10.4322/rbcv.2021.012

Parâmetros sanguíneos de potros da raça Crioula*

Blood parameters of crioulo breed horse

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Resumo

Com o propósito de estabelecer valores de hematócrito, proteínas plasmáticas totais, fibrinogênio, creatina quinase , aspartato transferase e lactato em potros da raça Crioula, do nascimento até os dois anos, utilizaram-se amostras sanguíneas de 85 animais, divididos pela estratificação etária: Grupo 1 (G1) Até 15 dias de vida (n=70); grupo 2 (G2), entre 16 dias até um mês (n=67); grupo 3 (G3), entre 1 e 3 meses (n=75); grupo 4 (G4), entre 3 e 6 meses (n=64); grupo 5 (G5), entre 6 e 9 meses (n=59); grupo 6 (G6), entre 9 e 18 meses (n=39); e grupo 7 (G7), entre 18 meses até 2 anos (n=17). Foi realizado estudo estatístico entre os grupos pela análise de variância unidirecional (one-wayANOVA), complementada pelo teste de Tukey. Para comparação das médias entre os sexos utilizou-se o teste t de Student. O hematócrito foi significativamente mais elevado até os 90 dias e nas fêmeas do G7. Para proteínas plasmáticas totais, notou-se aumento significativo nos grupos 3, 4, 6 e 7. Os valores de fibrinogênio foram maiores no G1. A CK apresentou maior concentração no G5 e a AST no G7. A AST assumiu valores semelhantes dos 30 dias até os 2 anos. A concentração de lactato foi mais elevada no G3. Conclui-se que na interpretação dos exames laboratoriais de potros da raça crioula, o gênero não interfere significativamente nos resultados, porém a idade deve ser considerada devido à ocorrência de variações relevantes. Recomenda-se que para interpretação sejam consultadas tabelas específicas para cada análise.

Palavras-chave: bioquímicos, enzimas, hematócrito.

Abstract

Plasma levels of hematocrit, total plasma protein, fibrinogen, creatine phosphokinase, aspartate transferase, and lactate were analyzed in blood samples of 85 Crioula breed foals, from birth to two years of age. The animals were divided into age groups: G1 (up to 15 days of age; n=70), G2 (from 16 days to one month of age; n=67), G3 (between one and three months of age; n=75), G4 (between three and six months of age; n=64), G5 (between six and nine months of age; n=59), G6 (between nine and 18 months of age; n=39), and G7 (between 18 months and two years of age; n=17). These groups were statistically analyzed by one-way variance analysis (ANOVA) and Tukey's test. Male and female means were compared by Student's t-test. Hematocrit levels were significantly higher up to 90 days of age and in G7 females. Total plasma proteins increased significantly in groups 3, 4, 6, and 7. The highest fibrinogen levels were found in G1. Yet for creatine phosphokinase, the highest concentrations were detected in G5, whereas those of aspartate aminotransferase in G7. The levels of this enzyme remained similar from 30 days to two years of age. Lactate concentrations were higher in G3. We concluded that the sex of the animal had no significant effect on laboratory test interpretations. By contrast, the age of the animal should be considered since relevant variations were observed with time. Nevertheless, specific tables for each analysis should be consulted for interpretation of results.

Keywords: biochemists, enzymes, PCV (packed cell volume).

Introduction

Laboratory tests are widely used in clinical routine of equine practitioners, providing useful information for diagnosis, treatment, and prognosis of diseases, while avoiding complex and invasive complementary procedures. However, laboratory analyses should be properly interpreted to obtain accurate and reliable information.

When interpreting laboratory results, some intrinsic factors shall be considered such as age, species, sex, and nutritional status (Veronesi et al., 2014). Region-specific reference ranges also have to be obtained, as extrinsic factors such as room temperature, humidity, and pasture type may influence results (Birgel et al., 2001).

In general, laboratory parameters are determined in Crioula breed horses to understand changes in exercise-induced and adult animals (Da Cás et al., 2001). In addition to the scarcity of studies on blood changes due to neonatal adaptations, young-animal blood tests have been interpreted using adult-animal parameters.

*Recebido em 4 de setembro de 2020 e aceito em 2 de março de 2021.
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Based on the above, this study aimed to analyze the levels of hematocrit, total plasma protein, fibrinogen, creatine phosphokinase, aspartate transferase, and lactate in Crioula breed foals under extensive rearing conditions, from birth to two years of age.

