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Influence of light intensity, pre-harvest fasting, and storage time on biochemical components in serum and plasma of broilers

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ABSTRACT - We determined the impacts of light intensity, fasting, and storage times on total protein (TP), albumin (ALB), globulin (Glb), uric acid (UA), creatinine (Cre), calcium (Ca), phosphorus (P), and alkaline phosphatase (ALP) in serum and plasma of broilers. At 42 days old, 140 broilers (3,123 ± 654 g) were assigned to two light intensities (5 or 20 lux m^{-2}) and seven fasting times (0, 2, 4, 6, 8, 10, and 12 h). At 45 days old, blood collection was performed in all the broilers. Serum and plasma were stored in a freezer at −20 °C and analyzed on 0, 15, 30, 60, and 120 days. Higher concentrations of Cre and plasma Ca were observed at 20 lux, while the other components were observed at 5 lux. Serum ALB and Ca decreased with each hour of fasting, whereas ALP increased. Uric acid had the lowest concentration at 4 h and 51 min of fasting. Peak serum concentrations of Glb, TP, and Cre were at 6 h, 4 h and 30 min, and 5 h and 15 min of fasting, respectively. Plasma UA, Ca, and P had the lowest concentration at 3 h and 48 min, 5 h and 45 min, and 30 min of fasting, respectively, and a reduction in ALP. Serum UA, TP, and Glb concentrations increased with increasing storage time. Peak serum concentrations of Cre, P, and Ca were at 42, 119, and lowest at 82 days, respectively. Plasma Glb and ALP showed an increase with each storage day, while Cre decreased. Plasma UA and P showed the highest concentrations at 101 and 62 days, respectively. Plasma Ca showed a lower concentration at 50 days. The factors studied significantly influence key blood components in broilers. Higher light intensity increases Cre and Ca concentrations, while fasting reduces serum ALB and Ca, with variable peaks in other components. Storage boosts serum UA, TP, and Glb, with component-specific peaks and declines over time.

Keywords: alkaline phosphatase, blood fraction, blood metabolites, lux, minerals, poultry

1. Introduction

Understanding the metabolic profile in broiler research involves factors such as standardizing the collection, analysis, and interpretation of these data. Storage time can cause variations in analyte concentrations (Wachholz et al., 2023). An ideal condition would be to adopt sample storage protocols

that reflect the best conditions for preserving the analytes (Peng et al., 2010). Literature data with other species such as dogs (Silva et al., 2015), lambs (Oliveira et al., 2011), goats (Divya and Jayavardhanan, 2010), rats (Spinelli et al., 2012), and humans (Cuhadar et al., 2013) showed distinct effects regarding the storage time of the samples.

Blood is taken from fasted animals because the biochemical reference values are established under this condition (Friedrichs et al., 2012), and when broilers are fed, their blood components may change (Wachholz et al., 2023). Therefore, each animal must be evaluated considering the fasting time it has received. However, there are methodological variations for blood collection among studies (fasting or non-fasting) (Córdova-Noboa et al., 2018) or variations of 2 to 12 h in the fasting time used (Behboudi et al., 2016; Zakaria et al., 2017; Swarna et al., 2018).

In addition to the aforementioned factors, the blood fraction (e.g., serum or plasma) to be used in biochemical analyses should be considered because there is variation, and data in the literature is limited for broilers (Burtis et al., 2012; Silva et al., 2015). In this regard, although there is a solid understanding of how photoperiod affects broilers production (Olanrewaju et al., 2016a) and metabolism (Olanrewaju et al., 2010), knowledge of how light intensity can alter blood components should be considered (Olanrewaju et al., 2016b), because this can act intensely at the physiological level (Fidan et al., 2017).

While light intensity can modulate stress and metabolism in broilers, fasting is known to impact nutrient contents and metabolic waste in blood. Additionally, storage time may alter blood component stability, impacting accuracy in studies. Therefore, the hypothesis that lower light intensity would increase the concentrations of blood biochemical components was reversed, as well as that increased fasting and storage time would decrease the concentration of blood biochemical components. The present study determined the impacts of light intensity, fasting, and storage times on total protein, albumin, globulin, uric acid, creatinine, Ca, P concentrations, and alkaline phosphatase (ALP) activity in serum and plasma of broilers.

2. Material and methods

The experiment was conducted at the Centro de Pesquisa em Avicultura (Poultry Research Center; Marechal Cândido Rondon, PR, Brazil; 24° 33' 24" South latitude, 54° 3' 24" West longitude, and average altitude of 420 m). Research on animals was conducted according to the institutional committee on animal use under number 23/20.

2.1. Broilers and handling

A total of 140 one-day-old Cobb 500® male broilers were raised in an experimental facility until they reached an average body weigth of 3,123 ± 654 g (42 days old). The broilers received water and feed *ad libitum*, and the same care in relation to management, lighting, and temperature recommended by the lineage manual (Cobb, 2021). The photoperiod used was 18 h of light and 6 h of dark. The diets provided up to 42 days old for each phase (starter, grower, and finisher) were formulated based on corn and soybean meal and supplemented with industrial amino acids, according to requirements proposed by Rostagno et al. (2017).

At 42 days old (start of experimental period), 70 broilers were redistributed into two environments from which the light intensity was different (5 or 20 lux m^{-2}), measured using an instrutherm LD-209 luxmeter. Incandescent lamps of 15 and 40 W were used, adjusting their height until obtaining the desired intensity at the level of the broilers' eyes. The facility was equipped with eletrical elements, hoods, evaporative plates to assist cooling, and cooling air changes. The choice of light intensities was based on the fact that they are used in commercial poultry farming.

Each environment consisted of seven pens (1.96 m^2), equipped with nipple drinkers and a tube feeder, totaling 14 pens, distributed in a completely randomized design, containing 10 broilers each with a

density of 5.1 broilers m^{-2}. The floor had a 10-cm layer of pine wood shavings. For blood collection, each broiler belonging to the same pen was considered as an experimental unit.

The final diet offered to the broilers (42 days to 45 days old) consisted of 62.65% ground corn (7.88% crude protein [CP]), 26.53% soybean meal (45% CP), 3% wheat bran (15.1% CP), 3.50% degummed soybean oil, 0.99% monobicalcium phosphate, 1.09% calcitic limestone, 0.44% common salt, 0.239% lysine sulphate (51.7%), 0.220% DL-methionine (98%), 0.037% L-threonine (99%), 0.016% L-valine (99%), 0.15% vitamin premix, 0.05% mineral premix, 0.06% choline chloride (60%), 0.02% BHT, and 1% celite®, according to requirements proposed by Rostagno et al. (2017) for finishing broilers.

2.2. Blood collection, preparation, and analysis

At 45 days old, broilers were fasted for a period of 1 h, then fed for 30 min (Wachholz et al., 2023). This procedure was adopted so that all broilers presented the same postprandial feeding condition. After this feeding period, the first blood collection was performed (time 0). The other collections were performed after 2, 4, 6, 8, 10, and 12 h of fasting.

