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Original Study

Generation of Reference Intervals and Evaluation of Seasonal Variation in Clinical Pathology Parameters of Backyard Laying Hens

Alison Cummings, Carolyn Cray, Fabiano Montiani-Ferreira, and Laurie Hess

Abstract: Backyard poultry have seen significant gains in popularity in the United States in recent years. Backyard chicken (*Gallus gallus domesticus*) breeds, selected for high egg production and friendly temperament, differ from those typically used in commercial egg and meat production, as well as in veterinary research studies. This increased interest in chickens as pets has led to a growing need for more breed-specific veterinary care, including clinical pathology references. In the present study, 48 clinically healthy, young laying hens from 4 different flocks, representing 7 popular backyard breeds, were evaluated. Reference intervals for complete blood count, plasma biochemistry panel, and plasma protein electrophoresis were established per the guidelines of the American Society for Veterinary Clinical Pathology. Paired samples (summer vs winter) were used to assess the effect of season on these results. Notably, significant seasonal differences were seen in all measured values of the complete blood count as follows: estimated white blood cell count ($P < 0.0001$) and lymphocyte ($P < 0.0001$), monocyte ($P = 0.0298$), eosinophil ($P = 0.0169$), and basophil ($P < 0.0001$) absolute counts were higher in the summer months, while packed cell volume ($P = 0.0006$) and heterophil count ($P = 0.0096$) were lower. When evaluating the results of the plasma biochemistry panel, samples from the summer months exhibited lower concentrations of glucose ($P < 0.0001$) and calcium ($P = 0.0008$) and aspartate transaminase activity ($P = 0.0046$), but higher creatine kinase activity ($P = 0.0069$) and phosphorus concentrations ($P = 0.006$). The plasma protein electrophoresis results demonstrated a lower albumin-to-globulin ratio ($P = 0.0012$) in the summer, with higher concentrations of alpha-1 ($P < 0.0001$) and gamma ($P < 0.0001$) globulins. These findings support the need for season-specific reference intervals when evaluating clinical pathology test results from backyard chickens.

Key words: avian, backyard chicken, biochemistry, clinical pathology, complete blood count, *Gallus gallus domesticus*, reference intervals, protein electrophoresis

INTRODUCTION

Backyard chicken (*Gallus gallus domesticus*) ownership in the United States, for both companionship and personal egg production, has increased in recent years.[1–3] These birds differ from those used in commercial egg and meat production not only in lifestyle, but also in the chicken breeds selected, with the main focus being on female birds of breeds with high egg production and docile temperament.[2,4] Backyard flocks are often all-female flocks of fewer than 10 birds of mixed egg-laying breeds.[2] Common backyard chicken breeds include Rhode Island Red, Plymouth Rock, Ameraucana, Orpington, and Wyandotte.[2] This shift in status from production animal to productive family member has created a need for veterinary care specific to this demographic with minimally invasive antemortem laboratory evaluation.[1–3]

Research in avian medicine has been largely driven by interest in the health of commercial poultry bred for rapid and efficient growth in a short lifespan.[1–3,5,6] Health assessment of chickens has traditionally been approached from a flock health perspective, with necropsy of deceased and/or culled birds being the common diagnostic starting point.[3,7] In contrast, backyard chicken

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owners are showing an increased desire for regular wellness examinations to assess the health of their birds before their pets show signs of illness.[1,2] Moreover, for birds seen as "pets" or "members of the family," culling and necropsying a sick bird for diagnostic purposes is not generally an acceptable option. In avian medicine, antemortem diagnostic tests, such as a complete blood count (CBC), plasma biochemical panel (PBP), and plasma protein electrophoresis (EPH), are commonly performed, affordable, reliable, and minimally invasive means to provide a diagnostic overview of overall health.[5,6,8–12] When species-specific parameters are accurately defined, these tests are invaluable tools for diagnosing and managing disease states.[5,6,11]

As the model avian research species, commercial chickens have been well-studied to develop diagnostic testing references. However, few references exist for the health and evaluation of backyard chickens bred for personality, appearance, and egg production.[1,3] Importantly, 2 recent regional studies have demonstrated that the PBP reference intervals of backyard chickens do not correspond to those previously established for commercial poultry,[1,13] indicating the need for the generation of new reference intervals specific to the growing number of chicken breeds in this backyard demographic.[1,2] Age has also been shown to affect normal physiologic values in chickens, with 1 study demonstrating broiler chickens' significant variation in physiologic electrophoretic protein fractions during different life stages.[14] With this in mind, it is hypothesized that for the most accurate health assessment, normal CBC, PBP, and EPH measurements are needed in the context of these specific factors, including the season in which they are measured. If seasonal changes in reproductive activity influence these measurements in reproductively active laying hens, reference values for reproductively active laying hens may be different during different seasons.

This study aimed to establish reference intervals for CBC, PBP, and EPH in backyard laying hens across different seasons and to investigate how seasonal metabolic fluctuations may impact these reference values in clinically healthy, reproductively active birds.