Material and methods

The experimental protocol performed in this study was approved by the Animal Use Ethics Committee (CEUA) of the Federal University of Pampa (protocol nº 018/2016).

Blood samples were collected from 85 Crioula breed foals, from birth to two years of age. These animals were reared and registered by the Brazilian Association of Crioula Horse Breeders (ABCCC), consisting of 36 males and 49 females born between 2014 and 2016. They originate from a horse farm in the city of Uruguaiana, Rio Grande do Sul State, Brazil (coordinates: -29.856737 S latitude and -57.020992 W longitude). Only clinically healthy animals were sampled, which were according to clinical examination, clinical history record, and anamnesis data provided by farm keepers and the veterinarian. All foals were handled uniformly, remaining infants until the seventh month of age and then being weaned in a native-grass paddock, with the predominance of *Eragrostis plana* (Annoni grass) and *ad libitum* water supply. The animals underwent no type of work or physical training and received helminthic treatment based on ivermectin (Iver Pasta Equina®, Ouro Fino, Brazil) at 60 and 210 days of age, and based on moxidectin and praziquantel (Moxi Duo®, Ouro Fino, Brazil) at 390 and 600 days.

Foals were divided into seven age groups for analyses: G1 - 70 animals up to 15 days of age (30 males and 40 females); G2 - 67 animals between 16 days and up to one month of age (29 males and 38 females); G3 - 75 animals between one and three months of age (32 males and 43 females); G4 - 64 animals between three and six months of age (32 males and 32 females); G5 - 59 animals between six and nine months of age (19 males and 40 females); G6 - 39 animals between nine and 18 months of age (19 males and 20 females); and G7 - 17 animals between 18 months and two years of age (8 males and nine females).

Blood samples were collected by jugular venipuncture, after injection site being previously sanitized with cotton soaked in 70% alcohol. Then, 5 mL blood was sampled per animal. An aliquot of 3 mL was stored in glass tubes containing 80 µL heparin anticoagulant, manually diluted (1:10) into a solution with sodium heparin (Hepamax–S® 5,000 IU/mL) and 0.9% sodium chloride (Fresenius Kabi Brasil Ltda®). The other 2 mL sample was stored in tubes with sodium fluoride anticoagulant (BdVacutainer®). Blood samples in heparin tubes were designed for hematocrit (Ht), total plasma protein (TPP), fibrinogen (Fib), creatine phosphokinase (CPK), and aspartate transferase (AST) analyses, while those in fluoride tubes were used for lactate (Lac) determination.

After collection, blood samples were conditioned in isothermal boxes with recyclable ice and transported to the Clinical Pathology Laboratory of the Federal University of Pampa, Campus of Uruguaiana – RS, Brazil. Thereafter, the collected material underwent visual inspection, and samples with hemolysis signs were immediately discarded. After, blood samples were homogenized and aspirated into two 1.2 x 75 mm capillary tubes, with one end sealed (modeling clay - Acrilex®).

Initially, Ht levels were determined by microhematocrit method (Goldenfarb et al. 1971), with capillary tubes subjected to 12,000 rpm centrifugation for five minutes (Spin 1000 – Spinlab®). After centrifuged, the tubes were read by a single examiner by microhematocrit method and values expressed in %. TPP levels were estimated in g/dL, using refractometry technique by depositing a plasma drop from the same tube onto the refractometer prism (RTP – 20ATC - Instrutherm®). Prior to each analysis, the equipment was washed with distilled water and dried with paper towels. Heat-precipitation technique (Schalm et al., 1975) was used to measure Fib. After the first centrifugation (Spin 1000 – Spinlab®) at 12,000 rpm for five minutes, the capillaries were kept in an oven at 56 ºC for three minutes and then centrifuged again at 12,000 rpm for five minutes for Fib precipitation. One drop of each capillary was once more extracted from the resulting plasma fib free and read on the same refractometer. Fib concentrations were estimated by the difference between TPP levels of the samples from both tubes, with and without heating; these values were expressed in g/dL. Afterward, the tubes were centrifuged at 2,500 rpm for five minutes (micro-processed NT 812 - Novatécnica®), and the separated plasma was stored in 1.5 mL microtubes (Eppendorf – Pró Análise®). These microtubes were identified with the animal number, collection date, and used anticoagulant, and then stored in a freezer at -18 ºC, controlled daily by a maximum and minimum freezer thermometer (Incoterm®), until enzymatic and Lac assays. After thawing, the samples were again homogenized and pipetted using a 200-µL-single-channel manual micropipette by an automated biochemical analyzer (Labmax® 400), which were performed by kinetic process using commercial kits (Labtest®, Lagoa Santa, Minas Gerais, Brazil). Concentrations of CPK enzyme were measured by CPK-NAC liquiform test - Labtest® (Reference 117, MS 10009010019) and activated CPK-NAC method. Yet, AST levels were assessed by the Labtest® liquiform kit (Reference 109, MS 10009010018). Finally, Lac concentrations were determined by the Labtest® enzymatic lactate kit (Reference 138, MS 10009010258), using the same device.

