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Sample storage and fasting times affect serum and plasma concentrations of metabolites in fasted and non-fasted broiler chickens

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ABSTRACT

Sample storage and fasting times leads to some changes of blood metabolite in broilers. Therefore, a study was conducted with the aim to assess the influence of storage and fasting times in serum and plasma fractions on glucose, total cholesterol, triacylglycerols, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyl transferase (GGT) concentrations in broilers. A total of 70 male broiler chickens fasted at 7 times (0, 2, 4, 6, 8, 10, and 12 h) to collect blood fractions (serum and plasma) stored at -20 °C for 0, 30, and 60 days. Glucose and GGT were affected by fasting times×blood fraction. Serum glucose concentration decreased linearly (≅ 2.48 mg dL^{-1}), whereas total cholesterol and plasma GGT increased linearly (≅ 0.92 mg dL^{-1} and ≅ 0.19 IU L^{-1}, respectively) with fasting time. There was a quadratic effect on plasma glucose and serum GGT (maximum at 3.95 h and minimum at 5.22 h of fasting, respectively), and triacylglycerol (minimum at 8.75 h of fasting) and ALT concentrations (maximum at 8.45 h of fasting). Glucose, total cholesterol, AST, ALT, GGT concentrations were higher in serum, while triacylglycerols were higher in plasma. Glucose concentration had the lowest values at 30 days, while ALT was higher on day 0. However, GGT concentrations were lower on days 0 and 30. Samples of plasma for glucose, ALT, and GGT stored at -20 °C for long periods should be avoided. In addition, serum samples and 6 h fasting are recommended for the assessment of blood biochemical metabolites in broilers.

Keywords: Blood fractions, Broiler, Fasting, Liver enzyme, Sample storage.

INTRODUCTION

The assessment of biochemical parameters in poultry research can be better targeted, allowing the measurement of the concentration of several blood biochemical constituents that can be used as indicators of metabolic disorders, nutritional status, and in the diagnosis of

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diseases. Experiments involving broiler chickens commonly aim to assess bird performance, promoting satisfactory feed conversion without compromising the metabolism and health integrity of the birds. The changes that occur in the metabolism of birds can be caused by the inclusion of ingredients, additives, chemotherapeutics, or other components of the diet (Hagan *et al.*, 2022), in addition to environmental effects, which can alter the nutritional status and health of the animals. Therefore, blood biochemical assessments are an important tool for the diagnosis of diseases and metabolic disorders, providing efficient, fast, and safe diagnoses.

According to Gattani *et al.* (2016), the measurement of blood concentrations of glucose, total cholesterol, triacylglycerol, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyl transferase (GGT) activities can be used to aid in the diagnosis of numerous metabolic disorders in broiler chickens. However, there is a wide divergence in the conditions under which blood samples are taken for analysis, as well as no standardization of the postprandial fasting period performed before blood collection. Córdova-Noboa *et al.* (2018) did not fast birds to collect blood samples, but Sadeghi *et al.* (2014), Behboudi *et al.* (2016), Zakaria *et al.* (2017), and Swarna *et al.* (2018) performed fasting between 2 to 12 h prior sample collection. Taking into account these variations in fasting times, it is not possible to state that the results obtained in biochemical blood analyses will not be influenced by a long postprandial fasting period, as recently demonstrated in a study conducted by Wachholz *et al.* (2023).

Biochemical parameters can be measured in serum samples, or plasma obtained with the use of anticoagulants. The difference between these two processes is that in plasma, a larger volume of supernatant is obtained compared to serum (Lumeij, 2008). Some divergence in the use of serum or plasma in research results in a lack of standardization of results. Some authors used samples of serum to measure blood biochemical parameters (Chand *et al.*, 2018, Gilani *et al.*, 2018, Rehman *et al.*, 2018, Subhani *et al.*, 2018), while others assessed in plasma (Sharideh *et al.*, 2016, Yang *et al.*, 2017, Zhang *et al.*, 2017, Kim *et al.*, 2019).

Regarding the storage times that each sample can be subjected to before the biochemical analyses, it is important to consider that the samples will usually be collected at different times from the day of analysis because factors such as distance between the laboratory and the experimental facility, time to obtain sufficient volume of samples, and transportation period, can delay the date of the measurements of the biochemical variables in blood (Livesey *et al.*, 2008). This is supported by the study conducted by Wachholz *et al.* (2023), who assessed the effect of time and storage condition on biochemical metabolites in serum or plasma samples of broiler chickens and observed significant changes in the concentrations of glucose, total cholesterol, triglycerides, AST, ALT and GGT.

