol/L)	-8.50 - 9.80	-9.00 - (-4.00)	7.80 - 10.00
	HCO_3 (mmol/L)	18.05 - 32.44	17.70 - 21.12	30.43 - 32.70
	pCO_2 (mm Hg)	24.69 - 53.22	24.50 - 27.89	46.94 - 53.90
	pH	7.24 - 7.61	7.23 - 7.30	7.56 - 7.62
	pO_2 (mm Hg)	38.50 - 84.10	38.00 - 43.00	76.40 - 85.00
	sO_2 (%)	72.70 - 97.00	72.00 - 78.00	95.00 - 97.00
	tCO_2 (mmol/L)	19.35 - 33.65	19.00 - 22.96	31.02 - 34.00
Vetscan VS2	AST (U/L)	133.30 - 826.10	133.00 - 146.30	644.00 - 828.00
	CK (U/L)	894.80 - 8000.00	879.00 - 1240.20	7264.00 - 8000.00
	UA (mg/dL)	1.93 - 11.12	1.90 - 2.70	8.31 - 11.20
	Glu (mg/dL)	206.50 - 270.80	206.00 - 217.90	261.40 - 271.00
	Ca (mg/dL)	10.11 - 13.19	10.10 - 10.39	12.77 - 13.20
	P (mg/dL)	5.41 - 8.29	5.40 - 5.61	7.99 - 8.30
	TP (g/dL)	2.35 - 4.79	2.25 - 2.46	4.02 - 5.88
	Alb (g/dL)	1.49 - 2.64	1.34 - 1.63	2.52 - 2.75
	K^+ (mmol/L)	*	*	*
	Na^+ (mmol/L)	143.80 - 161.10	141.90 - 146.20	157.00 -164.90

* Due to known interference from muscle damage, the calculated upper limit for K^+ is overestimated by the VS2 analyzer and the result is incompatible with life; thus, the intervals are not included.

Table 3.8. Reference Intervals for broilers at 35 days of age

		35 days of age (n=45)		
	Analyte	Reference Interval	90% Confidence Interval	
			Lower Limit	Upper Limit
i-STAT	Glu (mg/dL)	194.70 - 323.20	192.00 - 214.00	249.60 - 335.00
	Hct (%)	16.10 - 25.00	16.00 - 18.00	24.00 - 25.00
	iCa (mmol/L)	0.93 - 1.57	0.91 - 1.33	1.51 - 1.57
	K^+ (mmol/L)	4.36 - 6.47	4.30 - 4.72	6.07 - 6.50
	Na^+ (mmol/L)	140.20 - 155.70	140.00 - 142.00	150.90 - 156.00
	BE (mmol/L)	-3.00 - 7.00	-3.00 - (-2.00)	5.90 - 7.00
	HCO_3 (mmol/L)	22.38 - 32.39	22.30 - 23.33	29.88 - 32.60
	pCO_2 (mm Hg)	32.50 - 55.22	32.20 - 35.68	50.84 - 55.70
	pH	7.24 - 7.50	7.23 - 7.27	7.48 - 7.50
	pO_2 (mm Hg)	36.40 - 64.40	36.00 - 39.50	57.00 - 65.00
	sO_2 (%)	71.00 - 92.60	71.00 - 75.00	89.00 - 93.00
	tCO_2 (mmol/L)	23.20 - 33.90	23.00 - 24.20	31.90 - 34.00
Vetscan VS2	AST (U/L)	158.90 - 1109.70	147.40 - 172.40	691.00 - 1611.50
	CK (U/L)	2475.80 - >14000	2475.00 - 3431.60	above limit of detection (or >14000)*
	UA (mg/dL)	1.69 - 8.63	1.41 - 1.99	6.63 - 10.82
	Glu (mg/dL)	211.10 - 252.70	206.70 - 216.20	248.00 - 257.70
	Ca (mg/dL)	10.81 - 12.52	10.66 - 10.96	12.27 - 12.79
	P (mg/dL)	5.72 - 7.95	5.45 - 6.05	7.66 - 8.27
	TP (g/dL)	2.62 - 3.79	2.50 - 2.74	3.63 - 3.96
	Alb (g/dL)	1.89 - 2.54	1.77 - 2.03	2.49 - 2.60
	K^+ (mmol/L)	**	**	**
	Na^+ (mmol/L)	145.50 - 157.70	144.60 - 146.80	155.70 - 159.90

* CK upper interval is fixed at the VS2 upper limit of detection. Actual CK values may be higher.