Data were tabulated in BioEstat 5.3® spreadsheets for statistical analyses. Descriptive statistics was used to summarize the data in arithmetic means and standard deviations. One-way variance analysis and Tukey's test were adopted to compare means among the seven age groups. Student's t-test (sample data) was used to compare the means between the sexes (male and female). Pearson's linear correlation was performed between the means of variables in each age group. All tests were carried out considering a significant difference of $p < 0.05$.

Results and discussion

Jugular-venipuncture blood sampling succeeded well, and hemolysis rate was low (3.2%). This might have occurred due to the fast and easily accessible procedure, together with the lack of stress episodes at puncture time.

Regarding Ht, the highest concentration was found in the first three experimental groups. Animals between 90 and 180 days of age showed a decrease in Ht levels, and animals older than 180 days of age had contents significantly lower than those in younger groups (Table 1).

Table 1: Arithmetic means and standard deviations of hematocrit (Ht), total plasma protein (TPP), fibrinogen (Fib), creatine phosphokinase (CPK), aspartate aminotransferase (AST), and lactate (Lac) values in Crioulo foals. (G1: until 15 days of age; G2: 16-30 days; G3: 1-3 months; G4: 3-6 months; G5: 6-9 months; G6: 9-18 months; and G7: 18 months to two years of age) Born between 2014 and 2016 and originating from a horse farm in the city of Uruguaiana, RS, Brazil.

Group	Ht (%)	TPP (g/dL)	Fib (g/dL)	CPK (UI/L)	AST (UI/L)	Lac (mg/dL)
G1	38.46±3.17^{ab}	5.96±0.55^{d}	0.31±0.23^{a}	223.71±94.8^{c}	264.89±75.8^{c}	21.94±12.3^{ab}
G2	39.35±3.10^{a}	6.07± 0.71^{bcd}	0.22± 0.13^{b}	239.49±189.44^{bc}	286.40±46.1^{abc}	21.43±23.71^{ab}
G3	38.92±3.13^{ab}	6.48±0.77^{a}	0.19±0.10^{b}	288.28±183.05^{bc}	325.21±86.42^{ab}	42.72±28.33^{a}
G4	37.40±4.03^{b}	6.44±0.55^{a}	0.21±0.12^{b}	268.38±92.96^{bc}	313.47±64.80^{ab}	31.97±23.95^{ab}
G5	35.38±4.78^{c}	6.05±0.43^{cd}	0.24±0.15^{ab}	431.17±438.28^{a}	339.12±113.88^{a}	36.66±36.38^{ab}
G6	34.48±3.54^{c}	6.35±0.63^{abc}	0.20±0.11^{b}	382.23±470.96^{ab}	309.41±91.95^{abc}	28.59±24.35^{ab}
G7	33.04±2.20^{c}	6.17±0.39^{abcd}	0.24±0.11^{ab}	410.47±232.93^{ab}	358.47±70.36^{a}	17.70±14.19^{b}

Same letters without statistical difference and different letters with statistical difference. Unidirectional, complemented by Tukey's test, and significant at p <0.05.

Both G1 and G2 presented Ht contents above those reported in other breeds at the same age. According to Axon and Palmer (2008), in Thoroughbred (PSI) breed, these levels decrease sharply after 24 hours of age. However, we found a gradual decrease from 30 days onward. Such difference is believed to be due to breed since the other groups had contents quite close to those cited by the above authors, declining progressively and stabilizing near the minimum values of reference range 32 – 52% Smith (2006), corroborating the results found for Spanish and Lusitano breeds (Grondin & Dewitt, 2010).