Here, a study was conducted based on the hypothesis that both independent variables tested can significantly alter blood metabolite concentrations and the results in broiler chicken experiments. Therefore, this study aimed to assess the influence of storage and fasting times in serum and plasma fractions on the concentrations of glucose, total cholesterol, triacylglycerols, AST, ALT and GGT in broiler chickens.

MATERIALS AND METHODS

Place of study, birds, handling, housing, and diets

The present study was conducted at Universidade Estadual do Oeste do Paraná (Unioeste), Marechal Cândido Rondon, PR, Brazil. The University Animal Ethics Committee approved the experiment under number 23/20. A total of 70 45-day-old Cobb 500® male broiler chickens were used, with an average body weight of 3,072±859 g. The birds were raised from 1 to 42 days of age, receiving water and diet *ad libitum*, and the same care to management, lighting and ambient temperature recommended by the lineage manual. The diets for each phase (starter, grower, and finisher) were corn-soybean based supplemented with industrial amino acids, isonutritional and isoenergetic, and according to the nutritional requirements proposed by Rostagno *et al.* (2017).

At 42 days of age, the birds were assigned in a completely randomized design in 7 pens replicates (1.76 m^2), with a masonry floor covered with 10 cm of pine wood shavings. Each pen was composed of 10 birds and a density of 5.7 birds per m^2. The pens were equipped with a tubular feeder and nipple drinkers. The facility was equipped with electrical elements, hoods, evaporative pads to assist cooling and air exchange.

Blood sampling, preparation and analysis

After 3 days of adaptation at 45 days-old, the birds were fasted for 1 h, then fed for 30 min. This procedure was adopted so that all birds had the same postprandial feeding condition. After this period of feeding, the first blood collection was performed. At every 2-h interval, within a total period of 12 h (0, 2, 4, 6, 8, 10, and 12 h of fasting), blood was collected by puncture of the ulnar vein from 1 bird per pen (n = 7 birds per fasting time randomly selected). Every 7 birds were used only for a single sampling at a given fasting time. Blood collection was performed with the birds in the decubitus position lateral, using specific vacuum collection tubes (Vacutainer®, Curitiba, PR, Brazil) with a capacity of 10 mL, specific adapter and 25×0.8 mm needles (21G 1”) (Labor Import brand, Maringá, PR, Brazil).

Two tubes of approximately 4 mL each were collected within the allowable for species and body weight (Kelly and Alworth, 2013). The first blood sample was collected to obtain the serum in a tube (BD Vacutainer®, Curitiba, PR, Brazil) with clot activator (silica powder) blasted on the tube wall to accelerate the process of coagulation. The second sample collected was to obtain plasma in a tube (BD Vacutainer®, Curitiba, PR, Brazil) with 5 mg of sodium fluoride as a glycolysis inhibitor and 4 mL EDTA-K_3 anticoagulant. These two draws collected per bird were consecutive. After collection, the samples remained for 15 min at room temperature in a horizontal position and then were centrifuged (Centrifuge Kasvi K14-4000, Kasvi, São Paulo, SP, Brazil) at 2,500 *g* for 10 min at room temperature.

After centrifugation and separation of serum and plasma, the samples were identified and divided into three aliquots as technical triplicates, which were placed in 2 mL microtubes (Eppendorf® brand, Minispin®, Hamburg, Germany). An aliquot was immediately sent to the laboratory for analysis. The other aliquots (two microtubes as technical duplicates) were stored at -20 ºC for times of 30 and 60 days for further analysis (Wachholz *et al.*, 2023).

Within each storage times, the samples were thawed under refrigeration (4 °C) and kept in a refrigerator for 24 h. Before performing the analysis, the samples were centrifuged in an Eppendorf microcentrifuge (Eppendorf® brand, Minispin®, Hamburg, Germany) to remove fibrin formation. Biochemical analyzes were performed using commercial kits and 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) in automatic calibration spectrophotometer (Elitech® brand, Flexor EL200 model, Puteaux, France).