Each pen represented a fasting time for blood collection purposes, totaling seven pens with 10 broilers each. Blood collection was performed in one pen at each fasting time (10 broilers per fasting time randomly selected) and at each light intensity (70 broilers per light intensity). For each fasting interval, a new broiler was used, and each broiler was used only once for blood collection.

Blood was collected via ulnar veinipuncture, with the broilers in lateral recumbency, using specific vacuum collection tubes (Vacutainer®) with a capacity of 10 mL, specific adapters and 25 × 0.8 mm needles (21G 1", Labor Import®). Two samples of 5 mL of blood were collected in each tube. The first sample being collected to obtain the serum in a dry tube (BD Vacutainer®) for serum with clot activator (silica powder) blasted on the tube wall. The second one was collected to obtain plasma in tube (BD Vacutainer®) with 5 mg glycolytic inhibitor sodium fluoride and 4 mg anticoagulant ethylenediaminetetraacetic acid (EDTA)–K_3. After collection, the samples remained in a horizontal position for 15 min and then were centrifuged at 2,500 rpm (1,050 × *g*) for 10 min at room temperature. Fluoride EDTA–K_3 was chosen due to the fact that there are few data in the literature regarding its use in avian species.

After centrifugation and separation of serum and plasma, the samples were identified and divided into five aliquots per blood fraction as technical quintuplicate, which were placed in 2-mL microtubes (Eppendorf®) and an aliquot sent immediately to the laboratory for analysis. The other aliquots (four microtubes) were stored at −20 °C for 15, 30, 60, and 120 days.

To perform the readings, the samples were thawed under refrigeration (4 °C), remaining in a refrigerator for 24 h. Before performing the analysis, the samples were centrifuged in a microcentrifuge to remove possible fibrin formation. Analyzes were performed at 0, 15, 30, 60, and 120 days of storage, by spectrophotometry using an automatic biochemical analyzer (Elitech®, Flexor EL200 model, Puteaux, France) with reagents, calibrators (Elical II multiparametric Calibrator, ref. CALI-0550), and measurement standards for birds (Elitrol I normal multiparametric control, ref. CONT-0060) (Elitech Clinical Systems, ELITech Group, Paris, France). The total protein, albumin, globulin, uric acid, creatinine, Ca, and P concentrations, and ALP activity were determined.

Total protein readings were performed by the end-point biuret method. The principle of the method was: serum proteins form a colored complex in the presence of copper salts in an alkaline medium, being read at 546 nm (Rifai et al., 2018). For albumin, the readings were based on the colorimetric method bromocresol green, in which bromocresol green selectively fixes to albumin at acidic pH (4.20), giving it a blue color, with the reading performed at 620 nm (Doumas et al., 1972; Wu, 2006). Globulin concentrations were obtained from the difference between total protein and albumin. Uric acid readings were performed based on the Trinder method, end-point colorimetric enzymatic at 540 nm (Trinder, 1969). The determination of creatinine concentration was based on the kinetic colorimetric Jaffe method, in which the rate of formation of the colored complex formed between creatinine and

alkaline picrate is measured, readings taken at 500 nm. The effect of interfering substances was reduced using the kinetic procedure (Rifai et al., 2018).

The ALP activity (DEA SL) was measured using the enzymatic kinetic method, based on the recommendations of the German Society of Clinical Chemistry. In the presence of magnesium ions and diethanolamine as a phosphate acceptor, p-nitrophenylphosphate is broken down by ALP into phosphate and p-nitrophenol, forming a yellow compound, with readings taken at 405 nm (Henderson and Donald, 2001). Calcium (Ca arsenazo) values were obtained using the direct-complexometric (arsenazo III) end point colorimetric method. In a neutral medium, Ca complexes with arsenazo III (2,7-Bis(2-arsenophenylazo)-1,8-dihydroxynaphthalene-3,6-disulfonic acid), making the solution blue in color, whose absorbance is between 660 and 700 nm, is proportional to the total Ca concentration in the sample (Wu, 2006). The determination of P concentration was based on the endpoint ultraviolet phosphomolybdate method. Ammonium molybdate and sulfuric acid react in the presence of P contained in the sample forming an ammonium phosphomolybdate complex measured at 340 nm.

2.3. Statistical procedures

Data were analyzed considering blood fraction (serum or plasma), fasting (0, 2, 4, 6, 8, 10, and 12 h), storage times of the sample (0, 15, 30, 60, and 90 days), light intensities (5 or 20 lux), and their interactions of interest as fixed effects. Residual error was considered as a random factor. Data were subjected to normality analysis using the Shapiro-Wilk test. Results were analyzed as a three-way analysis of variance, considering the isolate effects and the interation among the studied parameters. F-test was performed for blood fraction (serum or plasma) and light intensities (5 or 20 lux per m^2), and regression analysis for fasting and storage times. The following overall model was used:

$$Y_{ijklm} = \mu + B_i + F_j + S_k + L_l + BF_{ij} + BS_{ik} + BL_{il} + FS_{jk} + FL_{jl} + SL_{kl} + \varepsilon_{ijklm}, \quad (1)$$

in which Y_{ijklm} = average observation of the dependent variable in each plot measured in the *i*-th blood fraction, in the *j*-th fasting time, in the *k*-th storage time, in the *l*-th ligth intensity, and in the *m*-th replicate; μ = overall average; B_i = fixed effect of blood fraction (*i* = 1 and 2); F_j = fixed effect of fasting times (*j* = 1, 2, 3, 4, 5, 6, and 7); S_k = fixed effect of storage times (*k* = 1, 2, 3, 4, and 5); L_l = fixed effect of light intensities (*l* = 1 and 2); BF_{ij} = interaction blood fraction × fasting time; BS_{ik} = interaction blood fraction × storage time; BL_{il} = interaction blood fraction × ligth intensity; FS_{jk} = interaction fasting time × storage time; FL_{jl} = interaction fasting time × ligth intensity; SL_{kl} = interaction storage time × ligth intensity; and ε_{ijklm} = random error of the plot associated with each Y_{ijklm} observation.

Additionally, the Scott-Kontt test was applied for fasting and storage times to separate the treatments into homogeneous groups, avoiding the superposition of means. Only the interactions of interest for this study were presented: blood fraction (serum or plasma) × fasting time, blood fraction × storage time, and blood fraction × light intensity. Triple and quadruple interactions were not analyzed. All statistical procedures were performed using the PROC GLM in SAS (Statistical Analysis System, University Edition) when P<0.05.

3. Results

3.1. Light intensities

A higher (P<0.05) concentration of total protein, albumin, and globulin was observed in broilers when using 5 lux (Table 1). A higher (P<0.05) serum uric acid concentration was obtained with 5 lux (Table 2). Plasma creatinine concentration was higher (P<0.05) with 20 lux. Plasma uric acid and serum creatinine concentrations were not affected by light intensity.