When compared by Pearson's correlation, Ht and CPK showed a strong negative correlation (Table 2). Diseased animals are supposed to present clinical correlation, mainly for renal lesions, associating decreased erythrocyte production by erythropoietin metabolism, or even hyperhydration cases associated with muscle damage (Zobba et al., 2011). However, the animals used in our study were sound; therefore, Ht tended to decrease as foals grew old and metabolism increased with intensification of animal movement. Thus, increased skeletal musculature stimulation would explain CPK extravasation.

Table 2: R-values of Pearson's linear correlation among the arithmetic means of hematocrit (Ht), total plasma protein (TPP), fibrinogen (Fib), creatine phosphokinase (CPK), aspartate aminotransferase (AST), and lactate (Lac) values in Crioulo foals. (G1: until 15 days of age; G2: 16-30 days; G3: 1-3 months; G4: 3-6 months; G5: 6-9 months; G6: 9-18 months; and G7: 18 months to two years of age) of the different age groups (0 day to two years of age) Born between 2014 and 2016 and originating from a horse farm in the city of Uruguaiana, RS, Brazil

	Ht	TPP	Fib	CPK	AST	Lac
Ht	-	-0.0016	0.0484	-0.8849*	-0.6982	0.2441
TPP	-0.0016	-	-0.8135*	0.0207	0.3248	0.5728
Fib	0.0484	-0.8135*	-	-0.2168	-0.4317	-0.517
CPK	-0.8849*	0.0207	-0.2168	-	0.8159*	0.1091
AST	-0.6982	0.3248	-0.4317	0.8159*	-	0.2343
Lac	0.2441	0.5728	-0.517	0.1091	0.1091	-

*significant at p<0.05.

Significant differences between sexes were only identified in G7 for Ht (p < 0,04). According to Smith (2006), castrated males PSI breed horses show a slight increase tendency in Ht compared to females, contrasting the findings of Van Heerden et al. (1990), observed higher levels of Ht in female PSI horses. In this case, we understand that the sampling difference with more than twice as many females may have influenced the results (12 females and 5 males), and such a difference was only observed in one of the groups.

Contents of TPP did not follow any pattern among age groups, increasing significantly in G3, G4, G6, and G7 and reducing in G5 during the weaning period (Table 1). Our findings differed from other studies since no significant increase in G1 was evidenced. According to Thomas (2000), significant increases would occur within this phase due to colostrum ingestion. However, Morresey (2005) stated that newborn-foal TPP contents vary widely, undermining it as an indicator of colostrum absorption. A significant increase in TPP was detected in G3 and G4, which represent one-to six-month-old foals. In this case, it may be related to lower water status during the summer, which might have influenced TPP values. On the other hand, as reported by Santos et al. (2014), such an increase may be related to the immune peak of PSI foals at this stage, associated with commensal flora formation and contact with microorganisms, besides being under diet adaptations. These findings corroborate Stoneham (2006), who explained the association of increased pasture intake with breastfeeding and handling changes as a possible explanation for TPP increases. Moreover, the decline found in G5 may be related to weaning period; this is because animals undergo stress at that time since they start feeding exclusively on pasture. Additionally, this stage coincided with the winter season when pastures undergo nutritional losses.

The TPP content we found are lower than those reported by Veiga et al. (2006), in which average for Crioula breed individuals under one year of age was 8.15 g/dL. Our findings were also smaller compared to those of Arabian breed, in which average for foals up to six months of age was 7.2g/dL (Favero et al.,

2011). The results of our study also differed from those of Thoroughbred breed, which ranged from 6.5g/dl at birth until reaching 8.0g/dl at six months of age (Santos et al., 2014). Even so, reference ranges for equines are 5.2g/dL and 7.9g/dL (Smith, 2006), which characterizes neither hypo nor hyperproteinemia in any of the age groups studied here. Stoneham (2006) asserted that proteins are more susceptible to changes in hypovolemic adults when compared to foals. However, below-reference values deserve attention, mainly in the first week of age, when an intensive follow-up is recommended due to protein loss risk from secondary diarrhea, enteropathy, passive immunoglobulin transfer failure, and sepsis. Moreover, we observed a strong negative correlation between TPP and Fib (Table 2). These findings may be related to the lack of acute signs of inflammation in the animals studied here; however, some protein changes were associated with nutritional factors, immunity development, and dehydration.

