Effect of Age, Sex, and Breed on the Blood Biochemistry and Physiological Constants of Dogs From 4 Weeks to > 52 Weeks of Age.

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Abstract

Background: Blood biochemistry and reference intervals help to differentiate between healthy and ill patients as well as to provide information for the prognosis, evaluation, and monitoring of a patient; however, these intervals are often obtained from adult animals. It is essential, hence, to understand that puppies and adults are physiologically different, which justifies the need to obtain age-specific biochemical reference intervals. The aim of this research was to assess the potential effect of age, sex, breed, and interaction on routine biochemical analytes and physiological constants (body temperature, heart rate, and respiratory rate) in addition to establish age-specific reference intervals. In order to carry out the research, we selected 197 healthy dogs of different sex and breed classified by age: group I (4-8 wk), group II (9-24 wk), group III (25-52 wk), and group IV (>52 wk). The biochemical analysis measured the enzymatic activity of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), and concentration of cholesterol, triglycerides, total proteins, albumin, globulins, glucose, urea, and creatinine. Statistical analyses used the Analysis of Variance (ANOVA) and General Linear Model (GLM), which allows the comparison of multiple factors at two or more levels ($p < 0.05$).

Results: The results of this study showed that ALT, total protein, albumin, globulin, urea, creatinine, and body temperature levels were lower in puppies compared to adult dogs ($p < 0.05$) while the enzymatic activity of ALP, LDH, glucose concentration, and heart rate were higher. Moreover, in small breeds, the serum creatinine levels were lower ($p < 0.05$) whereas sex and interaction did not show a significant effect ($p > 0.05$).

Conclusions: Some biochemical components evince influence by age. For this reason, this research offers specific reference intervals to help the veterinary clinician to interpret the biochemical results of puppies with accuracy.

Background

Blood biochemistry is useful to detect subtle abnormalities of organic systems (1). The reference intervals (RI) are a range that includes 95% of a healthy population, and they have become one of the most used tools in clinical decisions (2). However, just few laboratories do their reference studies, yet most RI consider values obtained from adult dogs; hence, those references should not be used to interpret test results in puppies (1,3).

On the other hand, clinical signs of disease in puppies often are unspecific. Furthermore, the scarce information about the biochemical values in puppies hinder the diagnosis (4–6). Evaluating biochemical components is a valuable tool in these situations, but it could be hampered by the lack of proper RI (5). The physiologic differences between puppies and adults dogs require an age-specific biochemical RI, as in most species (5,7); thus, the RI for adults should not be used in puppies since they lead to inaccurate interpretation of the results (2,5,8). When interpreting clinical data obtained from dogs during their first year of life, growth-related alterations and metabolism changes in biochemical limits should be considered (6,9).

Limited studies have examined the effect of age, sex, and breed on biochemistry variables in puppies, but most of these studies were performed in a controlled laboratory, environment, or with specific breeds where dogs experienced identical living conditions and followed the same diet (4,8–10). In most cases the puppies' history and physical examination are the same as for adults; however, there may be some variations in the

physiological constants. Therefore they must be carried out with full attention and following some guidelines (11,12).

This study aimed to assess the potential effect of age, sex, and breed on routine biochemical analytes measured in four groups of dogs from different ages (4-8, 9-24, 25-52, and >52 wk of age) as well as to establish age-specific reference intervals.

Results

A total of 197 blood samples were collected from pure and mixed breed dogs (small and medium to large size). Fifteen blood samples were excluded from the biochemical analysis: nine lipemic, four hemolyzed, and two with jaundice. In addition, seven observations were excluded from the statistical analysis by subclinical disease. Table 2 shows the mean, standard deviation, median, interquartile range, and significant difference with a 95% confidence level. It also shows a lower and upper limit of the reference intervals and the limits of reference calculated with a 90% confidence interval. Data from all the dogs were divided into four age groups: 4-8, 9-24, 25-52, and > 52 weeks of age.

This study evaluated the effect of age, sex, and breed on biochemical variables serum (AST, ALT, LDH, ALP, GGT, total protein, albumin, globulins, cholesterol, triglycerides, glucose, urea, and creatinine). Additionally, those effects on body temperature (rectal thermometer), heart rate, and respiratory rate were evaluated. In some response variables, we observed statistically significant differences.

Effect of age

No significant differences were observed in the results for AST concerning age ($F(3,158) = 0.59$, $p = 0.62$). On the other hand, the enzymatic activity of ALT had a significant effect ($F(3,158) = 22.11$, $p = 0.00$). The average of ALT in puppies from 4-8 wk of age was 26 U/L ($SD = 8.8$), significantly lower than the enzymatic activity in dogs from 9-24 wk of age ($p = 0.01$), 25-52 wk of age, and >52 wk of age ($p = 0.00$). Moreover, the activity was significantly lower in dogs from 9-24 wk of age ($M = 32$ U/L, $SD = 9.1$) than the adult's group IV ($p = 0.00$). Furthermore, ALT in young dogs of 25-52 wk of age ($M = 37$ U/L, $SD = 7.6$) was significantly similar to the enzymatic activity in adult dogs > 52 wk of age ($M = 44$ U/L, $SD = 12.6$; $p = 0.17$). Therefore, the ALT levels were lower in puppies of 4-24 wk of age; from 25 wk of age, the enzymatic activity is the same as that of adults (Figure 1).

The enzymatic activity of LDH had a significant effect on age ($F(3,158) = 19.44$, $p = 0.00$). The average of LDH in puppies from 4-8 wk of age was 244 U/L ($SD = 164.6$), significantly higher than the enzymatic activity in groups II ($M = 73$ U/L, $SD = 32.4$), III ($M = 63$ U/L, $SD = 32$), and IV ($M = 67$ U/L, $SD = 42$) $p = 0.00$. Our research showed that LDH decreases as age increases, and the values stabilize at 9 wk of age (Figure 1).