** Due to known interference from muscle damage, the calculated upper limit for K^+ is overestimated by the VS2 analyzer and the result is incompatible with life; thus, the intervals are not included.

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CHAPTER 4. Research Reflections and Practical Considerations

Value of data obtained. As previously discussed, there is very little information about clinical chemistry in commercial poultry species primarily because clinical pathology has commonly been used for diagnosis of individual patients instead of populations. Some complications that arise when using blood testing for commercial poultry include the sample transportation and conservation, the distance between farms and clinical laboratories, and the cost of the tests. Additionally, the minimum number of samples to obtain a reliable diagnosis on a population has not been calculated. The increased accessibility to portable analyzers such as the i-STAT and the Vetscan VS2 eliminates several disadvantages of conventional analysis and allows clinical chemistry testing to be a more routinely used in commercial poultry. This research compares the values obtained by i-STAT and the VS2 to conventional counterparts (GEM 3000 and Cobas c501, respectively) in meat type chicken (aka broilers) blood and has generated preliminary specific reference intervals for this type of birds at three different ages. Although there was information about blood values of laying hens and backyard chickens in the literature, it was necessary to create specific intervals for broilers because they have a completely different purpose and genetics, and their metabolism is not comparable to the other types of commercial poultry, even when they belong to the same species (1). Variability in blood profiles within the same species is recognized among different human races/ethnicities (2) and different dog breeds (3), and creating specific reference intervals according to each group allows a better understanding of results and diagnosis.

This study demonstrated that the i-STAT and the VS2 results are not directly comparable to the values obtained in benchtop analyzers when using blood from broiler chickens. This study can also serve as a guideline to perform further validations of portable biochemistry analyzers

and determination of reference intervals in other poultry species, as it is practical for application in field conditions. The generation of preliminary specific reference intervals using the portable analyzers will allow a more accurate interpretation of test results in the field. By knowing what the normal expected blood values are in broilers, we will be able to understand better the pathophysiology of some metabolic diseases that have a significant impact on modern broiler production and assess the impact of different management procedures in the organism of the birds and hopefully help to prevent or minimize these conditions in the field.

Blood analysis could be used as a tool to accomplish more thorough chick quality evaluations, since the concentration of certain analytes such as glucose, pH, sodium, and potassium, may be helpful to identify subtle issues that originated in the hatchery and/or the breeder flocks. Glucose levels of day-old chicks may serve as an indicator of the amount of nutrients that the embryo was able to utilize during its development. This can be influenced by the availability of oxygen throughout the incubation process, by the formulation of the breeders' diet, and by how efficiently the breeders assimilate the nutrients provided in their feed (4). Low glucose levels in day-old chicks may be linked to a condition known as "starve-out", which is characterized by the sudden death of chicks during the first week of life and that has been associated with chronic hypoglycemia (5). In 1-day old, pH may be useful to determine if the chicks were exposed to high temperatures and/or poor ventilation in the hatchery and during their transportation to the farms, as they will present a respiratory alkalosis caused by prolonged panting (6). It has been postulated that changes in pH observed in chicks less than 5 days old can be related to brooder temperatures (D. Venne, personal communication, August 30, 2020). Sodium and potassium levels could be used to determine the hydration status of the chicks, which may pinpoint issues with the incubation conditions, mainly in the hatchers (7). Prompt

detection of abnormalities in the chicks' blood would allow implementing corrective actions at the hatchery and breeder level, which would eventually result in better quality chicks able to develop all their genetic potential.