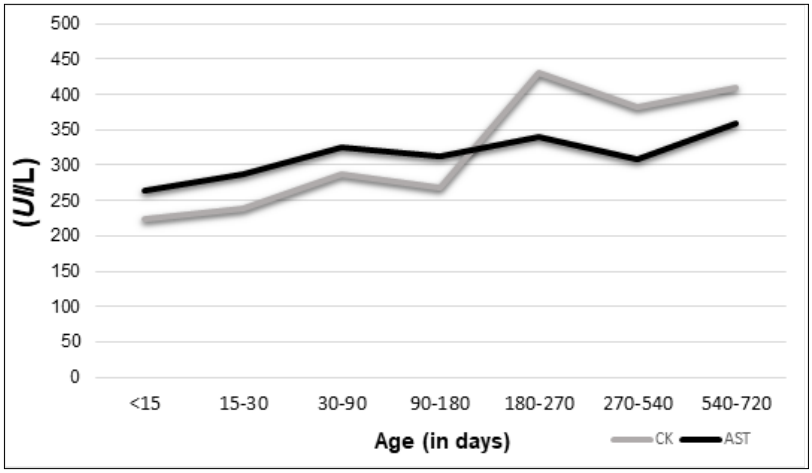

Figure 1: Evolution of arithmetic means of CPK enzyme and AST in Crioula breed foals according to the age group. (G1: until 15 days of age; G2: 16-30 days; G3: 1-3 months; G4: 3-6 months; G5: 6-9 months; G6: 9-18 months; and G7: 18 months to two years of age) Born between 2014 and 2016 and originating from a horse farm in the city of Uruguaiana, RS, Brazil.

The levels of Fib were higher in the first 15 days of age (G1) compared to the other age groups but differed statistically from G2, G3, G4, and G6. For Morresey (2005), higher values are common in the first days of age due to the first exposure to agents and challenges during neonatal period. The maximum values found here were below those reported in Thoroughbred and Quarter Horse breeds by Axon and Palmer (2008). On the other hand, Veiga et al. (2006) found higher values for Creole foals; however, these authors studied animals between four and nine months of age.

Despite the significantly higher Fib levels in G1, there was no evidence of hyperfibrinogenemia in the groups analyzed, which may have occurred because only healthy animals were included, in the same way we understand that the fact that it was not different from G5 and G7 reinforces the idea that it really rises in neonatal challenges and after stabilization occurs, in the end these groups were statistically equal to all the others as well.

Corroborating with Axon (2011), where increases in Fib levels would be considered a good prognostic indicator if normal values were found in animals submitted to and / or recovering from inflammatory and / or infectious processes, in addition to being a neonatal response.

The highest serum values of CPK were found in G5 and of AST in G7 (Table 1). According to Da Cás et al. (2001), CPK values remain balanced with no major changes from neonatal life to one year of age, while AST contents gradually increase in the first weeks after calving, owning to an increase in foal activity. We observed statistically different CPK values when comparing G1 to G5, G6, and G7, which showed higher values after six months of age (Fig. 1). Moreover, AST values did not differ statistically between G1 and G2, and remained balanced from 30 days to two years of age (Fig. 1).

Our findings for AST contents were similar to those in the study of Sales et al. (2013), who evaluated adult Arabian animals and reported values of 313.91 ± 67.48 UI/L. By contrast, Toledo et al. (2001) demonstrated levels of 178.9 – 215.2 UI/L for Thoroughbred breed equines. As enzymatic findings may vary widely, our study is relevant because there is a lack of parameters established for Crioula breed, for which biochemical findings are usually compared with those of other breeds at different age groups.

In our study, CPK values were quite close to those reported by Hodgson and Rose (1994), who described levels between 100 and 300 UI/L for adult animals. However, in Crioula breed foals over six months of age, the levels are reported to be 184.81±77.25 UI/L (Franciscato et al., 2006). Animal handling, room temperature, and horse nutritional status may explain the above-mentioned variations (Câmara and Silva et al., 2007).

Another relevant factor is the higher enzymatic values found in our study when compared to others with the same breed, as already cited (Franciscato et al., 2006**,** Veiga et al., 2006). Although our laboratory results have been compared to those studied with other breeds of horses, these studies lack rigor in group formation, sampling and handling intervals. In contrast, we use detailed age groups, an expressive number of animals combined in totally equal management, in addition to similar numbers between groups and males and females with the exception of G7.

We found the highest CPK levels during the weaning period, which suggests that the animals exercised more to search for food and interact with other animals. The time of year may also have been a relevant factor as the animal's metabolism increases in winter. One hypothesis is that CPK levels were influenced by animal handling for blood sampling since foals had to be taken to a hose in the morning, and blood sampling was sometimes performed in the afternoon. According to Soares et al. (2013), blood concentration peak could be reached from six to 12 hours after muscle injury. Conversely, AST concentrations only rise about 24 hours after injury (Zobba et al., 2011); therefore, AST correlation with animal handling could be discarded.