Light intensity affected (P<0.05) plasma ALP activity, with a higher activity with 5 lux (Table 3). Higher (P<0.05) plasma Ca concentration was obtained with 20 lux. Serum ALP activity and Ca, and serum and plasma P concentrations were not affected by light intensity.

Table 1 - Averages of total protein, albumin, and globulin concentrations in serum and plasma of broiler chickens at 45 days old subjected to different fasting and sample storage times and light intensity

	Total protein (g L^{-1})		Albumin (g L^{-1})		Globulin (g L^{-1})	
	Serum	Plasma	Serum	Plasma	Serum	Plasma
Fasting (h)						
0	34.90±3.47	34.54±2.75	15.44±1.42A	15.59±1.26	19.55±2.48Ba	18.66±1.85b
2	36.84±2.97	36.23±3.17	15.79±1.29Ab	16.20±1.23a	20.98±2.12Aa	19.94±2.25b
4	37.14±3.39	36.31±3.48	15.52±1.22Ab	16.31±1.29a	21.44±2.54Aa	20.20±2.66b
6	35.91±2.81	35.08±3.02	14.83±0.88Bb	15.84±1.41a	20.99±2.06Aa	19.18±1.90b
8	35.50±3.05a	34.30±2.94b	15.02±1.41B	15.39±1.22	20.91±2.56Aa	19.33±2.21b
10	35.21±3.05	35.23±3.33	14.85±1.21Bb	15.81±1.41a	20.68±2.38Ba	19.91±2.14b
12	34.63±2.75b	36.57±2.87a	14.47±1.08Bb	15.71±1.27a	20.32±2.21Bb	21.22±2.47a
Storage (days)						
0	35.13±2.97B	34.77±2.88	15.12±1.15Ab	15.59±1.18Ba	20.05±2.51Ba	19.17±2.17b
15	35.35±3.44B	35.24±3.08	15.01±1.18Bb	15.71±1.15Ba	20.31±2.50Ba	19.42±2.22b
30	36.59±3.11A	35.87±3.32	15.37±1.22Ab	16.19±1.30Aa	21.08±2.19Aa	19.84±2.43b
60	35.25±2.93B	35.47±3.26	15.14±1.18Ab	15.92±1.19Aa	20.11±2.23B	19.64±2.34
120	36.27±3.22A	35.74±3.37	15.04±1.77Bb	15.78±1.78Ba	21.94±1.98Aa	20.82±2.25b
Light intensity (lux m^{-2})						
5	37.03±2.92Aa	36.41±2.99Ab	15.64±1.15Ab	16.27±1.23Aa	21.58±2.28Aa	20.31±2.23Ab
20	34.44±2.90B	34.44±3.10B	14.68±1.25Bb	15.42±1.28Ba	19.83±2.18Ba	19.22±2.33Bb
SEM	2.91	3.04	1.21	1.26	2.23	2.28
P-value						
Fasting	0.191	0.294	<0.000	0.056	<0.000	0.413
Storage	0.000	0.569	0.028	0.002	0.033	0.864
Light intensity	0.028	0.012	<0.000	<0.000	0.010	0.020
Blood fraction × fasting	0.125		0.561		0.038	
Blood fraction × storage	0.339		0.263		0.364	
Blood fraction × light intensity	0.974		0.731		0.718	
Fasting × storage	0.080		<0.000		0.313	
Fasting × light intensity	0.287		0.007		0.400	
Storage × light intensity	0.029		0.919		0.114	

SEM - Pooled standard error of the mean.
a,b - Lowercase letters in the same row differ from each other by F test (serum vs plasma).
A,B - Capital letters in the same column differ by Scott-Knott test.
a,b - Lowercase letters in the column differ from each other by the F test for light intensity when P<0.05.

3.2. Fasting times

There was no effect of fasting times in serum and plasma on total protein concentrations (Table 1). The serum albumin concentration showed stability in its values up to 4 h of fasting, but the concentration was similar between 6 and 12 h of fasting. Serum globulin concentrations were higher (P<0.05) between 2 and 8 h of fasting and lower concentration among 0, 10, and 12 h of fasting. Plasma albumin and globulin concentrations were not affected by fasting times. However, when analyzed individually at each fasting time, there were higher (P<0.05) serum globulin and plasma albumin concentrations.

Fasting times affected serum and plasma uric acid and creatinine concentrations (Table 2). The highest concentrations of uric acid were obtained at 0, 8, 10, and 12 h of fasting, while the lowest concentrations were between 2 and 6 h of fasting in serum and plasma (P<0.05). Creatinine showed higher serum and plasma concentrations after 6 h of fasting, with the highest concentrations obtained in serum than in plasma among the fasting times (P<0.05).

Table 2 - Averages of uric acid and creatinine concentrations in serum and plasma of broiler chickens at 45 days old subjected to different fasting and sample storage times and light intensity

	Uric acid (mg dL^{-1})		Creatinine (mg dL^{-1})	
	Serum	Plasma	Serum	Plasma
Fasting (h)				
0	2.77±0.69Aa	2.43±0.86Ab	0.17±0.06Ba	0.15±0.07Ab
2	1.99±0.77B	2.08±0.74B	0.17±0.05Ba	0.14±0.06Ab
4	1.81±0.64B	1.83±0.70B	0.16±0.03Ba	0.11±0.03Bb
6	2.28±0.82B	2.11±0.81B	0.19±0.04Aa	0.16±0.04Ab
8	2.76±1.07A	2.59±1.01A	0.16±0.06Ba	0.14±0.06Ab
10	2.93±1.16A	3.13±1.09A	0.16±0.07Ba	0.13±0.06Bb
12	2.53±0.86Ab	3.02±1.03Aa	0.14±0.06Ca	0.10±0.05Bb
Storage (days)				
0	2.08±0.91C	2.05±0.91C	0.16±0.04Ba	0.13±0.03Bb
15	2.26±0.96B	2.32±0.93B	0.20±0.05Aa	0.18±0.06Ab
30	2.38±0.86B	2.38±0.95B	0.17±0.04Ba	0.12±0.04Bb
60	2.52±0.83B	2.52±0.97B	0.18±0.04Aa	0.15±0.05Bb
120	2.97±0.98A	2.95±1.03A	0.12±0.08Ca	0.09±0.06Cb
Light intensity (lux m^{-2})				
5	2.54±0.98A	2.39±0.97	0.16±0.06Ba	0.13±0.05Bb
20	2.34±0.92B	2.47±1.02	0.17±0.06Aa	0.14±0.06Ab
SEM	0.95	0.99	0.05	0.06
P-value				
Fasting	<0.000	<0.000	<0.000	<0.000
Storage	<0.000	<0.000	<0.000	<0.000
Light intensity	0.013	0.282	0.173	0.029
Blood fraction × fasting	<0.000		<0.000	
Blood fraction × storage	0.981		0.001	
Blood fraction × light intensity	<0.000		0.632	
Fasting × storage	<0.000		<0.000	
Fasting × light intensity	<0.000		<0.000	
Storage × light intensity	0.047		0.000	

SEM - Pooled standard error of the mean.
a,b - Lowercase letters in the same row differ from each other by F test (serum vs plasma).
A,B - Capital letters in the same column differ by Scott-Knott test.
a,b - Lowercase letters in the column differ from each other by the F test for light intensity when P<0.05.