One of the most striking findings of this research was the drastically high concentrations of CK in the broilers, for example, the 7-day-old chicks showed CK levels that would only be observed in conditions of severe ischemia and/or muscular damage in mammalian species (8), and the concentrations kept increasing as the birds grew older, reaching a point where the portable analyzers were not even able to measure them. Despite the extremely high CK concentrations, the chickens were not showing any clinical signs and the mortality remained within the expected rate for the breed (9). Once the flock reached 5 weeks of age, random birds (10-12 chickens) were found in dorsal recumbency and unable to right themselves almost every day; which is a characteristic feature of Wooden Breast myopathy (10). Associations between high CK concentrations and Wooden Breast have been evaluated (11–13), and the preliminary reference intervals provided in this study may be valuable to obtain a better insight of the systemic alterations that this condition induces in the organism of the birds affected, as variations in the concentrations of other blood analytes could be identified and associated to the elevations in the CK values and the overall pathophysiology of the disease. This data presents other future directions to explore, such as the possibility of performing dilutional studies to obtain a more accurate CK measurement. In one study, blood samples from thick-billed parrots were diluted up to 1:3.5 with distilled water and tested using the VS2. Significant differences between the undiluted and diluted blood samples were observed on all analytes except for AST, CK, and glucose (14). Since CK concentration in broiler blood is significantly more elevated than in other avian species, different detection limits may be needed to be established by the analyzer or the

samples may need to be diluted. However, to maintain the accuracy of the results, it would be important to use a diluent that does not interfere with the concentration of any of the analytes and to ensure that all the analytes behave linearly according to the dilutions used (15). Another potential avenue for research is to evaluate the CK values in broilers from various genetic lines and grown at different rates to determine if the high CK levels observed in this study are a normal feature of all meat-type birds or if it could be attributed to a particular strain of broilers and/or a certain growth rate. Other factors that cause muscular injury in chickens, such as ionophore toxicity and vitamin E deficiency (16,17), are also likely to increase the CK concentrations and additional information (i.e. clinical history, age of the birds, signs exhibited) would be needed to differentiate between these conditions.

Difficulties and Limitations. During this study, there were some difficulties and methodology limitations that are important to consider. The agreement between the portable and benchtop analyzers could not be measured following the CLIA guidelines (18) because the inherent imprecision values of the instruments were not available for chicken blood. Using the inherent imprecision obtained on blood from other species was considered, but it is not recommended by the CLIA because it significantly increases the bias of the results. Determination of those values as part of the study was also an option, but it was not feasible because a more controlled setting was needed. Due to the complications, the Bland Altman approach was utilized, which is another valid method comparison technique that is more suitable to the conditions of the experimental design of this study (19–21). A major difficulty faced during the study was the time required to analyze the samples and obtain the results, particularly in the Vetscan VS2. The i-STAT can run a sample in around 2 minutes (22), which allowed a good workflow between sample collection and analysis and made it possible to test at least 25

samples per hour. The VS2 on the other hand takes approximately 12 minutes to analyze each sample (23), causing significant delays between every test. For this study, it was required to wait any time needed to run all the samples, even though it was a tedious and time-consuming process. However, this would not be feasible in the field, as farm visits need to be concise, and any idle time cannot be afforded. Further research is necessary to determine the minimum number of blood samples required to get a representative overview of the flock's status. Some researchers have used two or more VS2 analyzers in an attempt to make the testing process more efficient, but the costs are higher, and it is less practical to do under farm conditions. The methodology used to measure the variability of the results over time had a significant limitation. It consisted of testing each of the blood samples at three different times after collection (immediately, 30 minutes, and 60 minutes), opening and closing the tubes every time, which allowed the blood gases to equilibrate with the gases in the air, and modifying their true concentration. This method was selected because it minimizes hemolysis by avoiding pushing the blood through the needle, but it was at the expense of exposure to the environmental air. Despite this issue, the variation through time of all the analytes was calculated and the reason for the significant alteration observed in the blood gases was explained. For future studies, the best methodology to assess the variability of the blood gases through time is to anaerobically divide each sample into 3 separate tubes, making sure there is no air exposure (no air bubbles, complete vacuum seal on tubes), followed by testing at each time point. It is critical to keep the samples completely anaerobic and to ensure that the portable analyzers are properly maintained and calibrated. There was also an issue regarding the sample size used in this study. The CLSI guidelines recommend using at least 120 samples from different healthy individuals to establish robust and reliable reference intervals (24), but the age groups used to create RIs in this study