We interpreted CPK and AST enzymatic values as increased cell permeability rather than as myocyte injury, as suggested by Andreazzi et al. (2014). This author observed AST values of 319.9±67.48UI/L for animals at rest. Differences in enzyme activity values may also be related to ease of CPK release, corroborating Hill et al. (2012), who postulated that CPK levels in the bloodstream

must be too high to indicate an injury. When assessing serum CPK activity in Quarter Horse breed, aged between two and 13 years of age, Bacalhao (2008) found values of 267.5UI/L for animals at rest, which were also not related to muscle injury. Our analysis revealed a strong positive Pearson's correlation between both enzymes (Table 2), which is in agreement with the findings in the literature (Da Cás et al., 2001; Franciscato et al., 2006). Although CPK and AST have distinct plasma extravasation specificities and mechanisms, depending on collection time, we could notice rise and decay of their serum levels, almost always together. AST showed no significant difference among groups at the level of ($p < 0.05$).

Lactate analysis revealed significantly lower values only in G7 compared to G3, however we did not find a plausible physiological relationship for this finding, it is important to note that other enzymes and health tests did not change as well. According to Smith (2006), sick and healthy foals can develop hyperlactatemia during the prenatal phase. This could be explained by the release of cortisol and catecholamines, but also related to physiological hypoxia at the time of delivery, which corroborates the findings of Kitchen and Rossdale (1975), where purebred and healthy English newborn foals showed values between 10 , 0 ± 44.14 mg / dL. Magdesian (2003) reported values of 11 ± 21mg / dL for foals after birth. No hyperlactatemia was detected in our samples (Figure 2), perhaps due to the fact that the G1 collection was performed in the animals up to 15 days after birth. According to Castagnetti et al. (2010), when healthy, animals are able to reduce the Lac content in the first hours of age to levels below 27.02 mg / dL. Other groups presented measurements above the reference values for adults, 10mg / dL (Smith, 2006). This analysis did not differ statistically ($p < 0.05$) between groups and genders.

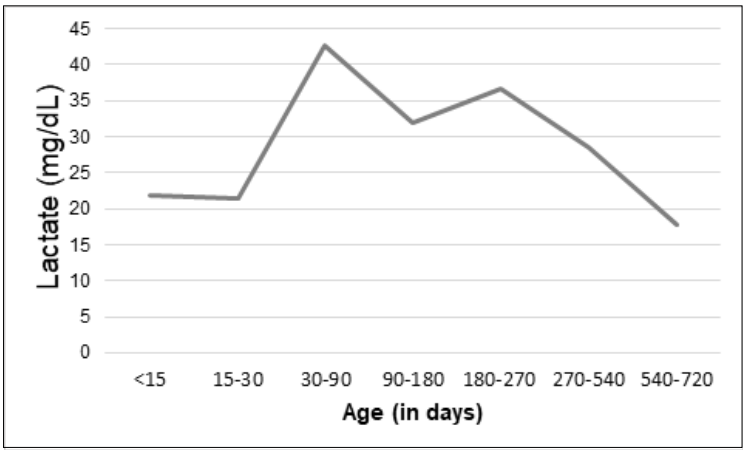

Figure 2: Evolution of arithmetic means of Lactate in Crioula breed foals according to the age group. (G1: until 15 days of age; G2: 16-30 days; G3: 1-3 months; G4: 3-6 months; G5: 6-9 months; G6: 9-18 months; and G7: 18 months to two years of age) Born between 2014 and 2016 and originating from a horse farm in the city of Uruguaiana, RS, Brazil.

Conclusion

After interpreting the laboratory tests of growing Crioula foals, we concluded that the sex of the animal does not significantly interfere with the results but the age is a factor to be considered, due to cases of relevant variations. Thus, the variables that must be interpreted a priori are hematocrit, total plasma protein, fibrinogen, aspartate aminotransferase, and creatine phosphokinase contents, referring to specific tables for this purpose.

Aknowledgements

To Igor Soares Leoni (*in memorian*) by his dedication and companionship in performing the study.

To the staff of the BIOSUL Laboratory in Uruguaiana-RS (Brazil) for performing the laboratory tests.

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