The enzymatic activity of ALP had a significant effect on age ($F(3,158) = 165.04$, $p = 0.00$). The average of ALP in puppies from 4-8 wk of age was 215 U/L ($SD = 71.6$), significantly greater than the enzymatic activity of groups III and IV ($p = 0.00$). The ALP of group II ($M = 193$ U/L, $SD = 39.2$) also showed an activity significantly higher than the one of groups III and IV ($p = 0.00$). Finally, the ALP enzymatic activity of group III ($M = 85$ U/L, $SD = 36.7$) is significantly greater than that of group IV ($M = 52$ U/L, $SD = 23.3$; $p = 0.00$). These results suggest that ALP enzymatic activity decreases as the age of dogs increases. The serum ALP activity at 4-24 wk of age

in puppies is four times higher than that in adults; in young dogs from 25-52 wk of age, the ALP activity is almost two times higher than the activity in adults (Figure 1). On the other hand, the enzymatic activity of the GGT did not have a significant effect on age ($F(3,158) = 1.33, p = 0.26$).

The concentration of total proteins showed a significant effect on age ($F(3,158) = 32.21, p = 0.00$). The average of serum total proteins in puppies from 4-8 wk of age ($M = 4.6$ g/dL, $SD = 0.6$) was significantly lower than the one in dogs from 9-24 wk of age ($p = 0.02$), 25-52 wk of age, and >52 wk of age ($p = 0.00$). Furthermore, that average was significant lower in dogs from 9-24 wk of age ($M = 5.1$ g/dL, $SD = 0.7$) compared to adults of group IV ($p = 0.00$). Finally, the concentration of group from 25-52 wk of age ($M = 5.7$ g/dL, $SD = 0.8$) was lower than in dogs of >52 wk of age ($M = 6.2$ g/dL, $SD = 0.9$; $p = 0.03$). Therefore, these results show that the total serum proteins in puppies from 4-8 wk of age are low while in dogs from 9-52 wk of age begin to increase until they stabilize after 52 wk of age (Figure 2).

The concentration albumin also had a significant effect on age ($F(3,158) = 21.38, p = 0.00$). The albumin levels in the group I ($M = 2.5$ g/dL, $SD = 0.7$) were significantly lower than the levels of groups II ($M = 2.7$ g/dL, $SD = 0.3$; $p = 0.01$), III ($M = 3.1$ g/dL, $SD = 0.3$), and IV ($M = 3.1$ g/dL, $SD = 0.4$) $p = 0.00$. From group II, puppies from 9-24 wk of age, the concentration was significantly lower than that of group IV ($p = 0.00$). These results show that serum albumin concentration increase as the dog's age advances. The albumin concentration is low in puppies from 4-8 wk of age while in dogs from 9 weeks of age begins to increase; however, until 25 weeks of age, the albumin concentration stabilizes at adult values (Figure 2).

Regarding the concentration of globulins, a significant effect on age was observed ($F(3,158) = 12.89, p = 0.00$). The average of serum globulins in puppies from 4-8 wk of age was 2.1 g/dL ($SD = 0.6$) and 2.3 g/dL ($SD = 0.6$) in puppies from 9-24 wk of age; significantly lower than that in the dogs from >52 wk of age ($M = 3$ g/dL, $SD = 0.7$; $p = 0.00$). The average of globulins in young dogs from 25-52 wk of age was 2.6 g/dL ($SD = 0.6$): the same as in groups I ($p = 0.25$), II ($p = 0.94$), and IV ($p = 0.06$). Therefore, these results indicate that the serum levels globulins in puppies from 4 to 24 wk of age are low whereas in dogs from 25 wk of age begin to increase the levels until they stabilize after 52 wk of age (Figure 2).

On the other hand, there were no statistically significant differences regarding age in the results of cholesterol ($F(3,158) = 1.49, p = 0.22$) and triglycerides ($F(3,158) = 2.52, p = 0.06$). Whilst, the concentration of glucose showed an effect on age ($F(3,158) = 4.14, p = 0.01$). This concentration was higher in puppies from 4-8 wk ($M = 89$ mg/dL, $SD = 21, p = 0.02$) and from 9-24 wk of age ($M = 90$ mg/dL, $SD = 18, p = 0.00$) than the levels observed in adults > 52 wk ($M = 77$ mg/dL, $SD = 21$). This result suggests that there is a decrease in glucose concentration as the age of the dog increases. In addition, it was established that puppies reached the glucose levels of an adult at 25 wk of age ($M = 81$ mg/dL, $SD = 21$) (Figure 2).

Our research evince an effect of age on the serum urea ($F(3,158) = 14.84, p = 0.00$). The urea concentration was significantly lower in puppies from 4-8 wk of age ($M = 23$ mg/dL, $SD = 7$) compared to that from group II ($p = .047$), III, and IV ($p = 0.00$). Similarly, puppies from 9-24 wk of age ($M = 27$ mg/dL, $SD = 8.7$) showed significantly lower urea levels than dogs from group IV ($p = 0.00$). Furthermore, urea in young dogs from 25-52 wk of age ($M = 36$ mg/dL, $SD = 9.5$) was equal to that of adult dogs > 52 wk of age ($M = 34$ mg/dL, $SD = 10$; $p = 0.99$). Therefore, the urea concentration was lower in puppies from 4-24 wk of age compared to adults, and it stabilized in week 25 (Figure 3).