had less than 50 values each. There are two alternatives for these cases; the first is doing an “a posteriori” or an “indirect” determination, where values from a databank are used to generate the reference intervals by a non-parametric method. This approach is a good option if a thorough selection process is performed to ensure that the values utilized come from healthy individuals that meet the characteristics of the population studied, and it has been used in various studies from mammalian species (25–27). Nonetheless, it was not a feasible option for this study, since there was no previous information on blood values in broiler chickens. The second alternative is using as many samples as possible whenever the recommended sample size cannot be achieved. This is common in studies involving exotic animal species, and for instance, reference intervals using small sample sizes ($n<50$) have been established in owls (28), wild turkeys (29), amazon parrots (30), and various psittacine species (31). Reference intervals for each of the age groups used in this study were generated using a non-parametric method since the data did not need to be transformed and all the groups had the minimum number of samples necessary to use it (32). Another limitation identified in this study was that the sample used may not be completely representative of the broader broiler population. There are several variations among modern broiler companies that could potentially have an impact on the blood biochemistry values and were not considered in this study, such as the breed of the birds, the diet formulations, inclusion of feed additives, water treatment systems, stocking densities, and management procedures. Therefore, the present reference intervals are only considered preliminary and further testing of different flocks is necessary to determine if there are any differences among complexes and/or companies that could be attributed to those variables and ultimately affect the interpretation of test results.

Practical considerations. Several factors need to be considered to successfully perform blood testing in a field setting, as the conditions are not as controlled as in a clinic or hospital. Point-of-care analyzers are characterized by their portability and ease-to-use, but most of them are not originally designed to be utilized outdoors as they are delicate instruments that require cautious handling and certain conditions to work properly (22,23). The i-STAT analyzer used has an estimated cost of $4,200, and each reagent cartridge has an approximate price of $18. The VS2 used in this study has an estimated cost of $5,000, with the reagent rotors valued at around $45 each. Both analyzers need to be kept in a protective case to prevent any bumps and must remain on a temperature range of -10 to 46°C (14-115°F), and unless those conditions can be always guaranteed, they should not stay inside a vehicle's cabin or in the back of a truck for prolonged periods of time. Ideally, they should be stored in an office or laboratory and transported to the field as needed, even when this could be tedious and time-consuming. The reagent cartridges and rotors must be stored at 2-8°C (36-46°F) so they need to be transported in a cooler and remain under the specified temperature until used. The i-STAT operates with two 9-volt lithium batteries which duration varies according to the brand and the testing frequency. It is recommended to always have a reserve of new batteries in case they run out during field testing. The VS2 on the other hand, cannot work with disposable batteries and it always requires a power supply, which can be problematic in the field, as some farms may not have electrical outlets available or may experience voltage fluctuations that can damage the analyzer. A potential solution is to use a car power inverter, which converts a vehicle's direct current (DC) into an alternate current (AC) that can be used to power the VS2 if needed. Before the sample collection and testing, the analyzers need to be set on a sturdy, leveled, and non-vibrating surface; in an environment free of dust or any other contaminants, within the required operating temperature

and humidity conditions (16-32°C, <90% humidity), and without direct contact to bright lights or sunshine. Since it is difficult to provide these conditions inside a poultry house, it is advised to conduct the testing in a separate area, such as the anteroom or the storage room. If there is no alternative but to do the testing inside the poultry house, the analyzers can be wrapped on a disposable hairnet and kept inside a box to avoid any contamination. Due to the relatively narrow range of operating temperature, there are geographical areas where the weather conditions may not be suitable on certain seasons of the year, so it is recommended to avoid the hottest/coldest times of the day when conducting a testing session. Whenever this is not possible and the conditions are too hot, the analyzers can be placed inside an open cooler or fridge to achieve the required temperature. If the conditions are too cold, a portable heater can be placed in the room where the analyzers are being used, or the testing can be conducted inside the poultry house with all the necessary precautions. The anticoagulant of choice for biochemical testing of chicken blood is lithium heparin since sodium citrate and EDTA can significantly alter the concentration of some electrolytes such as sodium, calcium, and potassium (33). A range of needle sizes can be used for the sample collection, from 25G for the youngest chicks to 18G for chickens over five weeks of age. The right jugular vein is the most common collection site for chicks from 0 to 3 weeks of age (34). It can sometimes be difficult to obtain blood from the jugular vein in chicks younger than five days, so a cardiac puncture method can be used as an alternative. If done correctly, this method yields larger volumes of blood rapidly and more cleanly, however, there is a high risk of lung perforation, resulting in fatal hemorrhage. If a fatal hemorrhage is suspected, the chick must be euthanized immediately (35). The brachial vein is the most accessible site to obtain the samples in chickens older than 3 weeks (36). Applying alcohol into any of the collection sites facilitates the visualization of the blood vessel and it is recommended for less