MATERIALS AND METHODS

Participant selection and study design

A total of 48 laying hens from 4 different flocks were evaluated between March and December of 2019. All birds were from the same geographic area in Westchester County, New York, USA, limiting the variability in day length and temperature that affects seasonal reproductive activity. Participating flocks were selected from among bird owners known to have good husbandry practices, including proper housing, sanitation, nutrition, and biosecurity. Only previously healthy flocks, without a history of illness or injury, were included. Flock size varied from 3 to 27 birds, with breeds representing popular varieties found in backyard laying and ornamental flocks,[1,2,4] ultimately including 16 Ameraucana/Ameraucana Cross ("Easter Eggers"), 16 Wyandottes, 8 Rhode Island Reds, 2 Marans, 3 Plymouth Rocks, 1 Orpington, and 2 Frizzles. Flocks that used artificial lighting in their coop environments, particularly in the winter to boost egg production, were excluded. All flocks included were confirmed by their owners to have variable seasonal egg production, with the highest production in July and August (>14 hours of daylight) and lowest production from December to March (<12 hours of daylight). To be included in the study, owners agreed to have their chickens evaluated and sampled on 2 separate occasions. Participating hens had to be confirmed egg layers within an optimal egg laying age range of 5 months to 2 years. Flock owners were not directly compensated for study participation; however, complementary physical examinations, fecal testing for parasites, and blood sampling for CBC, PBP, and EPH were performed.

Participant evaluation

Flocks were evaluated and sampled on site to minimize the stress of transport. To further minimize stress, chickens were initially restrained by either the owner or a veterinary professional experienced in handling poultry. A general physical examination was performed by an avian veterinarian (AC) to screen the birds for any apparent disease, defect, or injury. Hens found to have diseases, defects, or injuries were excluded from the study. To minimize variability, the same veterinarian performed the physical examination, blood collection, and sample handling for all birds. Further testing included flock fecal sampling for common gastrointestinal parasites and blood sampling for CBC and PBP to assess overall health and organ function according to previously established reference ranges, with major outliers excluded from the study.[15] Qualifying flocks had paired blood samples taken in 2 different seasons ("summer" vs "winter") to ensure there was no change in the health status of the birds from the first collection time point to the second. Blood samples were obtained both in the winter months (December–March; <12 hours daylight), when egg laying was decreased, and during the peak egg laying summer months (July and August; >14 hours daylight).

Sample collection and processing

All birds were restrained and identified by the owner, and their breeds and ages were confirmed. Any identifying markers were recorded and photographed so that the birds could be positively identified for repeated blood sampling at the second time point. Those without identifying leg bands were given temporary plastic leg bands with identification numbers to avoid misidentification when paired samples were collected.

Blood samples were drawn from the left or right brachial vein using a 1-mL syringe and a 21-G needle while the bird was held upright in a "football hold" with its head tucked under the restrainer's arm. The collected blood was immediately used to make 2 blood smears and to fill 2 heparinized microhematocrit tubes and a lithium heparinized green-top tube with a gel separator. These samples were transferred into a cooler for transport to the hospital for processing. The time from sampling to centrifugation and processing of all samples was less than 1 hour. The microhematocrit tubes were centrifuged for 5 minutes (10,000 rpm), and packed cell volume (PCV) and total solids were measured and recorded. Plasma was transferred into labeled bullet tubes, frozen (−20°C, −4°F), and shipped with the blood smears to the laboratory (University of Miami, Miller School of Medicine; Avian and Wildlife Laboratory, Miami, FL, USA) for CBC, PBP, and EPH testing.

Hematology

Smears were stained with Wright Giemsa and evaluated under oil (×1000). The total white blood count (WBC) count estimates were performed by averaging the total count of WBC in 10 fields at ×400. This number was multiplied by 2000. All estimated WBC counts and differential counts were performed by technical staff with more than 10 years of combined experience with avian samples. All blood smears were reviewed for cell distribution (ie, feathered edge) and found to be acceptable for analysis.

Plasma biochemical analysis

Testing was completed using a Vitros 250 analyzer (Ortho, Rochester, NY, USA), which was maintained according to the manufacturer's instructions. Reagents for the quantitation of glucose, total protein, calcium, phosphorus, aspartate transaminase (AST), and creatine kinase (CK) were purchased from Ortho. Bile acid concentrations were quantified using a radioimmunoassay (MP Biomedicals, Solon, OH, USA) as previously described.[16]

Plasma protein electrophoresis

Testing was completed using split beta gels on a Helena Laboratories electrophoresis system (Beaumont, TX, USA), as previously described.[17] Fractions were quantified as a percentage after the placement of fraction delimiters. Percentages were multiplied by the total protein to obtain absolute values. The albumin-to-globulin (A/G) ratio was calculated by dividing the albumin concentration by the sum of the globulin concentrations.

Statistical analysis

For seasonal comparisons, paired measures were examined using either a paired *t*-test or a Wilcoxon signed-rank test, based on the normality of the data. The latter was determined using the D'Agostino-Pearson test. For reference interval generation, the robust method was used with transformations as per the guidelines of the American Society for Veterinary Clinical Pathology.[18] All statistical analyses were conducted using MedCalc Statistical Software version 20.115 (MedCalc Software Ltd, Ostend, Belgium).