The determination of glucose concentration (Glucose PAP) was performed by Trinder's method, enzymatic colorimetric kinetic (Trinder, 1969), total cholesterol was performed by Trinder's method, enzymatic colorimetric endpoint (Allain *et al.*, 1974), triacylglycerols (Triglycerides SL New), enzymatic colorimetric endpoint (Fossati and Prencipe, 1982), AST and ALT were performed according to International Federation of Clinical Chemistry method without pyridoxal phosphate, kinetic, UV (Schuman *et al.*, 2002a, Shuman *et al.*, 2002b), and GGT (GAMMA GT plus) was performed according the procedure Glupa-C substrate method, kinetic (Schuman *et al.*, 2002c).

Statistical procedures

Data were analyzed considering blood fraction (serum and plasma), fasting (0, 2, 4, 6, 8, 10, and 12 h) and storage times of the sample (0, 30, and 60 days) as fixed effects. Residual error was considered as a random factor. Each bird belonging to the same pen was considered an experimental unit. Data were subjected to normality analysis using the Shapiro-Wilk test. Afterward, the two-way analysis of variance (ANOVA) was performed, considering the isolated effects and interactions between the studied factors: fasting or storage times×blood fractions (serum and plasma) as fixed factors. All tests were performed at 5% significance level. In case of significance, F-test was performed for the blood fraction, regression analysis for fasting times (linear or quadratic models), and Tukey's post hoc test for storage times. The triple interaction was not tested. All statistical procedures were performed using the GLM procedure of SAS University Edition.

RESULTS AND DISCUSSION

Glucose concentrations were influenced by the interaction between fasting times×blood fractions (Table 1). Serum glucose concentration decreased linearly with fasting time, reducing ≅ 2.48 mg dL^{-1} every 2 h of fasting. Total cholesterol concentrations increased linearly with prolonged fasting time, increasing ≅ 0.92 mg dL^{-1} (Figure 1). There was a quadratic effect on the concentrations of plasma glucose, with maximum concentrations at 3.95 h of fasting, and on triacylglycerol concentrations, with minimum concentration at 8.75 h of fasting. Glucose and total cholesterol concentrations were higher in serum than in plasma, while triacylglycerol concentrations were higher in plasma compared to serum. Glucose concentrations were influenced by storage times, with the lowest values at 30 days of storage compared to 0 and 60 days.

The linear decreasing effect shown in serum glucose and quadratic for plasma glucose is explained by the decrease of glycogen in the liver not being enough to maintain blood glucose concentrations, causing its gradual reduction (Rodrigues *et al.*, 2017). The increase in total cholesterol concentration and the decrease in triacylglycerols is due to lipid mobilization, as lipids enter the bloodstream and are consequently transported by very-low-density lipoproteins, causing a greater circulation of total cholesterol in the blood and, hence, the use of triacylglycerols by the tissues (Lumeij, 2008).

In addition, blood concentrations of triacylglycerols are also influenced by fasting times because when a negative energy balance occurs, bird metabolism is stimulated to mobilize circulating triacylglycerols to provide fatty acids and glycerol via β-oxidation and glycolysis, respectively, which are used for energy production, lowering blood triacylglycerols concentrations (Coelho *et al.*, 2013). These results are in agreement with Vosmerova *et al.*

(2010), who observed that birds exposed to prolonged fasting times showed reduced glucose and triacylglycerol concentrations.

The storage times of serum and plasma can lead to a reduction in glucose concentrations. The present study showed variation in glucose concentrations caused by storage even at -20 ºC. This suggested that the temperature used during storage was not sufficient to keep glucose concentrations stable for 60 days. The results of this study corroborate with the findings of Cuhadar *et al.* (2013) and Clark *et al.* (1990), who stored serum samples from humans at -20 ºC for 30 and 60 days, and observed a decrease in glucose concentrations.

The results observed for total cholesterol and triacylglycerols demonstrate that these analytes can remain stable for 60 days at a temperature of -20 ºC without any change. According to Stokes *et al.* (1986), and Tiedink and Katan (1989), cholesterol and triacylglycerols present in plasma and serum do not change their concentrations stored at -20 ºC for 19 to 27 weeks. The stability of triacylglycerols for long storage periods was also observed by Shimizu and Ichihara (2019) storing serum samples for 2 months at -20 ºC.

Although the differences found between the concentrations of biochemical analytes in the blood fraction are statistically significant, Picheth *et al.* (2001) reported that these differences between fractions for glucose do not represent chemically significant differences, pointing out that both fractions can be used analytically for glucose.