Regarding the concentration of creatinine, it showed an effect on age ($F(3,158) = 78.92$, $p = 0.00$). In puppies from 4-8 wk of age ($M = 0.45$ mg/dL, $SD = 0.09$), the creatinine levels were lower than the concentration from groups II, III, and IV ($p = 0.00$). Creatinine levels in puppies from 9-24 wk of age ($M = 0.59$ mg/dL, $SD = 0.16$) were lower than those from groups III and IV ($p = 0.00$). Furthermore, creatinine in young dogs from 25-52 wk of age ($M = 1.00$ mg/dL, $SD = 0.30$) was significantly similar to the concentration in adult dogs > 52 wk of age ($M = 1.03$ mg/dL, $SD = 0.25$; $p = 0.81$) while creatinine levels in puppies from 4 -24 wk of age were lower. from 25 wk of age, the concentration is the same as that of adults (Figure 3).

On the other hand, in body temperature, a significant effect on age was observed ($F(3,158) = 18.62$, $p = 0.00$). Body temperature in puppies from 4-8 weeks of age ($M = 37.9^\circ$ C, $SD = 0.5$) is lower than the temperature obtained from groups II, III ($M = 38.7^\circ$ C, $SD = 0.4$), and IV ($M = 38.8^\circ$ C, $SD = 0.4$) $p = 0.00$. Furthermore, in puppies from 9-24 weeks of age ($M = 38.5^\circ$ C, $SD = 0.6$), their body temperature is lower than that of adults > 52 weeks of age ($p = 0.01$). These results indicate that dogs from 4 to 24 weeks of age have a lower body temperature. In dogs from 25 weeks of age, the temperature begins to increase and it is similar to that from adults > 52 weeks of age ($p = 0.53$) (Figure 4).

Regarding heart rate, there was a significant effect of age ($F(3,158) = 5.44$, $p = 0.00$). In puppies from 4-8 ($M = 155$ bpm, $SD = 28.4$; $p = 0.00$) and from 9-24 ($M = 145$ bpm, $SD = 33$; $p = 0.01$) weeks of age the heart rate was higher than that of adults > 52 weeks ($M = 74$ bpm, $SD = 33$). Additionally, in the group from 25 weeks of age, the heart rate was similar to that of adults ($p = 0.86$). Therefore, our study shows a decrease in the heart rate as the animal's age increases (Figure 4). On the other hand, there was no statistically significant difference in respiratory rate ($F(3,158) = 1.21$, $p = 0.31$).

Effect of sex

No significant effect of sex was observed on the enzymatic activity of AST ($F(1,158) = 2.98$, $p = 0.09$), ALT ($F(1,158) = 1.24$, $p = 0.28$), LDH ($F(1,158) = 1.74$, $p = 0.19$), ALP ($F(1,158) = 2.75$, $p = 0.10$), and GGT ($F(1,158) = 0.12$, $p = 0.73$). Neither was there effect of sex on the serum concentration of total proteins ($F(1,158) = 0.14$, $p = 0.70$), albumin ($F(1,158) = 0.45$, $p = 0.50$), globulins ($F(1,158) = 0.62$, $p = 0.43$), cholesterol ($F(1,158) = 0.44$, $p = 0.51$), triglycerides ($F(1,158) = 1.11$, $p = 0.29$), glucose ($F(1,158) = 0.02$, $p = 0.90$), urea ($F(1,158) = 0.50$, $p = 0.48$), and creatinine ($F(1,158) = 1.40$, $p = 0.24$). Physiological constants did not have either an effect on sex: body temperature ($F(1,158) = 0.71$, $p = 0.40$), heart rate ($F(1,158) = 2.86$, $p = 0.09$), and respiratory rate ($F(1,158) = .80$, $p = 0.37$).

Effect of breed

From all the variables evaluated, the effect of breed showed statistically noteworthy differences in creatinine concentration ($F(3,158) = 4.00$, $p = 0.01$). The average creatinine in small breed dogs was 0.73 mg/dL ($SD = 0.31$): significantly lower than that in medium-large breed dogs ($M = 0.92$, $SD = 0.33$, $p = 0.01$). Nevertheless, the other variables did not show significant effects.

No statistical major differences were observed in enzymatic activity of AST ($F(3,158) = 1.34$, $p = 0.26$), ALT ($F(3,158) = 0.87$, $p = 0.46$), LDH ($F(3,158) = 2.61$, $p = 0.05$), ALP ($F(3,158) = 1.32$, $p = 0.27$), and GGT ($F(3,158) = 0.77$, $p = 0.51$). There was also no effect of breed on the serum concentration of total proteins ($F(3,158) = 0.21$, $p = 0.88$), albumin ($F(3,158) = 0.50$, $p = 0.68$), globulins ($F(3,158) = 0.59$, $p = 0.63$), cholesterol ($F(3,158) =$

2.39, $p = 0.07$), triglycerides ($F(3,158) = 1.85$, $p = 0.14$), glucose ($F(3,158) = 1.36$, $p = 0.26$), and urea ($F(3,158) = 1.36$, $p = 0.26$). Neither did breed have an effect on Physiological constants: body temperature ($F(3,158) = 0.47$, $p = 0.70$), heart rate ($F(3,158) = 1.94$, $p = 0.13$), and respiratory rate ($F(3,158) = 1.77$, $p = 0.15$).