Fasting times affected serum and plasma ALP activity and Ca concentrations (Table 3). The highest ALP activity was obtained in serum after 8 and 12 h of fasting (P<0.05). In plasma, the highest activity was obtained after 2 h of fasting, after which there was a reduction in its activity (P<0.05). The highest serum Ca concentrations were obtained after 2 and 4 h of fasting and, after this time, a reduction in its concentration was observed (P<0.05). Plasma Ca concentrations showed a different dynamic. Serum P concentrations were higher (P<0.05) at 4 and 6 h of fasting and, after this time, the concentrations were stable. Plasma P concentrations were not affected by fasting times.

There was a linear effect (P<0.05) of fasting times in serum albumin and ALP (Table 4). Quadratic and cubic effects were observed (P<0.05) for uric acid, total protein, globulin, and creatinine. In addition, linear and cubic effects (P<0.05) were obtained for Ca concentration. Albumin decreased its concentration by 0.097 g L^{-1} every hour of fasting. Conversely, ALP increased its activity by 38.55 IU L^{-1}. By the quadratic model, serum uric acid concentrations showed the lowest concentration of 2.20 mg dL^{-1} at 4 h and 51 min of fasting. For the cubic effect, there was a lower concentration of 1.85 mg dL^{-1} at 3 h and 13 min, a peak of 2.83 mg dL^{-1} at 9 h and 48 min, and an average concentration of 2.33 mg dL^{-1} at 6 h and 30 min of fasting.

Table 3 - Averages of alkaline phosphatase activity and calcium and phosphorus concentrations in serum and plasma of broiler chickens at 45 days old subjected to different fasting and sample storage times and light intensity

	Alkaline phosphatase (U L^{-1})		Calcium (mg dL^{-1})		Phosphorus (mg dL^{-1})	
	Serum	Plasma	Serum	Plasma	Serum	Plasma
Fasting (h)						
0	3497±969B	3394±1064B	8.59±1.64Ba	1.47±0.61Ab	5.66±0.64a	5.10±0.51b
2	3705±1052B	4005±1072A	8.78±1.20Aa	1.01±0.42Bb	5.56±0.58a	4.89±0.47b
4	3488±1046B	3265±1126B	8.76±1.28Aa	1.03±0.49Bb	5.82±0.60a	5.15±0.55b
6	3671±793Ba	3103±922Cb	8.37±1.39Ba	1.01±0.37Bb	6.04±0.57a	5.37±0.45b
8	3969±1025Aa	3435±1046Bb	7.59±1.67Ca	0.92±0.54Bb	5.48±0.58a	5.17±051b
10	3687±992Ba	3036±1226Cb	7.73±1.26Ca	1.74±1.04Ab	5.61±0.59b	5.92±0.79a
12	3994±1060Aa	3169±1534Cb	7.79±1.14Ca	1.84±1.02Ab	5.73±0.57	5.84±0.96
Storage (days)						
0	3595±942Ba	3262±1222b	9.64±1.00Aa	1.21±0.66Bb	6.15±0.58Aa	5.14±0.72b
15	3929±969Aa	3388±1103b	8.56±1.63Ba	1.20±0.67Bb	5.79±0.48Ba	5.24±0.70b
30	3590±974Ba	3260±1081b	7.96±1.03Ba	1.19±0.78Bb	5.61±0.56Ba	5.35±0.66b
60	3564±1120B	3340±1268	7.49±1.00Ba	1.17±0.75Bb	5.68±0.60Ba	5.49±0.65b
120	3909±968Aa	3469±1233b	7.49±1.28Ba	1.67±0.88Ab	5.29±0.52C	5.37±0.71
Light intensity (lux m^{-2})						
5	3749±1052a	3508±1056Ab	8.28±1.54a	1.07±0.57Bb	5.69±0.62a	5.18±0.56Bb
20	3684±959a	3179±1278Bb	8.18±1.37a	1.50±0.89Ab	5.71±0.61a	5.46±0.80Ab
SEM	1007	1172	1.46	0.74	0.61	0.69
P-value						
Fasting	0.003	<0.000	<0.000	<0.000	0.061	1.000
Storage	0.012	0.535	0.000	0.699	0.036	1.000
Light intensity	0.443	0.000	0.449	0.044	0.133	1.000
Blood fraction × fasting	<0.000		<0.000		0.276	
Blood fraction × storage	0.422		0.030		0.286	
Blood fraction × light intensity	0.030		0.361		0.263	
Fasting × storage	0.001		0.874		0.459	
Fasting × light intensity	<0.000		0.026		0.422	
Storage × light intensity	0.368		0.350		0.405	

SEM - Pooled standard error of the mean.
a,b - Lowercase letters in the same row differ from each other by F test (serum vs plasma).
A,B - Capital letters in the same column differ by Scott-Knott test.
a,b - Lowercase letters in the column differ from each other by the F test for light intensity when P<0.05.

Table 4 - Regression equations for prediction of uric acid, total protein, albumin, globulin, creatinine, and calcium concentrations and alkaline phosphatase (ALP) activity in serum samples of broilers at 45 days old as a function of fasting time

Variable	Regression equation	CP	R^2	P-value
Uric acid	$0.01084\times UA^2 - 0.10497\times UA + 2.45114$	4.84	0.30	0.000
Uric acid	$-0.00702\times UA^3 + 0.13696\times UA^2 - 0.66216\times UA + 2.77668$	6.50	0.99	<0.000
Total protein	$-0.03199\times TP^2 + 0.28940\times TP + 35.61535$	4.52	0.63	0.001
Total protein	$0.01015\times TP^3 - 0.21448\times TP^2 + 1.0956\times TP + 35.14432$	7.05	0.91	0.001
Albumin	$- 0.09738\times ALB + 15.68917$		0.77	<0.000
Globulin	$-0.02799\times GLB^2 + 0.33905\times GLB + 20.00464$	6.06	0.75	0.000
Globulin	$0.00869\times GLB^3 - 0.18412\times GLB^2 + 1.02877\times GLB + 19.60167$	7.06	0.94	0.000
Creatinine	$-0.00048792\times CRE^2 + 0.00513\times CRE + 0.16783$	5.26	0.57	0.002
Creatinine	$-0.00014543\times CRE^3 + 0.00213\times CRE^2 - 0.00642\times CRE + 0.17458$	4.89	0.61	0.003
Calcium	$-0.08725\times Ca + 8.75509$		0.72	<0.000
Calcium	$0.00531\times Ca^3 - 0.09849\times Ca^2 + 0.3707\times Ca + 8.44906$	6.18	0.94	<0.000
ALP	$38.54876\times ALP + 3460.62057$		0.55	0.000

CP - Critical point obtained from the derivation of the quadratic and cubic equation; R^2 - coefficient of determination.