RESULTS

A total of 48 laying hens between 6 and 15 months of age were sampled during the previously defined "summer" and "winter" periods. Breeds represented popular varieties found in backyard laying and ornamental flocks,[1,2,4] including 16 Ameraucana/Ameraucana Cross ("Easter Eggers"), 16 Wyandottes, 8 Rhode Island Reds, 2 Marans, 3 Plymouth Rocks, 1 Orpington, and 2 Frizzles. Two of 48 sampled chickens were excluded from any seasonal comparative evaluation because these animals died from predation before paired samples could be collected. All flocks remained free from clinical illness throughout the study and for at least 3 months after the final blood sampling.

Seasonal differences observed for CBC, PBP, and EPH are reported in Tables 1, 2, and 3, respectively. Reference intervals are shown in Tables 4 and 5. Significant seasonal differences ($P < 0.05$) were noted in all measured values for the CBC (Table 1). Total estimated WBC count ($P < 0.0001$) and lymphocyte ($P < 0.0001$), monocyte ($P = 0.0298$), eosinophil ($P = 0.0169$), and basophil ($P < 0.0001$) absolute counts were higher in the summer, while PCV ($P = 0.0006$) and heterophil counts ($P = 0.0096$) were lower.

Table 1. Descriptive statistics for seasonal hematologic parameters from backyard chickens (*Gallus gallus domesticus*). Because the measures were not normal, the medians and interquartile ranges are reported. Significant differences were found between seasons for all hematologic parameters.

Parameter	Summer	Winter	*P* value
Estimated WBC $\times 10^3/\mu l$	15.0 (13.0–21.0)	11.0 (9.0–15.0)	<0.0001
PCV, %	39.8 (35.0–42.0)	45.5 (42.0–50.0)	0.0006
Heterophils $\times 10^3/\mu L$	3.5 (2.3–4.9)	4.5 (3.2–5.7)	0.0096
Lymphocytes $\times 10^3/\mu L$	10.4 (7.3–13.6)	5.0 (3.6–8.2)	<0.0001
Monocytes $\times 10^3/\mu L$	0.7 (0.4–1.3)	0.3 (0–0.9)	0.0298
Eosinophils $\times 10^3/\mu L$	0.3 (0.2–0.5)	0.2 (0–0.3)	0.0169
Basophils $\times 10^3/\mu L$	0.4 (0.2–0.6)	0.1 (0–0.3)	<0.0001

Abbreviations: WBC, total white blood cell count; PCV, packed cell volume.

Evaluation of the PBP results (Table 2) also showed significant seasonal variation. Samples from the summer exhibited lower glucose concentrations ($P < 0.0001$), calcium concentrations ($P = 0.0008$), and AST activity ($P = 0.0046$), but higher CK activity ($P = 0.0069$) and phosphorus concentrations ($P = 0.006$).

Representative electrophoretograms are shown in Figure 1. The EPH results (Table 3) demonstrated significant seasonal differences, including lower A/G ratios ($P = 0.0012$) and higher alpha-1 ($P < 0.0001$) and gamma ($P < 0.0001$) globulin concentrations during the summer.

DISCUSSION

The growing popularity of backyard chickens has created a need for accurate antemortem diagnostic hematologic and biochemical reference values.[1,2,13] Veterinarians commonly use the CBC, PBP, and EPH to evaluate the health of birds, and specific reference intervals have been established for many species.[5,6,9,10,12,19,20] Extrapolation of normal reference values from 1 species to another is used when necessary; however, such extrapolation is often inaccurate and may lead to misinterpretation and misdiagnosis.[5,6,9,11,20,21] Although much of the pioneering research in avian medicine was performed using chickens as a model,[3,6,9] many of the reference values established through this research are now outdated or were established using commercial chicken populations.[5,14] These reference ranges may not accurately represent the currently popular backyard breeds and ages of birds.[1,3,19]

A 2018 evaluation of PBP values of backyard chickens in Washington state demonstrated that several backyard chicken values differed from those previously established for commercial flocks.[1] This study showed that globulin, potassium, and sodium concentrations correlated well between backyard and commercial chickens; however, albumin, calcium, phosphorus, total protein, and uric acid concentrations did not.[1] Samples in our study were collected from backyard chickens from June through August, mirroring the summer month sampling performed in the previous study; however, a direct comparison between the 2 studies was not possible because of differences in the ages of the birds. In the current study, the reference values measured in the summer months for calcium, uric acid, total protein, AST, CK, and phosphorus correlated well with these parameters from the 2018 study. However, measurements of PCV, glucose, and bile acids did not. A more direct comparison

Table 2. Descriptive statistics for seasonal plasma biochemistry parameters from backyard chickens (*Gallus gallus domesticus*). Because the measures were not normal, the medians and interquartile ranges are reported, except uric acid.

Parameter	Summer	Winter	*P* value
Glucose, mg/dL	229 (214–243)	266 (252–277)	<0.0001
Calcium, mg/dL	11.2 (10.0–14.2)	12.5 (11.2–15.3)	0.0008
Uric acid, mg/dL[a]	5.8 (5.3–6.3)	5.5 (4.8–6.3)	0.4627
Total protein, g/dL	4.3 (3.9–5.8)	4.3 (4.0–4.8)	0.2082
AST, U/L	177 (146–204)	221 (175–267)	0.0046
CK, U/L	872 (598–1327)	784 (484–996)	0.0069
Phosphorus, mg/dL	4.2 (3.3–5.0)	3.5 (3.2–4.3)	0.0060
Bile acids, μmol/L	22.5 (19.3–66.8)	53.5 (40.7–97.6)	0.0696

Abbreviations: AST, aspartate aminotransferase; CK, creatine kinase.