Age, sex and breed interaction

No statistical remarkable effects were observed in enzymatic activity of AST ($F(9,158) = 0.76$, $p = 0.65$), ALT ($F(9,158) = 0.87$, $p = 0.55$), LDH ($F(9,158) = 1.41$, $p = 0.19$), ALP ($F(9,158) = 0.44$, $p = 0.91$), and GGT ($F(9,158) = 1.54$, $p = 0.14$). There was not effect of interaction on the serum concentration of total proteins ($F(9,158) = 0.81$, $p = 0.61$), albumin ($F(9,158) = 0.78$, $p = 0.64$), globulins ($F(9,158) = 0.77$, $p = 0.64$), cholesterol ($F(9,158) = 0.79$, $p = 0.62$), triglycerides ($F(9,158) = 1.29$, $p = 0.25$), glucose ($F(9,158) = 0.54$, $p = 0.84$), urea ($F(9,158) = 1.92$, $p = 0.05$) and creatinine ($F(9,158) = 0.79$, $p = 0.63$). Body temperature ($F(9,158) = 0.69$, $p = 0.71$), heart rate ($F(9,158) = 0.55$, $p = 0.84$), and respiratory rate ($F(9,158) = 0.74$, $p = 0.67$) did not show effects either.

Discussion

Regarding the effect of age on the enzymatic activity of AST and ALT, research show contrasting results. The enzymatic activity of AST from this research did not evince a significant effect, which agrees with the studies from other authors (5,13). Nonetheless, other research did find age-related differences in AST enzyme activity (9,10). On the other hand, this study shows that the enzymatic activity of the ALT tends to increase with age. The effect of age on ALT is consistent with prior results (9,10,14,15). However, some researches did not report significant differences in their results in puppies when compared to adults (4,5). These changes in ALT activity derive from physiological variations related to age, hormonal action, and reproductive stages (gestation, lactation) (14). Despite the early embryogenic differentiation of the liver, many of its metabolic functions are incompletely developed at birth. The fetal liver has a lower capacity for gluconeogenesis, glycogen storage, bile acid metabolism, detoxification, and elimination processes, making it more susceptible to toxins and transplacental and postnatal infections that may not have consequences in adults (16). Although ALT predominates in the liver, AST is also present in cardiac muscle, skeletal muscle, liver, and kidneys. The age-dependent activities in both enzymes appear to correlate well with tissue growth (9).

The enzymatic activity of LDH evaluated in the present study was higher in puppies from 4-8 wk old. However, a study conducted in 2013 did not observe age-related differences in LDH enzyme activity (10). In puppies, LDH is at the highest levels during suckling, likely because of the enhanced use of lactose as a glucose precursor during the neonatal period. Adult values are obtained soon postweaning (1). Early increases in LDH probably reflect muscle trauma associated with delivery (17). Nevertheless, the information about this enzyme is limited and few studies include it in their research.

Regarding ALP enzyme activity, it remained elevated from week 4 to week 52, compared to adults, but it began to decrease since week 25. Numerous reports indicate that in young animals there is an increase in ALP compared to adults (5,6,10,11). This is the result of the activity of the ALP bone isoenzyme, which is increased in serum during bone development and growth (1,28). While the GGT did not have a significant effect of age, postweaning GGT values slightly decreased to below adult values; then, it increased to reach adult values at approximately 6 months of age. Moreover, in puppies postweaning, serum GGT activity is believed to reflect the enzyme derived from other tissues, mainly in liver (1). On the other hand, due to the high levels of

ALP and GGT contained in colostrum, the evaluation of these enzymes in serum or plasma provide some important information about the transfer status of passive immunity in puppies, when used as a marker of the adequate ingestion of colostrum. However, these differences are short-lived and are determined within the first 2 wk of age (1,6).

The serum concentration of total proteins and albumin showed an evident effect on age. These results agree with previous research (4,5,9,14). Age-associated increase of total protein and albumin are attributed to normal immune stimulation, what results in an elevated globulin fraction and albumin production derived from improved liver function and intestinal absorption (1). The low serum concentration of albumin in dogs younger than 24 wk of age derives from the increased demand for albumin during this phase of intense growth (14). In the same way, the concentration of globulins showed a tendency to increase as age advances. This result agrees with previous researches (5,15). Puppies are born hypogammaglobulinemic with only a small amount of IgG and IgM and no detectable IgA in serum at birth. Therefore, total protein concentration in puppies is initially low, particularly precolostral intake. Protein concentration, then, steadily increases during the first year of life, and it stabilizes from 1 year old onward. On the other hand, during the first 6 weeks of life, a decrease in globulins concentration occurs due to the degradation of maternal antibodies; meanwhile, an increase in albumin concentration occurs because of normal liver function development (1).

Regarding cholesterol and triglycerides, no effect of age was observed. While a study from 2008 did not find an effect of age on triglycerides levels, the cholesterol concentration was higher in dogs from < 52 weeks of age (18). Other researches from 2012 and 2015 found no significant difference in the cholesterol concentration of puppies and adults (4,5). However, in a longitudinal study conducted in 2016 in Labrador and miniature schnauzer dogs, the researches obtained blood samples from 8-52 weeks of age, and they found higher concentrations of cholesterol at week 26 and triglycerides at week 20 and 36 (9). The neonatal liver has a lower capacity to synthesize triglycerides and cholesterol, so neonates depend on the lipids absorbed through the diet; that is why breastfeeding is an important source of lipids in newborns (6).

On the other hand, the glucose concentration was higher in puppies from 4 to 24 weeks of age compared to adults. Some research developed in dogs from different ages showed that the concentration of glucose had a decrease with growth (1,5,10). A study found blood glucose values to be similar to the adults on day 4, but significantly higher at all the other time points with a peak on week four (8). However, in another study there was not a significant difference in the glucose concentration of puppies compared to adult dogs (4). Glucose in the blood is closely regulated and normally maintained by three major mechanisms: intestinal absorption, hepatic production, and, to a lesser degree, renal production. In young animals, there is a reduced potential for gluconeogenesis and glycogenolysis (1). Furthermore, the inability of puppies to recover quickly from hypoglycemia or hyperglycemia can be attributed to their insensitivity to endogenous insulin, and the low response of counter-regulatory hormones (epinephrine and cortisol) (17). Although glucose regulation improves with age, puppies up to 16 wk of age should be considered as predisposed to hypoglycemia when they are anorexic or dehydrated (6).