experienced handlers. It is advised to heparinize the needle before collection to avoid clots that can plug the needle and/or interfere with the testing. Even when the analyzers only need 0.1 mL of blood to conduct a test, it is ideal to collect at least 0.5 mL from each bird in case there is a cartridge/rotor error, and the testing needs to be repeated. Once the sample is collected, the reagent cartridges/rotors can be filled directly from the syringe, or the blood can be carefully transferred to green top tubes and kept there until analysis. It is very important to not fill the cartridges/rotors ahead of time since they must be used within 10 minutes of applying the sample. The used reagent cartridges/rotors are considered biohazardous waste and they may need special treatment before disposition according to each company's standards. After each test, the analyzers show a summary of the results and automatically store them in the internal memory, so there is no need to manually record the results as they can be reviewed and transferred to a computer later as needed. Once the testing is finished, the analyzers should be cleaned with a soft cloth moistened with a mild, non-abrasive detergent or cleaning solution and stored back in their case. The VS2 has an air filter in the back that must be checked at least once a month and replaced as needed to ensure proper ventilation and temperature control of the analyzer (23). These recommendations are based on the issues faced during the collection process of this study and will hopefully be useful for other clinicians and researchers that wish to perform blood testing in poultry farms. They are also helpful to maintain the analyzers in a good condition and to extend their operating life as much as possible.

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Summary and Conclusions

Although many of the metabolic diseases that affect commercial poultry have been extensively studied, their etiology is not well understood. This lack of understanding may be in part because the routine diagnostic tests used in commercial poultry emphasizes infectious diseases. Also, in most cases, the tools used for investigating metabolic diseases are ineffective for detecting them before clinical signs appear. The utilization of point-of-care analyzers has increased dramatically in the last two decades in human and veterinary medicine due to their convenience, accessibility, and short turnaround time, which eliminate the disadvantages of conventional laboratory testing. Given their portability, these analyzers can potentially be used for field testing, and studies in laying hens, broiler breeders, and backyard chickens have been performed in the past. Therefore, the goal of this thesis was to evaluate the performance of the i-STAT and Vetscan VS2 portable analyzers using broiler blood and to determine preliminary reference intervals for broilers of different ages. This information would help to advance the knowledge of metabolic conditions of broilers and contribute to their early diagnosis and potential treatment.

The first manuscript in this thesis, concluded that even though there were statistically significant differences in the concentration of some of the analytes measured by the portable analyzers after one hour of collection, they were not clinically relevant and did not affect the interpretation of the results. Therefore, if the samples have minimal contact with the environmental air and are kept at 4°C, they can be tested within an hour without adverse consequences. It also demonstrated that the portable analyzers are not directly comparable to their conventional counterparts, as some of the analytes failed to reach the 95% of agreement. Based on these results, a subsequent study to determine reference intervals for broilers at

different ages using the i-STAT analyzer with the CG8+ cartridge, and the VS2 analyzer with the Avian/Reptilian Rotor was developed. This second manuscript showed that the concentration of certain analytes is age-dependent in broilers, , opening a new path of research for the potential association of these fluctuations with common metabolic diseases such as Wooden Breast and Sudden Death Syndrome. Additionally, preliminary reference intervals for 7-, 21-, and 35-days old broilers were determined.

Overall, this work demonstrates great potential that point-of-care analyzers have as a diagnostic tool for improving poultry health and welfare and lays the foundations for further studies that could provide critical information needed to develop strategies for better insight and control of metabolic conditions.