For total protein, the highest serum concentration (P<0.05; 36.18 g L^{-1}) was observed at 4 h and 30 min of fasting, estimated by the quadratic model (Table 4). As for the cubic model (P<0.05), it estimated a peak of 36.79 g L^{-1} at 3 h and 22 min, a lower concentration of 34.75 g L^{-1} at 10 h and 43 min, and a midpoint of 35.76 g L^{-1} obtained at 7 h of fasting. The quadratic model for globulin predicted the highest concentration of 21.03 g L^{-1} at 6 h and 4 min of fasting (P<0.05). The cubic model predicted a higher concentration of 21.33 g L^{-1} at 3 h and 51 min, a lower concentration of 20.19 g L^{-1} at 10 h and 18 min, and an average concentration of 20.75 g L^{-1} at 7 h of fasting (P<0.05). For creatinine concentration, an opposite behavior to globulin was observed (P<0.05), with the lowest concentration of 0.17 mg dL^{-1} at 1 h and 52 min of fasting, a higher concentration of 0.19 mg dL^{-1} at 7 h and 57 min, and an average concentration of 0.18 mg dL^{-1} achieved at 4 h and 54 min of fasting.

The linear model for the Ca variable estimated a drop (P<0.05) in its serum concentration of 0.09 mg dL^{-1} every hour of fasting (Table 4). The cubic regression model estimated the highest serum concentration of 8.85 mg dL^{-1} at 2 h and 19 min, a lower concentration of 7.62 mg dL^{-1} at 10 h, and an average concentration of 8.23 mg dL^{-1} at 6 h and 11 min of fasting (P<0.05). Data for the P variable did not fit any of the proposed regression models.

There were quadratic and cubic effects (P<0.05) of fasting times on plasma uric acid concentration (Table 5). We observed a cubic effect for total protein, albumin, and creatinine concentrations (P<0.05). There were linear and cubic effects for globulin concentration and ALP activity (P<0.05). There was a quadratic effect on Ca and P (P<0.05). By the quadratic model, uric acid had the lowest concentration of 2.08 mg dL^{-1} at 3 h and 48 min of fasting. The cubic regression model predicted the lowest concentration of 1.88 mg dL^{-1} at 3 h and 8 min, a peak of 2.92 mg dL^{-1} at 11 h and 11 min, and average of 2.40 mg dL^{-1} at 7 h and 9 min of fasting.

According to the cubic regression model (P<0.05), plasma total protein showed the highest concentration of 36.22 g L^{-1} at 3 h of fasting, a lower concentration of 34.48 g L^{-1} at 8 h and 45 min, and average of 35.35 g L^{-1} achieved at 5 h and 52 min of fasting (Table 5). Plasma albumin concentration showed a similar behavior, with the highest concentrations of 16.28 g L^{-1} at 2 h and 51 min, a lower concentration of 15.45 g L^{-1} at 9 h and 17 min, and average of 15.87 g L^{-1} at 6 h and 4 min of fasting. Creatinine showed an opposite behavior in relation to total protein and albumin, with a peak concentration of 0.161 mg dL^{-1} at 8 h and 39 min, a lower concentration of 0.128 mg dL^{-1} at 2 h and 37 min, and an average concentration of 0.144 mg dL^{-1} at 5 h and 38 min of fasting (P<0.05).

Plasma ALP activity decreased linearly (P<0.05) by 24.92 IU L^{-1} for each hour of fasting (Table 5). However, the model explains only 33% of the variability that occurred with the data for this variable. The cubic model indicated a higher activity of 3702 IU L^{-1} with 2 h of fasting, a lower concentration of

Table 5 - Regression equations for prediction of uric acid, total protein, albumin, globulin, creatinine, calcium, and phosphorus concentrations and alkaline phosphatase (ALP) activity in plasma samples of broilers at 45 days old as a function of fasting time

Variable	Regression equation	CP	R^2	P-value
Uric acid	$0.01502\times UA^2 - 0.11445\times UA + 2.30164$	3.81	0.78	<0.000
Uric acid	$-0.00401\times UA^3 + 0.08603\times UA^2 - 0.42081\times UA + 2.47348$	7.15	0.96	<0.000
Total protein	$0.01809\times TP^3 - 0.31789\times TP^2 + 1.41041\times TP + 34.36136$	5.86	0.92	<0.000
Albumin	$0.00622\times ALB^3 - 0.11324\times ALB^2 + 0.49448\times ALB + 15.64616$	6.07	0.71	<0.000
Globulin	$0.07125\times GLB + 19.12838$		0.40	0.002
Globulin	$0.01187\times GLB^3 - 0.20449\times GLB^2 + 0.91498\times GLB + 18.71675$	5.74	0.93	<0.000
Creatinine	$-0.00029765\times CRE^3 + 0.00503\times CRE^2 - 0.02012\times CRE + 0.15123$	5.63	0.64	<0.000
Calcium	$0.01576\times Ca^2 - 0.18108\times Ca + 1.43202$	5.75	0.83	<0.000
Phosphorus	$0.00502\times P^2 + 0.00487\times P + 5.03092$	0.49	0.79	0.006
ALP	$-24.92117\times ALP + 3587.80220$		0.33	0.038
ALP	$3.6163\times ALP^3 - 58.09088\times ALP^2 + 183.42613\times ALP + 3538.50442$	5.35	0.42	0.000

CP - Critical point obtained from the derivation of the quadratic and cubic equation; R^2 - coefficient of determination.

3119 IU L^{-1} at 8 h and 47 min, and an average activity of 3411 IU L^{-1} at 5 h and 38 min of fasting (P<0.05). There was a quadratic effect (P<0.05) in plasma Ca and P concentrations. Plasma Ca concentration decreased with the lowest concentration of 0.91 mg dL^{-1} at 5 h and 45 min of fasting. After this time, its concentration increased. Phosphorus had the lowest concentration of 5.03 mg dL^{-1} at 30 min of fasting.

3.3. Storage times

There was an effect (P<0.05) of storage time on serum total protein and globulin. The highest concentrations were obtained at 30 and 120 days of storage (Table 1). For albumin, the highest concentrations were at 30 and 60 days of storage in serum and plasma (P<0.05).

Uric acid concentration showed (P<0.05) an increase as the storage time increased. Creatinine concentration showed (P<0.05) a reduction as the storage time increased (Table 2).