Gamma-glutamyl transferase concentrations were affected by the interaction between fasting times×blood fractions (Table 2). Plasma GGT concentrations increased linearly with prolonged fasting time, increasing $\cong$ 0.19 IU L^{-1} (Figure 1). There was a quadratic effect on the concentrations of serum GGT, with a minimum concentration at 5.22 h of fasting. In addition, there was a quadratic effect on ALT concentrations, with a maximum concentration at 8.45 h of fasting. Aspartate aminotransferase, ALT and GGT concentrations were higher in serum than in plasma. Alanine aminotransferase and GGT concentrations were influenced by storage times. It was observed that ALT concentration was higher on day 0 compared to the other storage times. However, GGT concentrations were lower on days 0 and 30 than on day 60.

The quadratic response for ALT enzyme activity corroborates the results described of Veiga *et al.* (1978), who reported that fasting time increases ALT concentrations in birds due to the greater hepatic influx of gluconeogenic substrates, but less correlated with hepatic gluconeogenesis than AST activity. The occurrence of a quadratic effect with the increase in serum GGT concentration, and the linear increase in plasma GGT is because the metabolism of the animals in prolonged fasting triggers the protein catabolism and, consequently, increases the transport of amino acids. This highlights the main function of GGT, which according to Yu and Long (2016), has the function of acting in the extracellular environment by catalyzing the conversion of glutathione into glutamate dipeptides or cysteine.

Another metabolite stable to storage is AST, which did not change its concentration during the 2 months of storage at -20 ºC. According to Kaneko *et al.* (2008), this enzyme is stable to storage during freezing. This response was also observed by Thoresen *et al.* (1995) and Oliveira *et al.* (2011), who studied the same enzyme, but in blood samples from dogs and lambs, respectively. However, ALT concentrations tend to decrease with storage times (Kaneko *et al.*, 2008). This decrease in blood ALT concentration is the result of enzyme denaturation as reported by Ikeda *et al.* (2015) in a study conducted with human blood samples, who observed enzyme denaturation and a decrease in its concentrations at -20 ºC due to its instability at this temperature.

Storage times can negatively interfere with the results of blood analyses in experiments because researchers can often make conclusions based on results influenced by storage times. The concentrations of glucose, total cholesterol, AST, ALT, and GGT evaluated in the current study were higher in the serum sample compared to those observed in the plasma. This higher concentration in serum can be attributed to the action that low molecular weight anticoagulants, such as sodium fluoride, can exert on the samples. The osmotic effect of these anticoagulants tends to remove an amount of water from inside the erythrocytes and transfer it to the plasma, which would cause a dilution of the plasma concentrations of some metabolites (Alper *et al.*, 1974). According to Grande *et al.* (1964), this can cause dilution of the plasma constituents, resulting in lower values in this blood fraction and, therefore, an anticoagulant that does not interfere with the analysis results should be chosen. In addition, another important point to note is that an osmotic redistribution between cells and plasma can occur, and this can interfere with the analyte results.

The use of sodium fluoride as an anticoagulant can interfere analytically in spectrophotometric assessments because this anticoagulant can cause fibrin formation in samples during their collection (Fernandez *et al.*, 2013, Al-Kharusi *et al.*, 2014, Bonetti *et al.*, 2016). Thus, after plasma separation, the formation of hemolysis must be observed and, if possible, to perform a new collection or make a higher centrifugal force.

Gamma-glutamyl transferase concentrations showed different effects on blood fraction during the storage times. The difference in GGT concentrations between serum and plasma, as well as a decrease in metabolite values in plasma analysis, are due to the use of sodium fluoride anticoagulant, which reduces GGT activity (Burtis *et al.*, 2012).

Du *et al.* (2023) examined serum blood metabolites in broilers at 49-day-old on restricted feeding and 16-h fasting and found lower average values than our findings for total cholesterol (67.14 vs. 136 mg dL^{-1}), triacylglycerols (7.92 vs. 42 mg dL^{-1}), and glucose (151 vs. 233 mg dL^{-1}) and ALT (2.06 vs. 9.51 IU L^{-1}) compared to the 12-h fasting in our study. However, the values for AST (587 vs. 412 IU L^{-1}) and GGT (24.78 vs. 22.59 IU L^{-1}) were higher than those of the present study. Corroborating the results of the present study, Hagan *et al.* (2022) reported serum reference values of 197 to 299 mg dL^{-1} for glucose, 129 to 297 mg dL^{-1} for total cholesterol, and average values of 96.15 mg dL^{-1} for triacylglycerol concentrations in 56-day-old Cobb and Ross broiler chickens fasted for 12 h.