Blood urea and creatinine are the most commonly assessed indices of glomerular filtration in mammals. As these components are freely filtered by the glomerulus, any reduction in the glomerular filtration rate (GFR) results in increases in the concentration of these analytes in serum. However, both urea and creatinine are affected by other body systems, which may affect their rate of production and their rates of excretion. Age

variations have been noted for both parameters (1). The urea concentration evaluated in the present study increased as age advanced. These results agree with previous studies (4,8,9). One previous study found a lower concentration of urea in puppies compared to adult values although it did not find statistically significant differences (5). This increase may derive from a higher rate of protein metabolism since puppies are in the growth stage (14). Some proposals explain the low urea concentration in puppies such as the increment in protein synthesis by the influence of growth hormone, or the increase in metabolic status with the glomerular filtration rate (GFR) (1). The GFR increases with age postnatally. Glomerular capillary surface area and pore density increase between the first and sixth weeks after birth. Studies suggest that GFR and renal blood flow increase up to 11 weeks of age in the puppy before reaching adult levels (19). Serum creatinine levels also tended to increase as age advanced. Our results are consistent with some studies (4,5,10,20). Moreover, the lower creatinine in young animals, in relation with adults, correlates with smaller body size and decreased muscle mass (1). It is important to have an age-specific reference interval to identify an increase in creatinine concentration in puppies.

On the other hand, body temperature and heart rate had an effect of age. In the second and third weeks of life, before the puppy is actively crawling and walking consistently, normal body temperature oscillates from 37.0° to 38.2° C (12). However, one previous research mentions that weaned and adolescents have the same normal body temperature as adult animals (11). The normal heart rate in puppies is about 220 beats per minute (bpm) during the first week of life (12). On the other hand, the respiratory rate had no age-related effect. Respiratory rate is the same as that in adults by 4 weeks of age (11).

Regarding sex, no significant effect was observed. These results coincide with some studies from 2013 and 2016 where researchers did not find an effect of sex on alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, alkaline phosphatase, total protein, albumin, cholesterol, triglycerides, glucose, creatinine, and urea (9,10). In other research from 2008, they did not find an effect of sex on triglycerides, yet the cholesterol concentration was higher in females than in males (18). Even though some researchers report statistically significant differences according to sex, in some biochemical parameters such as cholesterol and aminotransferases (13). However, our research shows that for the tested variables in this study, there is no need to establish sex-specific RI, but it is important to consider its effect when interpreting results.

Breed had a significant effect only on serum creatinine concentration. Some research informs that German Shepherd puppies up to 8 weeks of age have higher creatinine values than other breeds. In addition, adult Greyhounds have been shown to have higher creatinine concentration because of their increased muscle mass compared to other breeds of dogs (1). Other research, on the contrary, found no significant effect of breed on creatinine concentration (13). On the other hand, the rest of the evaluated variables did not show any breed. One previous study reported an effect of breed on the total protein, albumin, AST, ALT, ALP, and triglycerides while the concentration of cholesterol and urea did not show any significant effect related to breed (9). Another study reports a breed effect evident in the concentration of urea, total proteins, albumin, glucose, and the enzymatic activity of ALT (13). Regarding the physiological constants statistically evaluated in this study, we did not observe any effect of sex and breed. Nevertheless, it is necessary to have a greater number of studies that provide information about the effects related to age, sex, and breed on these variables.

Finally, age, sex, and breed did not have any significant interaction on the evaluated variables. However, one study reported a significant interaction of breed and age on biochemical tests of young Labrador retrievers and

miniature schnauzers between weeks 8 and 52 of age (9). Another study from 2014 observed a significant interaction between breed and sex for plasma cholesterol concentration and ALT enzymatic activity (13).

Conclusions

Our study showed that ALT, total protein, albumin, globulin, urea, creatinine, and body temperature levels were lower in puppies from 4-8 weeks of age compared to adult dogs. In most of these variables from 25 weeks of age, their values were similar to those of adult dogs; these variables began to increase until reaching adult values except for total proteins, where their concentration began to increase from 9 weeks of age and remained stable after 52 weeks of age. On the other contrary, the enzymatic activity of ALP, LDH, the glucose concentration, and the heart rate were higher in puppies from 4-24 wk of age than in adult dogs. Moreover, the values of ALP, glucose, and heart rate began to decrease from week 25 while the enzymatic activity of LDH decreased from week 9.

On the other hand, the results of AST, GGT, cholesterol, triglycerides, and the respiratory rate did not show an effect of age while only the creatinine concentration showed an evident effect of breed. In small breeds, the serum creatinine levels were lower.

Thus, it is evident that some biochemical components are influenced by age. For this reason, our research offers specific reference intervals that can help the veterinary clinician to accurately interpret the biochemical results obtained from puppies. It is also important to consider sex and breed when interpreting the results; therefore, we suggest more research like this to keep the information up-to-date, including the evaluation of other biochemical, vital, and hematological variables.

Methods

Study area and population

This study was carried out in compliance with the provisions established in the Ethics Regulations for the Use of Animals in Teaching and Research at the Autonomous University of Aguascalientes (CEADI-UAA) Code: DI-PL-NO-37 (21). A non-experimental transverse design was used (22). We selected 197 healthy dogs of different sex and breed classified by age: group I (4-8 wk), group II (9-24 wk), group III (25-52 wk), and group IV (>52 wk). A variety of small and large breed dogs as well as pure and mixed breeds were represented in each group. The inclusion criteria based on history and physical examination that assess weight, body temperature, hydration status, behavior, sensory organs, heart rate, respiratory rate, abdomen, skin, musculoskeletal system, and reproductive tract (11,12) to identify clinically healthy dogs with optimal conditions of vaccination, deworming, and fasting. On the other hand, we excluded dogs with a) records of disease in the last month, b) administration of any medication or recent vaccination, c) clinical signs of apparent disease, d) females in estrus, gestation or lactation period, e) over 6 years of age, and f) without fasting (2,6,9). All dogs selected for this study were privately owned and reported to be free of disease by the owner and were normal on physical examination. All owners signed an informed consent form.