There was an effect (P<0.05) of storage times on serum ALP activity, with higher activity at 15 and 120 days (Table 3). The highest serum Ca concentration was obtained at time 0 of storage and, after this period, the concentration tended to decrease. Serum and plasma P concentrations were not affected by storage times.

Storage times showed (P<0.05) a linear effect on serum uric acid concentrations (Table 6). We observed linear and cubic effects on total protein and globulin concentrations (P<0.05). There was a quadratic effect on creatinine, Ca, and P concentrations (P<0.05). The linear model indicated an increase in serum uric acid, total protein, and globulin concentrations as the storage time of the samples increased. The cubic model for total protein predicted the highest concentration of 36.06 g L^{-1} at 30.5 days, a lower concentration of 34.77 g L^{-1} at 85 days, and an average concentration of 35.41 g L^{-1} at 58 days of storage. For globulin, a peak concentration of 20.83 g L^{-1} was observed at 28 days, a lower of 19.79 g L^{-1} at 84 days, and an average concentration of 20.27 g L^{-1} at 56 days of storage.

The quadratic effect predicted (P<0.05) the highest serum creatinine concentration of 0.184 mg dL^{-1} at 42 days of storage, after which the serum creatinine concentration decreased (Table 6). Calcium and P concentrations had the opposite behavior: Ca had the lowest concentration of 7.17 mg dL^{-1} at 82 days of storage, and P reached the lowest concentration of 5.40 mg dL^{-1} at 119 days and, after this period, its concentration increased. The data for serum albumin and globulin for the storage period did not fit the linear, quadratic, or cubic regression models.

There was an effect (P<0.05) of storage time on plasma, with a linear effect for uric acid, globulin, creatinine concentrations, and ALP activity (Table 7). We found a quadratic effect on Ca and P concentrations (P<0.05) and observed linear and cubic effects on total protein concentrations (P<0.05). By the linear model, the uric acid and globulin concentrations and ALP activity showed an increase in their values of 0.0045 mg dL^{-1}, 0.0097 g L^{-1}, and 2.76 IU L^{-1}, respectively, as one unit on

Table 6 - Regression equations for prediction of uric acid, total protein, globulin, creatinine, calcium, and phosphorus concentrations in serum samples of broilers at 45 days old as a function of storage time

Variable	Regression equation	CP	R^2	P-value
Uric acid	0.00534×UA + 2.15506		0.99	<0.000
Total protein	0.01101×TP + 35.22616		0.22	0.001
Total protein	$0.00001367\times TP^3 - 0.00237\times TP^2 + 0.1031\times TP + 34.734$	57.81	0.68	0.001
Globulin	0.01161×GLB + 20.08578		0.61	<0.000
Globulin	$0.00001189\times GLB^3 - 0.00201\times GLB^2 + 0.08542\times GLB + 19.74873$	56.35	0.91	0.000
Creatinine	$-0.00000665\times CRE^2 + 0.00055736\times CRE + 0.17233$	41.91	0.79	<0.000
Calcium	$0.00033445\times Ca^2 - 0.05510\times Ca + 9.44293$	82.37	0.97	<0.000
Phosphorus	$0.00004333\times P^2 - 0.01034\times P + 6.01190$	119.32	0.84	0.011

CP - Critical point obtained from the derivation of the quadratic and cubic equation; R^2 - coefficient of determination.

sample storage days. On the other hand, creatinine concentrations decreased by 0.00018 mg dL^{-1}. Calcium showed the lowest concentration of 1.02 mg dL^{-1} at 50 days of storage. Phosphorus showed a peak concentration of 5.47 mg dL^{-1} at 62 days of storage. Total protein concentrations increased by 0.0144 g L^{-1} as storage time increased. As for the cubic model, it estimated a peak of 35.56 g L^{-1} at 38 days, a lower concentration of 35.32 g L^{-1} at 78 days, and an average concentration of 35.44 g L^{-1} at 58 days of storage time.

Table 7 - Regression equations for prediction of uric acid, total protein, globulin, creatinine, calcium, and phosphorus concentrations and alkaline phosphatase (ALP) activity in plasma of broilers at 45 days of age as a function of storage time

Variable	Regression equation	CP	R^2	P-value
Uric acid	$-0.00005389 \times UA^2 + 0.01095 \times UA + 2.08153$	101.60	0.97	0.044
Total protein	$0.01437 \times TP + 34.81924$		0.40	<0.000
Total protein	$0.00000723 \times TP^3 - 0.00126 \times TP^2 + 0.06421 \times TP + 34.54292$	55.98	0.91	0.057
Globulin	$0.00972 \times GLB + 19.12720$		0.89	<0.000
Creatinine	$-0.00017711 \times CRE + 0.14927$		0.40	0.001
Calcium	$0.00008025 \times Ca^2 - 0.00799 \times Ca + 1.21783$	49.67	0.98	<0.000
Phosphorus	$-0.00009113 \times P^2 + 0.01140 \times P + 5.11032$	62.64	0.99	<0.000
ALP	$2.75686 \times ALP + 3345.40744$		0.60	0.020

CP - Critical point obtained from the derivation of the quadratic and cubic equation; R^2 - coefficient of determination.

4. Discussion

4.1. Light intensities

Light management and intensity are important factors in broiler production. Commonly used lighting programs start at 20 lux, reducing to 5 lux or less. Olanrewaju et al. (2010) did not observe the effect of different light intensities (0.5, 3.0, and 20 lux) on hematological components and total protein concentration, with an increase in the concentrations of triglycerides and high-density lipoprotein analytes of the glycolytic pathway in broilers from 21 to 56 days old.

In contrast, in the present study, there was an effect of light intensity on the concentrations of the evaluated metabolites. Higher concentrations of analytes were observed for the intensity of 5 lux, which may be due to the lower stimulus and physical activity of the broilers compared with 20 lux. Broilers exposed to 20 lux would spend more energy for physical activities and not for growth (Olanrewaju et al., 2008; Olanrewaju et al., 2010). This can be observed in the creatinine concentrations of this study, which is an indicator of muscular activity, and its values were higher in broilers exposed to 20 lux.

Total protein, albumin, and globulin concentrations were higher at 5 lux, which may explain the better growth of the broilers under this light intensity, as there is an increase in circulating proteins, and as albumin is a transporter protein, there is higher supply of nutrients for muscle growth. This justifies the use of an intensity of 5 lux in industrial poultry.

Olanrewaju et al. (2012) evaluated different light intensities (0.2, 2.5, 5, 10, and 25 lux) in broilers aged 28 to 56 days old on hematological components, and observed that only for the intensity of 0.2 lux there was an increase in pH, Na^+, K^+, Cl^- and a reduction in CO_2 pressure, hemoglobin, and hematocrit. Such results are related to less broiler movement and higher respiratory rate. It is suggested that the pH of the blood may act in the regulation of breathing and that it may also influence the chemical control of breathing, as the respiratory system participates in the blood acid-base balance. Olanrewaju et al. (2013) did not observe effect of light intensity on serum concentrations of total protein and Ca, with photoperiod being more important, but this was not a factor evaluated in this study.