[a] Mean and 95% confidence interval.

Table 3. Descriptive statistics for seasonal protein electrophoresis results from backyard chickens (*Gallus gallus domesticus*). Because the measures were not normal, the medians and interquartile ranges are reported, except the alpha-2 globulins.

Parameter	Summer	Winter	*P* value
Total protein g/dL	4.3 (3.9–5.8)	4.3 (4.0–4.8)	0.2082
A/G ratio	0.77 (0.58–0.93)	0.81 (0.62–1.03)	0.0012
Albumin, g/dL	1.88 (1.76–2.02)	1.84 (1.71–2.14)	0.3126
Alpha-1, g/dL	0.23 (0.20–0.32)	0.17 (0.14–0.20)	<0.0001
Alpha-2, g/dL[a]	0.66 (0.62–0.70)	0.68 (0.63–0.73)	0.3205
Beta, g/dL	1.06 (0.84–1.68)	1.19 (1.08–1.43)	0.9581
Gamma, g/dL	0.43 (0.34–0.50)	0.31 (0.23–0.37)	<0.0001

Abbreviation: A/G, albumin/globulin.

[a] Mean and 95% confidence intervals.

of the data, excluding those from outside the age limits sampled in the current study, is needed to determine the significance of these differences. In addition, further data collected from different geographic regions would be useful in determining whether geographic location (and subsequent effects on light intensity and temperature) have any effect on hematologic or biochemical reference values.

A second study conducted in Colorado in 2022 used healthy backyard chickens of mixed breeds, sexes, and ages (sexually mature adults >4 months of age) and also found discrepancies with previously published biochemical reference ranges established for commercial flocks.[13] Specifically, there were differences in the measured concentrations of sodium, calcium, total protein, potassium, phosphorus, uric acid, and glucose compared with the previous publications. Further evaluation of the collected data revealed significant differences in reference ranges established when the results of hens and roosters were evaluated independently. These differences were presumptively attributed to factors associated with egg laying. However, when the data from the hens were separated seasonally, based on seasonal variation in egg production, any differences in biochemistry measurements noted were deemed statistically insignificant. As a result, the authors suggested that separate reference intervals for roosters versus hens should be established, and results for egg-laying hens versus non-egg-laying hens should be considered separately, rather than specifically focusing on seasonality.

Although the large sample size (N = 123) in the Colorado study was advantageous for generating reference ranges according to the American Society of Veterinary Clinical Pathologists guidelines,[18] and the diversity of breeds in this study well represented the general backyard chicken population, this study was not without flaws. The birds in this study were only briefly examined for obvious signs of disease, and CBCs were not performed to evaluate any of the chickens for underlying illness. Additionally, although data were separated into both summer (n = 69) and winter (n = 54) categories, the Colorado seasonal samples were not paired from the same individuals. Moreover, these individuals were not divided into egg-laying versus non-egg-laying groups, an important distinction when the youngest chickens in the study are below the average age of reproductive maturity (5 to 5.5 months).[22] The Colorado study also did not assess whether artificial light was being used in winter to boost egg production. The variables introduced by using data from birds of varied breeds and ages, as well as from different flock conditions, limit the reliability of the Colorado study's assessment of seasonal differences in PBP reference ranges. Therefore, a direct comparison of the Colorado study's results to ours is not possible. Our study's reference intervals and those previously published in the literature are noted in Table 6.

The physiological demands egg laying places on chickens are substantial, with a recent investigation demonstrating a clear increase in blood cortisone concentrations in egg-laying hens compared with non-egg-laying hens under the same conditions.[23] Chickens are considered continuous egg layers, demonstrating a 24- to 28-hour ovulation/oviposition cycle. This cycle continues uninterrupted in sequence over several days, with ovulation usually occurring within 15 to 75 minutes after the last egg has been laid, followed by intermittent "rest days."[22,24] Oviposition rate varies by breed.[4] In peak egg-laying season, when maximum photostimulation occurs with 12 to 14 hours of light per day,[24] birds bred for meat production (eg, Cornish cross) lay an average of 1 small egg per week, in contrast with the high-production egg-laying breeds evaluated in this study. High-production egg-laying breeds produce larger eggs more frequently, with Ameraucanas, Marans, and Orpingtons averaging 3 eggs per week; Plymouth Rock and Wyandottes 4 eggs per week; and Rhode Island Reds 5 eggs per week.[25] These production numbers

Table 4. Hematologic and plasma biochemistry reference intervals for backyard chickens (*Gallus gallus domesticus*) during the summer. The *P* value is associated with the test for normality.