Based on the criteria assessed in the current study, the results indicated that the determination of AST is not influenced by the sample storage and fasting times. Total cholesterol and triacylglycerol measurements can be performed on samples stored for up to 60 days at -20 ºC. Plasma glucose, ALT, and GGT analyses stored at -20 °C for long periods should be avoided. In addition, serum samples and 6 h fasting are recommended for the assessment of blood biochemical metabolites in broilers.

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Table 1. Concentrations (mg dL^{-1}) of glucose, total cholesterol and triacylglycerols in serum and plasma stored at different times in 45-day-old broiler chickens subjected to different fasting times.

	Glucose		Total cholesterol		Triacylglycerols	
Fasting times (h)	Serum	Plasma	Serum	Plasma	Serum	Plasma
0	260±12	233±15	130±13	114±10	76±19	78±19
2	260±18	236±13	134±13	118±13	48±10	52±12
4	258±21	255±17	141±14	136±13	47±12	53±13
6	247±21	239±12	138±12	123±12	41±14	42±11
8	241±11	232±10	132±11	121±12	38±6	41±08
10	231±03	219±10	146±14	127±11	32±7	33±06
12	238±21	228±17	145±14	128±13	42±5	43±06
Storage times (days)						
0	251±20	238±16^{A}	137±14	124±14	44±19	49±20
30	243±17	230±15^{B}	138±14	125±12	47±17	50±71
60	249±23	235±19^{A}	138±14	122±15	47±16	48±18
Average	248±20^{a}	234±17^{b}	138±14^{a}	124±14^{b}	46±17^{b}	49±18^{a}
SEM	0.961		0.765		0.847	
Fasting times	<0.001		<0.001 (L)		<0.001 (Q)	
Blood fraction	<0.001		<0.001		0.017	
Storage times	<0.001		0.501		0.364	
Fasting times×blood fraction	<0.001		0.061		0.899	
Linear	<0.001	<0.001				
Quadratic	<0.001	<0.001				
Storage times×blood fraction	0.848		0.594		0.355	

$^{a-b}$ Lowercase letters in the same row differ by F test (P< 0.05); $^{A-B}$ Capital letters in the same column differ by Tukey's post hoc test (P< 0.05); SEM: Pooled standard error of the mean; L: Linear effect; Q: Quadratic effect.

Table 2. Concentrations (IU L^{-1}) of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyl transferase (GGT) in serum and plasma stored at different times in 45-day-old broiler chickens subjected to different fasting times.

	AST		ALT		GGT	
Fasting times (h)	Serum	Plasma	Serum	Plasma	Serum	Plasma
0	353±60	322±56	9.17±2.43	7.18±2.13	23.95±6.97	14.57±2.62
2	408±87	407±81	9.34±2.29	8.28±2.83	21.10±6.78	16.36±4.86
4	450±76	440±70	9.95±2.84	9.59±2.85	21.32±5.49	18.05±4.01
6	436±63	403±71	10.25±2.45	9.12±2.15	19.55±6.01	15.08±2.67
8	413±66	380±73	9.93±2.00	8.80±2.30	20.96±5.10	18.57±4.02
10	460±67	417±70	10.65±2.33	8.85±2.91	23.25±6.07	16.14±4.22
12	436±71	387±65	10.65±3.01	8.38±2.75	27.04±5.63	18.15±2.99
Storage times (days)						
0	409±71	388±65	11.14±2.40	9.76±2.26^{A}	16.80±3.85	16.66±4.08^{B}
30	431±74	397±71	9.40±2.42	8.02±2.64^{B}	16.48±3.69	16.03±4.43^{B}
60	426±85	396±94	9.44±2.37	8.04±2.64^{B}	24.22±6.45	16.82±4.32^{A}
Average	422±77^{a}	394±77^{b}	9.99±2.52^{a}	8.60±2.64^{b}	22.46±6.41^{a}	16.70±3.94^{b}
SEM	3.826		0.130		0.296	
Fasting times	0.140		<0.001(Q)		<0.001	
Blood fraction	<0.001		<0.001		<0.001	
Storage times	0.135		<0.001		0.009	
Fasting times×blood fraction	0.483		0.352		0.002	
Linear					0.032	0.004
Quadratic					<0.001	0.010
Storage times×blood fraction	0.703		0.998		0.005	
Unfolding					<0.001	0.851

$^{a-b}$ Lowercase letters in the same row differ by F test (P< 0.05); $^{A-B}$ Capital letters in the same column differ by Tukey's post hoc test (P< 0.05); SEM: Pooled standard error of the mean; L: Linear effect; Q: Quadratic effect.