Blood collection

Samples of 5 ml of blood were collected using venipuncture jugular with tubes vacuum and coagulation activator (BD Vacutainer; BD Medical Technology, Franklin Lakes, NJ). Separation of serum performed via centrifugation (Ultra-8 digital, LW Scientific, Lawrenceville, GA) from 5 to 10 minutes at 2,500 RPM (846 RCF) when coagulation occurred at room temperature, within the first hour after blood collection. The serum was transferred to a 1.5 ml tube (Eppendorf, Hamburg, Germany). Biochemical analysis performed the same day; when it was not possible to perform, the serum was frozen at -20°C (-4°F) and protected from light until its analysis the next day, avoiding several cycles of freezing and thawing (23,24).

Biochemical analysis of blood samples

We analyzed the blood samples obtained in the Laboratory of Diagnostic Pathology using the spectrophotometer BTS-350 (BioSystems, Barcelona, Spain) and reagents (Pointe Scientific, Canton, MI). The analysis was performed according to the manufacturer's instructions and utilizing standardized methods (Table 1).

Monitoring recommendations for clinical chemistry are addressed in the general American Society for Veterinary Clinical Pathology (ASVCP) quality assurance and laboratory standards guidelines (25,26). We evaluated the functioning of the analytical instrument with calibration curves for each blood analyte in GraphPad Prism version 6 (GraphPad Software, La Jolla, CA). In addition, the spectrophotometer BTS-350 (BioSystems, Barcelona, Spain) has an internal quality control system based on the Levey-Jennings chart; this analysis allowed us to apply the Westgard rules. Before analyzing each determination, the analytical methods were calibrated according to the manufacturer's instructions with the help of a chemical calibrator and commercial controls (levels I and II) (Pointe Scientific, Canton, MI) (25). Lipemic, hemolyzed, and icteric blood samples were excluded from the biochemical analysis (2). The biochemical analysis measured the enzymatic activity of AST, ALT, LDH, GGT, ALP, and the concentration of cholesterol, triglycerides, total proteins, albumin, globulin, glucose, urea, and creatinine (Table 1). Globulins were determined by subtracting albumin concentration from total protein concentration (5,27).

Statistical analysis

Statistical analysis was performed with Minitab 17 (Minitab Statistical Software, State College, PA); $p < 0.05$ was considered significant. We evaluated the distribution of the variables by examining the histograms and using a goodness of fit test (Anderson–Darling) (28). In order to determine if the variance of two or more groups are statistically different, we used test of equality of variances with multiple comparisons and Levene's methods. These methods are valid in non-normal distributions while in normal distributions Bartlett test is used. All tests of variances used a confidence level of 95%(29).

Statistical analyses employed the Analysis of Variance (ANOVA) General Linear Model (GLM), which allows the comparison of multiple factors at two or more levels ($p < 0.05$). Biochemical analytes, body temperature, heart rate, and respiratory rate represent response variables (dependent) while age, sex, and breed represent factors (independent variables). The interaction between factors was also evaluated. When the data did not meet the normality assumptions and homoscedasticity, we performed a Box-Cox transformation using Minitab's optimal lambda (λ) with a confidence level of 95%. Subsequently, a multiple comparison method (Tukey) was performed with a confidence level of 95% (30).

Reference intervals were calculated using the software Reference Value Advisor (RefValAdvV.2.1, <http://www.biostat.envt.fr/reference-value-advisor/>) based on the recommendations of International Federation of Clinical Chemistry (IFCC) and the Clinical and Laboratory Standards Institute (CLSI) (31). This software detected the outliers with Tukey and Dixon tests, showing the distribution (dot plot and histograms) and QQ plot for visual inspection (2,31). Out-of-bounds values were examined and excluded from the dataset when a blood sample contributed to more than one observation since it could indicate subclinical disease (5).

Abbreviations

ALP: alkaline phosphatase; ALT: alanine aminotransferase; ANOVA: Analysis of variance AST: aspartate aminotransferase; bpm: beats per minute and breaths per minute; ASVCP: American Society for Veterinary Clinical Pathology; BCG: bromocresol green dye binding; CI: confidence interval; G: Gaussian; GFR: glomerular filtration rate; GGT: gamma-glutamyl transferase; GLDH: glutamate dehydrogenase; GLUPAC: Ly-glutamyl-3-carboxy-4-nitroanilide; IFCC: International Federation of Clinical Chemistry; IQR: inter quartile range; LDH: lactate dehydrogenase; LL: low limit; NG = not Gaussian; p-NPP: p-Nitrofenil phosphate; SD = standard deviation; UL = upper limit.

Declarations

Ethics approval and consent to participate

This study was carried out in compliance with the provisions established in the Ethics Regulations for the Use of Animals in Teaching and Research at the Autonomous University of Aguascalientes (CEADI-UAA) Code: DI-PL-NO-37. The owner's written consent was obtained before the dog was enrolled in the study.

Consent for publication

Not applicable.

Availability of data and materials

The dataset analyzed in the current study is available from the corresponding author on a reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

ALMN and TQT conceived this study, participated in its design, upon performing and coordination, and helped to draft the manuscript. SLS, ROM, AGVF, LMM and MCLL made significant contributions to conception, design, and the analysis of the results. ALMN also carried out laboratory analysis and statistical analysis. All authors have read and approved the final version of this manuscript.