Parameter	Units	N	Mean	SD	Median	Min	Max	*P* value	Dist	Method	LRL	URL	CI 90% LRL	CI 90% URL
Estimated WBC	$\times 10^3/\mu L$	47	17.2	6.6	15.0	8.0	39.0	<0.0001	NG	R, T	0.6	28.6	0–5.5	24.5–33.4
PCV	%	36	39.5	6.5	38.8	25.0	58.5	0.0092	NG	R, T	24.9	51.8	21.5–29.0	47.7–55.7
Heterophils	$\times 10^3/\mu L$	47	3.7	1.9	3.5	0.8	9.4	0.0111	NG	R, T	0	7.4	0–0.4	6.4–8.4
Lymphocytes	$\times 10^3/\mu L$	47	11.8	5.9	10.7	4.9	32.0	<0.0001	NG	R, T	0	22.5	0–1.6	18.6–26.0
Monocytes	$\times 10^3/\mu L$	47	0.8	0.5	0.7	0	1.9	0.0185	NG	R, T	0	1.9	0–0.2	1.6–2.1
Eosinophils	$\times 10^3/\mu L$	47	0.4	0.4	0.3	0	2.1	<0.0001	NG	R, T	0	1.1	0–0.2	0.8–1.4
Basophils	$\times 10^3/\mu L$	47	0.4	0.3	0.4	0	1.5	0.0001	NG	R, T	0	1.0	0–0.1	0.8–1.2
Glucose	mg/dL	47	227	21	228	175	269	0.9957	G	R, T	180	266	169–191	258–273
Calcium	mg/dL	47	12.8	4.8	11.0	8.1	31.5	<0.0001	NG	R, T	3.5	22.1	1.5–5.5	20.1–24.1
Uric acid	mg/dL	47	5.9	1.6	5.7	2.3	9.7	0.9941	G	R, T	2.6	9.1	2.0–3.3	8.4–9.8
Total protein	g/dL	47	4.5	0.9	4.3	3.2	8.3	0.8102	G	R, T	3.4	6.8	3.3–3.5	6.0–8.0
AST	U/L	47	169	123	177	58	774	0.2040	G	R, T	65	512	54–81	388–654
CK	U/L	47	860	683	892	342	4661	0.9896	G	R, T	331	2891	275–404	2232–3802
Phosphorus	mg/dL	47	4.1	1.4	4.3	2.0	9.3	0.6975	G	R, T	2.2	7.7	1.9–2.5	6.8–8.6
Bile acids	µmol/L	47	21.0	57.9	20.0	2.0	350.6	0.0740	G	R, T	4.1	154.4	3.1–6.0	84.6–298.8
A/G ratio		47	0.75	0.19	0.77	0.30	1.15	0.1095	G	R, T	0.35	1.14	0.27–0.43	1.08–1.23
Albumin	g/dL	47	1.88	0.21	1.86	1.05	2.21	0.9790	G	R, T	1.34	2.18	1.11–1.50	2.12–2.22
Alpha 1	g/dL	47	0.25	0.08	0.25	0.13	0.40	0.0492	NG	R, T	0.13	0.44	0.11–0.14	0.40–0.48
Alpha 2	g/dL	47	0.64	0.12	0.63	0.44	1.03	0.6583	G	R, T	0.45	0.97	0.43–0.48	0.89–1.07
Beta	g/dL	47	1.31	0.70	1.06	0.52	4.69	<0.0001	NG	R, T	0	2.66	0–0.26	1.99–3.24
Gamma	g/dL	47	0.42	0.11	0.43	0.23	0.68	0.6999	G	R, T	0.23	0.67	0.20–0.27	0.62–0.72

Abbreviations: Min, minimum; Max, maximum; Dist, distribution; LRL, lower reference limit; URL, upper reference limit; CI, confidence interval; WBC, white blood count; G, Gaussian; NG, non-Gaussian; R, robust; T, transformed; PCV, packed cell volume; AST, aspartate aminotransferase; CK, creatine kinase; A/G, albumin/globulin.

Table 5. Hematologic and plasma biochemistry reference intervals for backyard chickens (*Gallus gallus domesticus*) during the winter. The *P* value is associated with the test for normality.