It is believed that higher concentrations of total protein are related to the higher concentration of the hormone triiodothyronine, as found by Olanrewaju et al. (2013), when they evaluated long and regular or intermittent photoperiods. In addition, these results may be associated with higher protein deposition or higher feed intake during the light period. In the present study, the photoperiod used (18L:6D) was the same for both intensities, and these had an effect on the evaluated metabolites. As the broilers were exposed to different intensities for only three days before blood collection, it is questionable whether this time was enough to cause metabolic changes in the broilers.

Olanrewaju et al. (2014) when evaluating light intensities similar to those in this study (0.2, 2.5, 5, 10, and 25 lux), found differences in Ca concentrations in two broiler strains (Ross 308 and Ross 708). Creatinine and plasma Ca in this study had a higher concentration when using 20 lux, being 6.9 and 40.18% compared to 5 lux, respectively. Serum concentrations of total protein, albumin, globulin, and uric acid were 7.5, 6.5, 8.8, and 8.5% lower at 20 lux than at 5 lux. Plasma total protein, albumin, globulin concentrations, and ALP activity were lower at 20 lux, being 5.7, 5.5, 5.7, and 10.3%, respectively.

The reduction or increase in the concentration of biochemical components are dependent on several factors (e.g., physiological and metabolic). Variations in the concentrations of some analytes evaluated in this study may be related to analytical variability, because even with a statistical difference, it is not possible to state that this difference is clinically significant and that it has compromised the results in biological and physiological terms.

4.2. Fasting times

Blood metabolites immediately reflect the nutritional and physiological status of broilers, but can also be affected by the storage time of the serum or plasma samples and the environmental conditions of the barn (e.g., light intensity). There are some differences among authors regarding the type and age at which broilers are subjected to fasting or feed restriction, or even the intensity and duration of this restriction (Rahimi et al., 2015).

Here, serum and plasma concentrations of total protein, as well as plasma concentrations of albumin and globulin were not influenced by fasting times of up to 12 h. This can be attributed to the fact that the concentration of total protein reflects the balance of circulating proteins; therefore, prolonged fasting time did not influence the mobilization of proteins from muscle tissues and their synthesis or degradation by the liver.

Differences in the values of biochemical components in the blood found in this study compared with those reported in the literature are probably due to the physiological changes considered normal among the different growth stages in broilers (Wachholz et al., 2023). Plasma and serum total protein concentrations, plasma albumin, and globulins concentrations did not have large fluctuations, which may be a reflection of the *ad libitum* intake of the broilers prior to the fasting times evaluated (Silva et al., 2007). Total protein and albumin can undergo variations in broilers, and changes in their concentrations are dependent on intrinsic factors of the animal and the physiological function. Possible variations in total protein and albumin concentrations in the blood may be a direct effect of the high demand for amino acids required by the intense growth of broilers (Piotrowska et al., 2011).

Demir et al. (2004) reported that blood albumin may increase by 25% in its concentration, or even suffer a decrease of up to 50% depending on age and time of feed restriction, prior to blood collection and analysis. In the present study, the data for albumin in relation to fasting time did not have a behavior that corroborates those found by the authors.

The period up to 45 days old is characterized by an ample supply of amino acids for growth, and the liver tends to maintain serum protein synthesis for muscle protein synthesis. Variations in total protein and albumin concentrations may be associated with alterations in nutrition, as the withdrawal feed has a lower protein content, or even changes in feed intake or intense protein deposition may influence the concentration of proteins in the blood (Tóthová et al., 2019).

The total protein concentrations in the present study are consistent with the literature, and it is not possible to confirm that the nutritional composition of the final diet influenced the serum and plasma total protein and albumin concentrations. The biological explanation for the non-reduction of total protein and albumin is not entirely clear, but it may be related to the time course of postprandial metabolism for these analytes (Mora et al., 2008). Also, the decrease in the concentration of total protein would probably be followed by a reduction in the blood concentrations of glucogenic amino acids, such as lysine, alanine, serine, and glutamine (Goodman et al., 1980; Le Ninan et al., 1988), although this was not evaluated in the present study.

Fasting can cause oxidative stress and the formation of reactive oxygen species (ROS) (Khan et al., 2012; Majid et al., 2015; Chand et al., 2018). This would result in denaturation of biomolecules such as nucleic acids, proteins, and enzymes, due to reduced protein intake, either by fasting or diet reduction, which could cause deficiency of essential amino acids and lead to changes in total protein (Laudadio et al., 2012) and uric acid concentrations in the blood (Simoyi et al., 2002).

Changes in the concentrations of certain blood metabolites may be indicative of the type of energy reserves used during fasting. The concentration of uric acid in the blood is indicative of protein catabolism, as broilers excrete uric acid as a final product of nitrogen metabolism (Scanes, 2015). In addition, uric acid can also act as an effective antioxidant, helping to neutralize ROS and protecting cells from oxidative stress (Machín et al., 2004).

When comparing the results of uric acid and total protein, it is not possible to state that protein was mobilized and used as an energy source during the 12-h fast. For a more in-depth analysis, it would be necessary to assess the serum and plasma β-hydroxybutyrate (βHB) concentrations, which is one of the ketone bodies produced after oxidation of fatty acids resulting from the hydrolysis of triglycerides, to verify whether there was mobilization of proteins or fatty acids as the main source of energy during fasting (Cherel et al., 1988; Boismenu et al., 1992). In this sense, the analysis of serum and plasma triglycerides concentrations is also necessary. However, the βHB concentrations in poultry are subject to higher variation than acetoacetate, and both are related to catabolic reactions.

Uric acid values in avian species are considered normal from 2 to 15 mg dL^{-1} (Benez, 2004). Blood concentrations higher than 15 mg dL^{-1} suggest changes in renal function (Campbell, 2004) or are even associated with the diet and hydration status of broilers. However, it is believed that these factors did not compromise the uric acid results of this study, which can be considered normal, with no evidence that renal activity has been compromised by the fasting time of up to 12 h, since the average concentration of uric acid was 2.45 mg dL^{-1}.

According to Rezende et al. (2019), higher values of uric acid may reflect more intense protein metabolism in broilers. This may be due to the genetic improvement of broilers that are currently selected for intense protein deposition. The results of the present study for uric acid and total protein in serum and plasma of broilers at 45 days old were 60.50 and 39.38% lower than those reported by Rahimi et al. (2015), who found an average concentration of 3.90 mg dL^{-1} and 49.80 g L^{-1}, respectively.

Possible changes in uric acid concentrations may be due to its antioxidant function. The exposure of the sample to room temperature until its centrifugation or during the laboratory analysis can result in degradation (Elliott and Peakman, 2008). However, as the samples were collected and processed, it is believed that this time between sample collection and processing did not influence the results of the evaluated analytes.