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Tables

Table 1. Analytical methods for blood biochemistry using spectrophotometry.

Analyte	Method/Principle	Reaction type	Reading mode	Type of reaction	Wavelength
AST	Modified IFCC	Kinetic	-	Decreasing	340 nm
ALT	Modified IFCC	Kinetic	-	Decreasing	340 nm
LDH	Modified IFCC	Kinetic	-	Increasing	340 nm
ALP	p-NPP	Kinetic	-	Increasing	405 nm
GGT	GLUPAC	Kinetic	-	Increasing	405 nm
Total protein	Biuret	Endpoint	Monochromatic	Increasing	540 nm
Albumin	BCG	Endpoint	Monochromatic	Increasing	630 nm
Cholesterol	Enzymatic	Endpoint	Monochromatic	Increasing	500 nm
Triglycerides	Enzymatic	Endpoint	Monochromatic	Increasing	500 nm
Glucose	Enzymatic	Endpoint	Monochromatic	Increasing	500 nm
Urea	Urease, GLDH	Fixed time	-	Decreasing	340 nm
Creatinine	Jaffe, acid picric	Kinetic	-	Increasing	510 nm

ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; BCG = bromocresol green dye binding; GGT = gamma-glutamyl transferase; GLDH = glutamate dehydrogenase; GLUPAC = Lγ-glutamyl-3-carboxy-4-nitroanilide; IFCC = International Federation of Clinical Chemistry; LDH = lactate dehydrogenase; p-NPP = p-Nitrofenil phosphate.

Table 2. Reference intervals by age.

Variables	Distribution	(I) 4 - 8 wk (n = 35)		(II) 9 - 24 wk (n = 48)		(III) 25 - 52 wk (n = 21)		(IV) > 52 wk (n = 71)	
		Mean (SD)	LL (90% CI)	Mean (SD)	LL (90% CI)	Mean (SD)	LL (90% CI)	Mean (SD)	LL (90% CI)
		Median (IQR)	UL (90% CI)	Median (IQR)	UL (90% CI)	Median (IQR)	UL (90% CI)	Median (IQR)	UL (90% CI)
AST (U/L)	G	39 (9.9) ^a 40 (16)	18 (13- 23) 60 (55- 64)	40 (8.6) ^a 40 (11)	21 (21- 27) 57 (54- 57)	38 (10.2) ^a 38 (15.5)	16 (11- 23) 60 (53- 66)	38 (9.1) ^a 37 (16)	21 (20- 24) 54 (52- 54)
ALT (U/L)	NG	26 (8.8) ^a 26 (11)	8 (4- 12) 45 (40- 49)	32 (9.1) ^b 31 (15.7)	15 (14- 20) 54 (46- 55)	37 (7.6) ^{b, c} 36 (14.5)	25 (24- 27) 60 (52- 80)	44 (12.6) ^c 41 (19)	22 (21- 26) 70 (65- 70)
LDH (U/L)	NG	244 (164.6) ^a 166 (228)	43 (43- 48) 695 (526- 861)	73 (32.4) ^b 74 (55.5)	25 (24- 34) 139 (131- 140)	63 (32) ^b 62 (51.5)	6 (6- 13) 131 (112- 150)	67 (42) ^b 56 (39)	9 (8- 17) 185 (143- 187)
ALP (U/L)	NG	215 (71.6) ^a 186 (96)	101 (91- 115) 394 (332- 456)	193 (39.2) ^a 187.5 (58)	111 (110- 123) 281 (253- 286)	85 (36.7) ^b 90 (72)	18 (10- 36) 179 (150- 202)	52 (23.3) ^c 47 (36)	15 (15- 23) 112 (92- 113)
GGT (U/L)	NG	5 (1.7) ^a 5 (2)	2 (2- 3) 10 (9- 12)	5 (1.3) ^a 5 (2)	2 (2- 3) 8 (7- 8)	5 (1.9) ^a 6 (2)	2 (0.3- 3) 9 (8- 10)	6 (2.1) ^a 6 (4)	2 (2- 3) 10 (9- 10)
Total protein (g/dL)	NG	4.6 (0.6) ^a 4.4 (1.2)	3.5 (3.3- 3.7) 6.4 (5.8- 6.9)	5.1 (0.7) ^b 5.1 (1.1)	4.0 (4.0- 4.0) 7.1 (6.3- 7.3)	5.7 (0.8) ^b 5.5 (1.4)	4.3 (4.1- 4.6) 7.9 (6.9- 8.8)	6.2 (0.9) ^c 6.3 (1.3)	4.4 (4.2- 4.8) 8.2 (7.7- 8.2)
Albumin (g/dL)	NG	2.5 (0.3) ^a	1.9 (1.9- 2.0)	2.7 (0.3) ^b	2.1 (2.1- 2.2)	3.1 (0.3) ^{b, c}	2.2 (2.0- 2.5)	3.1 (0.4) ^c	2.4 (2.3- 2.5)