Parameter	Units	N	Mean	SD	Median	Min	Max	*P* value	Dist	Method	LRL	URL	CI 90% LRL	CI 90% URL
Estimated WBC	$\times 10^3/\mu L$	46	11.5	3.4	11.0	6.0	17.0	0.0001	NG	R, T	4.5	18.4	2.9–5.6	17.1–19.6
PCV	%	45	44.8	7.0	46.0	30.0	57.0	0.2473	G	R, T	31.4	60.4	27.7–35.1	57.4–62.9
Heterophils	$\times 10^3/\mu L$	46	4.6	1.8	4.4	2.1	9.2	0.1376	G	R,T	0.7	8.2	0–1.4	7.3–9.1
Lymphocytes	$\times 10^3/\mu L$	46	5.8	2.8	5.0	1.4	12.3	0.1227	G	R, T	0.0	11.4	0–0.6	9.7–12.7
Monocytes	$\times 10^3/\mu L$	46	0.6	0.7	0.3	0	3.0	<0.0001	NG	R, T	0.0	1.9	0–0	1.4–2.3
Eosinophils	$\times 10^3/\mu L$	46	0.3	0.5	0.2	0	2.9	<0.0001	NG	R, T	0.0	1.1	0–0	0.6–1.6
Basophils	$\times 10^3/\mu L$	46	0.2	0.2	0.1	0	0.8	0.0120	NG	R, T	0.0	0.6	0–0	0.5–0.7
Glucose	mg/dL	46	264	20	265	229	308	0.7130	G	R, T	223	305	216–231	296–313
Calcium	mg/dL	46	15.0	6.1	12.5	9.0	32.7	0.0003	NG	R, T	3.0	27.0	0.4–5.6	24.4–29.6
Uric acid	mg/dL	46	5.7	2.6	5.5	1.2	11.6	0.1568	G	R, T	0.6	10.7	0–1.8	9.6–11.8
Total protein	g/dL	46	4.4	0.5	4.3	3.5	5.4	0.3435	G	R, T	3.4	5.4	3.2–3.6	5.1–5.5
AST	U/L	46	219	66	214	100	402	0.3931	G	R, T	80	349	54–108	317–379
CK	U/L	46	934	867	787	222	4939	<0.0001	NG	R, T	0	2525	0–155	1358–3288
Phosphorus	mg/dL	46	3.7	0.8	3.5	2.1	5.8	0.1589	G	R, T	2.0	5.2	1.6–2.3	4.7–5.6
Bile acids	µmol/L	21	73.4	51.0	49.8	24.6	223.9	0.0010	NG	R, T	0	175	0–0	114–216
A/G ratio		46	0.86	0.28	0.82	0.49	1.58	0.0314	NG	R, T	0.24	1.40	0.14–0.35	1.23–1.56
Albumin	g/dL	46	1.94	0.31	1.85	1.50	2.82	0.0310	NG	R, T	1.23	2.54	1.09–1.38	2.35–2.71
Alpha 1	g/dL	46	0.18	0.06	0.17	0.08	0.40	<0.0001	NG	R, T	0.06	0.28	0.02–0.09	0.24–0.32
Alpha 2	g/dL	46	0.68	0.16	0.68	0.41	1.14	0.0638	G	R, T	0.33	1.00	0.26–0.40	0.92–1.08
Beta	g/dL	46	1.23	0.31	1.19	0.54	1.91	0.9552	G	R, T	0.58	1.87	0.46–0.72	1.72–2.00
Gamma	g/dL	46	0.31	0.09	0.31	0.19	0.61	0.0275	NG	R, T	0.12	0.49	0.08–0.16	0.44–0.54

Abbreviations: Min, minimum; Max, maximum; Dist, distribution; LRL, lower reference limit; URL, upper reference limit; CI, confidence interval; WBC, white blood count; G, Gaussian; NG, non-Gaussian; R, robust; T, transformed; PCV, packed cell volume; AST, aspartate aminotransferase; CK, creatine kinase; A/G, albumin/globulin.

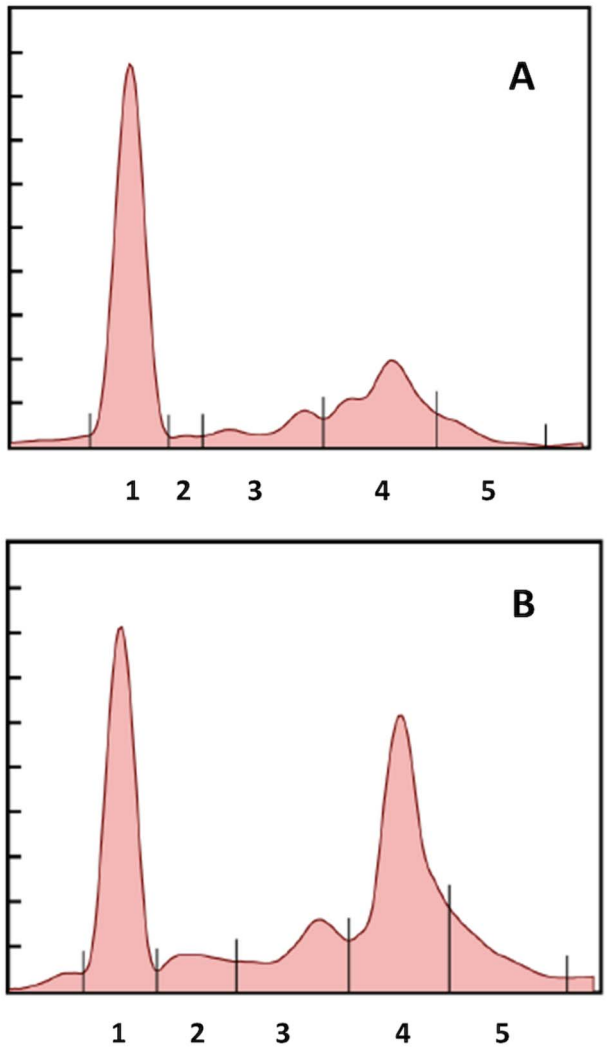

Figure 1. Representative electrophoretograms from winter (A) and summer (B) sampling. (A) Ameraucana Cross ("Easter Egger") hen (*Gallus gallus domesticus*), albumin/globulin (A/G) ratio = 1.31; (B) Rhode Island Red hen, A/G ratio = 0.49. X-axis labels as follows: 1, albumin; 2, alpha-1 globulins; 3, alpha-2 globulins; 4, beta globulins; 5, gamma globulins.