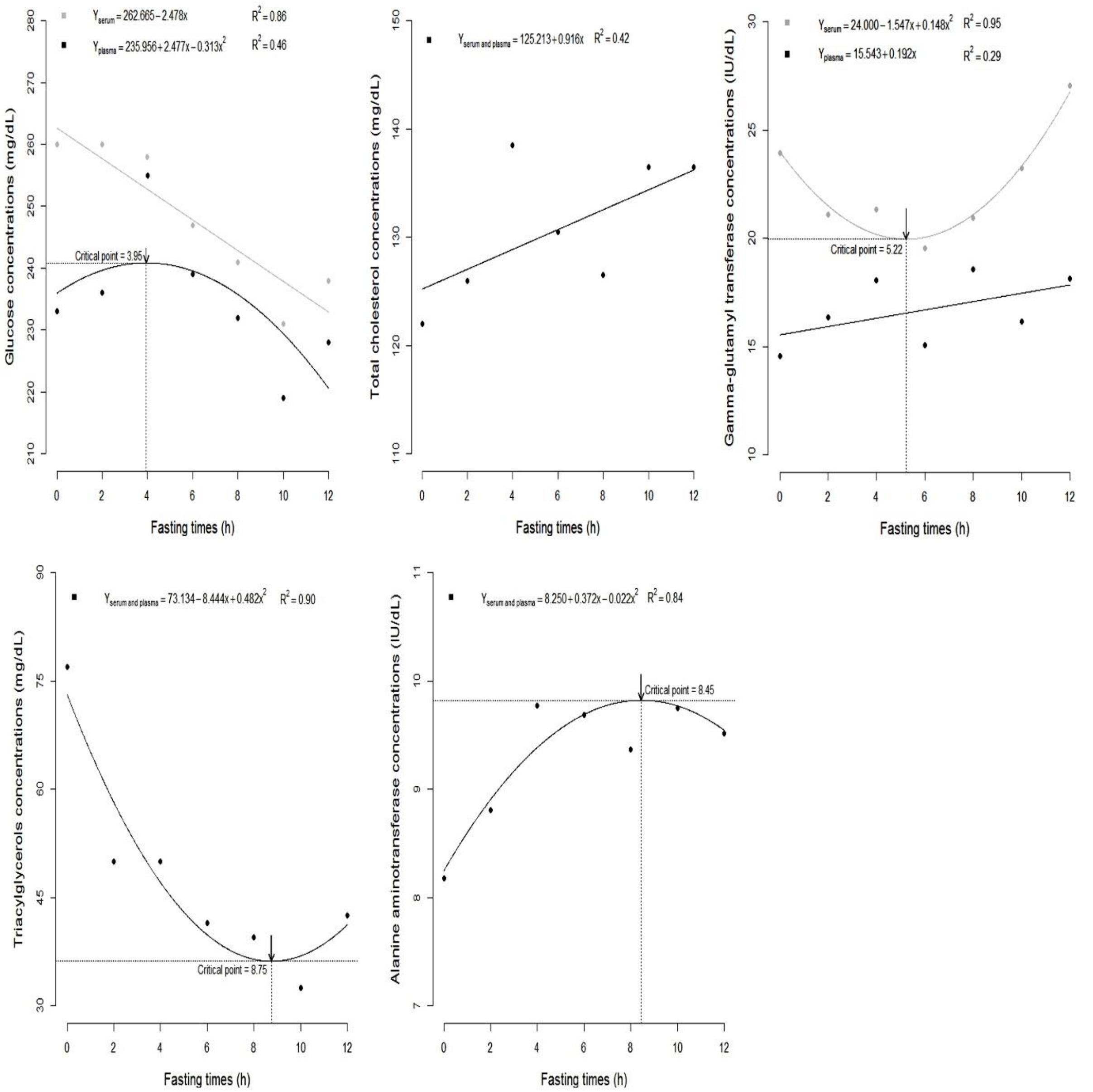

Figure 1. Serum and plasma concentrations of glucose, total cholesterol, triacylglycerols, alanine aminotransferase and gamma-glutamyl transferase in broiler chickens at 45-day-old subjected to different fasting times (R^2: coefficient of determination).