		2.4 (0.4)	3.5 (3.2-3.9)	2.7 (0.6)	3.7 (3.4-3.8)	3.1 (0.5)	3.9 (3.7-4.2)	3.1 (0.6)	4.2 (3.9-4.4)
Globulins (g/dL)	NG	2.1 (0.6) ^a 2.1 (0.7)	1.0 (0.9-1.2) 3.5 (3.1-4.0)	2.3 (0.6) ^a 2.3 (0.8)	1.0 (1.0-1.4) 3.9 (3.6-3.9)	2.6 (0.6) ^{a, b} 2.7 (1.1)	1.3 (0.9-1.8) 4.0 (3.6-4.4)	3.0 (0.7) ^b 3.0 (1.3)	1.7 (1.6-1.9) 4.3 (4.1-4.6)
Cholesterol (mg/dL)	G	169 (86.3) ^a 124 (144)	62 (60-68) 418 (309-493)	199 (60.9) ^a 199 (105)	92 (88-113) 317 (306-318)	185 (41.1) ^a 179 (54)	97 (73-123) 272 (245-299)	186 (51) ^a 185 (55)	80 (76-90) 288 (283-291)
Triglycerides (mg/dL)	G	37 (18) ^a 28 (22)	14 (13-16) 91 (65-114)	41 (13) ^a 40 (18)	20 (20-23) 76 (67-76)	43 (14) ^a 43 (16)	(12 (4-21) 73 (64-82)	45 (14) ^a 44 (18)	14 (14-25) 79 (66-80)
Glucose (mg/dL)	G	89 (21) ^a 89 (26)	47 (38-56) 132 (122-142)	90 (18) ^a 92 (23)	49 (47-61) 135 (121-137)	81 (21) ^{a, b} 78 (33)	43 (37-53) 131 (114-148)	77 (21) ^b 74 (32)	36 (31-50) 122 (114-128)
Urea (mg/dL)	NG	23 (7) ^a 20 (12)	12 (11-13) 42 (35-48)	27 (8.7) ^b 27 (10)	9 (8-14) 47 (43-48)	36 (9.5) ^{b, c} 35 (15)	15 (10-21) 56 (50-62)	34 (10) ^c 34 (15)	14 (11-18) 55 (49-56)
Creatinine (mg/dL)	NG	0.45 (0.09) ^a 0.45 (0.15)	0.3 (0.3-0.3) 0.7 (0.6-0.8)	0.59 (0.16) ^b 0.54 (0.26)	0.4 (0.4-0.4) 1.0 (0.9-1.1)	1.00 (0.30) ^c 0.99 (0.50)	0.3 (0.0-0.5) 1.6 (1.4-1.7)	1.03 (0.25) ^c 1.05 (0.43)	0.6 (0.5-0.6) 1.6 (1.4-1.7)
Temperature (°C)	NG	37.9 (0.5) ^a 38.0 (0.7)	36.7 (36.3-37) 39 (38.8-39.2)	38.5 (0.6) ^b 38.5 (0.8)	37.3 (37.3-37.6) 39.6 (39.4-39.6)	38.7 (0.4) ^{b, c} 38.7 (0.7)	37.7 (37.5-38) 39.6 (39.3-39.7)	38.8 (0.4) ^c 38.9 (0.7)	37.9 (37.9-38.1) 39.7 (39.5-39.7)
Heart rate (bpm)	NG	155 (28.4) ^a	86 (64-107)	145 (33) ^a	85 (84-92)	129 (26) ^{a, b}	74 (58-90)	125 (27) ^b	80 (80-89)

		160 (44)	207 (196- 216)	140 (38.5)	200 (200- 200)	132 (28)	185 (168- 202)	120 (40)	180 (180- 180)
Breathing frequency (bpm)	NG	30 (6.6) ^a	15 (13- 19)	33 (5.9) ^a	22 (22- 24)	34 (6.6) ^a	20 (16- 24)	32 (7) ^a 32 (16)	15 (15- 20)
		30 (12)	44 (41- 46)	31 (8)	44 (40- 44)	36 (12)	44 (44- 44)		44 (44- 46)

CI = confidence interval; °C = degrees Celsius G = Gaussian; IQR = inter quartile range; LL = low limit; NG = not Gaussian; SD = standard deviation; UL = upper limit. Groups that do not share a letter are significantly different (a, b, c) $p < 0.05$

Figures

Figure 1

Enzymatic blood activity at different stages of the dog's life. Comparison of mean is seen (red line) of (A) ALT, (B) LDH, and (C) ALP between groups of different age clinically healthy dogs: group I (n = 35, 4-8 wk), group II (n = 48, 9-24 wk), group III (n = 21, 25-52 wk), and group IV (n = 71, >52 wk). Horizontal blue dotted lines represent the RI for adult dogs with a 90% CI (see Table 2). Means that do not share a letter are significantly different ($p < 0.05$).

Figure 2

The serum concentration of some analytes at different stages of the dog's life. Comparison of mean is seen (red line) of (A) protein total, (B) albumin, (C) globulins, and (D) glucose between groups of different age clinically healthy dogs: group I (n = 35, 4-8 wk), group II (n = 48, 9-24 wk), group III (n = 21, 25-52 wk), and group IV (n = 71, >52 wk). Horizontal blue dotted lines represent the RI for adult dogs with a 90% CI (see Table 2). Means that do not share a letter are significantly different ($p < 0.05$).

Figure 3

The serum concentration of some analytes at different stages of the dog's life. Comparison of mean is seen (red line) of (A) urea, and (B) creatinine between groups of different age clinically healthy dogs: group I (n = 35, 4-8 wk), group II (n = 48, 9-24 wk), group III (n = 21, 25-52 wk), and group IV (n = 71, >52 wk). Horizontal blue dotted lines represent the RI for adult dogs with a 90% CI (see Table 2 to age-specific reference intervals). Means that do not share a letter are significantly different ($p < 0.05$).

Figure 4

Values of the physiological constants at different stages of the dog's life. Comparison of mean is seen (red line) of (A) body temperature, and (B) heart ratio between groups of different age clinically healthy dogs: group I (n = 35, 4-8 wk), group II (n = 48, 9-24 wk), group III (n = 21, 25-52 wk), and group IV (n = 71, >52 wk). Horizontal blue dotted lines represent the RI for adult dogs with a 90% CI (see Table 2 to age-specific reference intervals). Means that do not share a letter are significantly different ($p < 0.05$).