decrease, and some individuals stop laying altogether, because the day length decreases over the winter months and the environmental photoperiod is less than 12 hours of light per day.[24] This reduced photoperiod triggers ovarian follicle regression and molting.[22,24] In young laying hens, the initiation, peak, and cessation of egg production vary with both seasonal photoperiod and age of the hen. Egg laying generally starts at 20 to 22 weeks of age (5–5.5 months old), peaks 6 to 10 weeks after the start of laying (6.5–8 months old), and then, starting at about 18 to 20 weeks after peak (11–13 months old), gradually decreases over the next 10 to 12 months (21–25 months old). Subsequently, egg laying continues over a varied number of years at intermittent intervals, while reproductive diseases and age-related illnesses increase.[22]

The drastic escalation in physiological stress that occurs in young birds from egg laying causes variations in normal hematologic reference values.[6,8,10,14,22] However, these variations have not yet been correlated to seasonal variations in hematologic reference values in backyard laying hens. The current study assessed seasonal differences in hematologic and biochemical reference values during the peak reproductive age of laying hens and found that the time of year (likely through the effects of light exposure) impacts the measured hematologic and biochemical reference values. Specifically, the results of CBC, PBP, and EPH vary in healthy laying hens depending on the season. Further research is needed to determine whether similar changes are observed in older hens whose egg production rates are declining with age.

Normal variations in the avian CBC have been reported based on age, sex, hormonal status, environmental conditions, and species.[5,21,26] In the current study, all parameters measured in the CBC between the paired winter and summer samples were statistically different. Notably, the summer data demonstrated a significant decrease in heterophil count and significant increases in lymphocyte and basophil counts when compared with similar counts in the winter. The elevation in total estimated WBC count in the summer samples is likely indicative of an increase in leukocytes in response to stress and/or inflammation. A typical avian stress leukogram involves heterophilia and lymphopenia.[3,26] However, the pattern of decreased heterophils and increased lymphocytes and basophils correlates with the pattern expected in chickens undergoing a more severe or a chronic stress rather than the acute stress typically reflected in a classic stress leukogram.[5,27] This shift in leukocyte pattern in healthy young chickens was unexpected and may be reflective of increased physiologic stress on hens from both egg laying and high summer temperatures, and/or inflammation from reproductive activity. The total WBC counts in this study were determined using the manual estimate method; therefore, the reference intervals are best applied to data obtained using the same method. As estimated total WBC counts in avian species have been reported to both correlate and not correlate with the phloxine-based hemacytometer method, future studies could seek to address such a comparison in chickens.[28,29]

The PCV in birds has also been noted to vary with species, age, sex, chronic states of inflammation, and hormonal influences. Female birds are reported to have lower PCVs compared with males of the same species,

Table 6. Comparison of biochemical parameters from backyard chickens (*Gallus gallus domesticus*) from the current study and 3 historic studies.

Parameter	Current study summer	Current study winter	Kaiser et al[13]	Board et al[1]	Greenacre et al[15]
Calcium, mg/dL	3.5–22.1	3.0–27.0	10.4–32.7	>10.9	13.2–23.7
Phosphorus, mg/dL	2.2–7.7	2.0–5.2	1.9–6.5	1.6–7.2	6.2–7.9
Uric acid, mg/dL	2.6–9.1	0.6–10.7	2.9–10.5	0.9–8.9	2.5–8.1
AST, U/L	65–512	80–349	116–241	118–298	–
CK, U/L	331–2891	0–2525	316–1792	107–1780	–
Glucose, mg/dL	180–266	223–305	210–295	174–239	–
Total protein (g/dL)	3.4–6.8	3.4–5.4	3.6–6.5	3.9–7.0	–

Abbreviations: AST, aspartate aminotransferase; CK, creatine kinase.

a difference that is more pronounced during the breeding season.[5,21,26,30] This physiologic difference is commonly attributed to the decreased erythropoiesis associated with increased circulating concentrations of estrogens and/or androgens. Osmoregulatory adjustments and the subsequent hemodilution that occurs in response to elevated concentrations of the yolk precursor vitellogenin may also contribute to the decreased PCV noted in reproductively active hens.[21,26,30] In addition, chronic inflammation associated with reproductive activity may further suppress red blood cell production, as seen with anemia of chronic disease.[27] The significant decrease in PCV noted in this study during peak egg-laying season may be a consequence of all of these factors.

Biochemical testing is a key antemortem diagnostic tool for evaluation of organ system health and function. Major variations from established normal values typically indicate dysfunction; however, minor physiological variations may occur with changes in nutrition, hydration status, and reproductive activity. Variations in biochemical analytes have been previously described in reproductively active birds. In general, reproductive activity is associated with physiologic increases in blood concentrations of total protein, total calcium, globulins, phosphorus, cholesterol, and alkaline phosphatase.[8,10,31] The results of the current study agreed with the previously reported increase in phosphorus concentration typically noted with egg laying, but increases in total protein and total calcium concentrations, also typically seen with egg laying, were not observed. Total calcium concentration typically increases in laying hens several weeks before the onset of egg laying and then remains elevated throughout the ovulation-oviposition cycle with minimal change. This is in opposition to ionized calcium concentration, which peaks 4 hours after oviposition and decreases significantly during shell calcification.[22] Additional studies should be performed to evaluate differences in ionized calcium concentrations in blood between backyard laying hens and commercial chickens, accounting for time of day and stage in the reproductive cycle. In this study, alkaline phosphatase and cholesterol concentrations were not evaluated; therefore, it is unknown whether these values increased in egg-laying hens during summer, as observed in previous studies.