Comparing other species due to limited information on broilers, for example, in evaluations in the human clinical area, serum Ca concentrations were not influenced by the postprandial period when blood samples were collected at 1, 2, and 3 h after feed intake (Thode et al., 1985). In the present study, P had the lowest concentration at 30 min of fasting, but metabolically 30 min of fasting is believed to have no impact on plasma P concentrations.

4.3. Storage times

Peng et al. (2010), evaluating samples of serum and plasma of rats, observed that storage time can increase the variations in the concentrations of the analytes, and that these variations can affect research conclusions and decision-making. The interval between blood collection and sample analysis should be a methodological concern, as it may influence the accuracy of data in animal studies. Each blood analyte may respond differently to storage time and conditions, as well as factors related to the animal species. Cray et al. (2009) observed stability for total protein, albumin, ALP, Ca, and P in rat serum samples at −20 °C for 90 days. In blood samples from broilers, Nunes et al. (2018) confirmed this stability for the blood metabolites mentioned above.

In the present study, plasma concentrations of total protein and globulin, ALP activity, and serum and plasma P concentrations showed stability during storage times. Thoresen et al. (1995) also stated that storage time did not interfere with the concentrations of total protein, albumin, Ca, and P in serum samples of dogs, showing stability for up to 240 days when kept between −20 and −70 °C; and even with oscillations among them, they remained within the reference concentrations.

In the human clinic, Hostmark et al. (2001) evaluated the effect of a long period (up to 25 years) of serum storage at −25 °C and found that there were no large fluctuations in albumin concentrations. Through regression analysis, they estimated an increase of 0.28 g L^{-1} per year. A possible explanation for the increase in concentrations for long periods of storage is the unfolding of proteins, exposing aromatic amino acids that react (bind) with the bromocresol green used in the analysis of albumin. Based on the serum and plasma concentrations of albumin obtained in this study, it seems unlikely that the albumin concentration in the evaluated samples was influenced by storage times. It is suggested that the binding properties of bromocresol green may vary between human and broiler albumin.

A common theory is that blood metabolism can lead to changes in the sample. In this sense, freezing aims to interrupt the metabolism of erythrocytes and leukocytes (Knowles et al., 2006). However, it is not entirely clear how the dynamics of sample freezing and thawing can affect the concentration of certain analytes. For the uric acid that showed an increase in its concentration as the storage time increased, this effect may have been caused by changes in the water concentration in the sample (Laderson et al., 1974). In addition, it is suggested that with the increase in storage time, protein or enzyme breakdowns may have occurred, influencing the results.

In the human clinic, Clark et al. (2003) evaluated the storage of whole blood samples in EDTA under refrigeration (4 °C) and room temperature (21 °C) for seven days before centrifugation and observed stability for total protein and albumin compared with samples immediately processed. The reduction in the concentration of these two analytes was 4%. Less stability was observed in creatinine with a 20% increase in its values in relation to the refrigerated sample. They also did not observe significant alterations for the analytes considered stable in plasma samples stored at −80 °C for up to five years.

Jensen et al. (2008) recommended some pre-analytical conditions to guarantee better quality results, such as maintaining the temperature between 20 and 25 °C during storage and transport, and that this storage time without centrifugation should not exceed 6 h. The samples in this study were processed 15 min after collection, and it is not possible to confirm whether the time between collection and centrifugation affected the results. Leino and Koivula (2009) confirmed the stability of non-centrifuged samples for albumin, creatinine, ALP, and Ca (with the exception of P) for up to 6 h at 8 to 22 °C, consistent with the results found by Peakman and Elliott (2008) and Zwart et al. (2009).

In a similar study, Tanner et al. (2008) evaluated three different times (4, 12, and 24 h) and storage temperatures (15, 25, and 35 °C) before sample processing. The authors observed that creatinine, Ca, and P showed less stability at this pre-analytical stage compared with time 0. Given the above, the pre-analytical stage can have some impact on the results, which can lead to possible interpretation and reliability errors. Therefore, the standardization of the processes is essential for the reliability of the results. These precautions were taken so that the possible effects of the pre-analytical stage were minimized as much as possible.

Oliveira et al. (2011) observed a significant difference for total protein in sheep serum samples stored at −20 °C for up to 28 days. The values were increased compared with fresh serum (time 0). Similarly, Comis (2006) observed an increase in total protein concentrations in equine serum samples after 30 days of storage and a decrease in values after 60 and 90 days of storage at −20 °C.

The Ca concentrations found in the plasma fraction suffered interference from the anticoagulant used. Ethylenediaminetetraacetic acid contains four acid groups and two amine groups with an electron pair that chelate Ca and other divalent metals. Calcium is necessary for the activation of a wide range of enzymes necessary for the coagulation cascade to occur, and its removal prevents coagulation (Banfi et al., 2007). This explains the differences in concentrations between the serum and plasma fractions. In this sense, the use of EDTA to measure plasmatic Ca concentration is not recommended.

A possible explanation for the higher concentration of certain analytes in serum compared with plasma can be attributed to the action that low molecular weight anticoagulants (e.g., sodium fluoride) can exert on the sample. The osmotic effect of low molecular weight anticoagulants tends to remove a quantity of water from the interior of the erythrocytes, transferring it to the plasma, which would cause a dilution of the plasmatic concentrations of some metabolites (Alper, 1974). According to Grande et al. (1964), this removal of water from the erythrocytes may lead to a higher dilution of plasma constituents, resulting in a lower concentration of analytes in this blood fraction. It should also be noted that osmotic redistribution between blood cells and plasma may occur, interfering with the results of the analytes (Grande et al., 1964).

The use of sodium fluoride anticoagulant can analytically interfere with spectrophotometry, as it can cause the formation of fibrin in samples during collection (Fernandez et al., 2013; Al-Kharusi et al., 2014; Bonetti et al., 2016). After separating the plasma, fibrin formation should be observed and, if possible, the sample should be collected again or centrifuged with higher force of gravity.

5. Conclusions

Both fractions can be used for total protein. Plasma can be stored for up to 120 days, while serum storage is recommended for up to 60 days at −20 °C. For serum globulin, the quadratic and cubic regression models estimated 6 and 7 h of fasting, respectively. Serum and plasma fractions can be used to measure uric acid and creatinine, but immediate analysis after sample collection is recommended. Serum and plasma P concentrations are not affected by storage time, and samples can be stored for up to 120 days. Except for creatinine and plasma Ca, the other variables show higher concentrations when using 5 lux in serum and plasma. The use of ETDA anticoagulant for measuring Ca and ALP activity is not recommended.

Data availability

The entire dataset supporting the results of this study was published in the article itself.

Author contributions

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Conflict of interest

The authors declare no conflict of interest.

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