Refractometric measurement of total protein concentration can be affected by the presence of other blood components, including glucose, lipemia, and hemolysis. In this study, lipemia and hemolysis were not observed in the study samples and can therefore be excluded as factors impacting total protein concentration.[11,12] Glucose concentrations in the summer and winter samples were statistically different; however, these differences were not considered large enough to have impacted the total protein concentrations in this study.

Creatine kinase activity, which typically increases with muscle damage,[8,10,16] was higher in the summer samples. Although CK activity was not initially expected to increase in response to changes in reproductive activity, this increase is not surprising in egg-laying birds undergoing the increased physical demand and muscle strain of egg laying during peak season. Creatine kinase activity is often evaluated in conjunction with AST activity. Aspartate aminotransferase, an enzyme primarily found in the liver and skeletal muscle (as opposed to CK, which is predominantly found in muscle), is typically measured simultaneously with CK activity to differentiate between liver disease and skeletal muscle damage when both enzymes are elevated. This differentiation is made based on CK's shorter half-life relative to AST, such that elevations in AST activity without elevations in CK activity suggest liver disease, while simultaneously increased activities of both enzymes cannot distinguish between liver disease and muscle damage.[8,10] The finding of lower AST activity in summer versus winter in this study was unexpected because increased AST activity typically accompanies increased CK activity associated with soft tissue damage

(presumed to occur in laying hens straining to lay eggs during summer).[3]

The decreased total calcium concentrations measured in this study were also unexpected because this mineral generally increases or remains unchanged in reproductively active birds.[8,10] The total calcium concentration measured in a standard PBP includes ionized calcium, protein-bound calcium, and calcium anion complexes.[8,32] Additional studies should be performed to evaluate the changes in these individual calcium components for backyard laying hens in different reproductive stages. Calcium has an important role in avian reproduction, both in the calcification of the eggshell and in uterine contraction for oviposition. Reproductive activity and subsequent estradiol secretion increase total blood calcium concentrations in poultry several weeks before a laying cycle. These values generally remain static with minimal variability during the ovulation-oviposition cycle.[8,10,32] However, chronic egg-laying conditions typically cause the opposite finding, with lower calcium concentrations in the blood due to increased consumption. Increased calcium consumption may explain the lower total calcium concentrations measured in the summer in this study.[8,10] However, even in birds undergoing increased demands for calcium, blood calcium concentrations may remain within the normal reference range because the body typically extracts calcium from the medullary cavity of bones to maintain constant circulating blood calcium concentrations.[8,32] The reference intervals included in this study are to be considered preliminary because they are based on a moderate sample size obtained from a small number of backyard flocks. The American Society of Veterinary Clinical Pathologists recommends the robust method of reference interval generation for these types of data, and an increased sample size may help strengthen the intervals of this and other measurements.

Physiological and environmental stress typically increase blood glucose concentrations due to the release of endogenous corticosteroids.[20,31] However, changes in blood glucose concentrations are not historically noted in birds undergoing normal reproductive activity, making the significant decrease in glucose concentration observed in this study during the summer months unexpected.[31] Decreased glucose concentrations can occur as a result of decreased feed intake,[10] commonly noted in laying hens in response to higher temperatures during the summer. While decreased feed intake may have led to decreased glucose concentrations in laying hens in the summer, this decrease in blood glucose was not found to be physiologically significant in the current study.

Plasma protein electrophoresis has proven to be a highly reliable method of assessing avian protein concentrations in both healthy and sick birds.[7,9–11,20,27] Although EPH is a non-specific test for disease, it is commonly used as a diagnostic screening tool for both acute and chronic inflammatory conditions. Plasma protein electrophoresis is also recommended to monitor response to treatment, disease progression, and prognosis.[6–8,10–12,20,33] Reproductively active birds typically have increased blood albumin and total globulin concentrations, notably alpha or beta globulins,[6,9] leading to an overall physiologic decrease in the A/G ratio that is not indicative of disease.[8,10–12,21,33] This change begins immediately before ovulation due to an increase in both estrogen and the yolk precursors vitellogenin and lipoproteins.[22,26] These changes may be reflected in the inverse A/G ratio and increased alpha-1 and gamma globulin concentrations observed in this study.[8,9,11] However, the typical increase in the globulin fraction observed in reproductively active birds with chronic inflammation associated with egg laying was not appreciated in the present study. It may indicate breed-specific differences or a lack of seasonal influence on this parameter.

The seasonal effects on hematologic and biochemical reference intervals noted in this study should be considered when assessing the health of backyard laying hens. Seasonal differences should be considered as normal physiological variations when evaluating egg-laying hens for disease. Additional studies should be undertaken to determine whether these same seasonal variations are present in birds in other geographic locations, in hens past peak egg-laying age, or in roosters. Additional studies may also be warranted to evaluate whether breed-specific considerations should be taken into account in addition to the effects of sex, age, and season.

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