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EFFECTS OF AGE ON THE HAEMATOLOGICAL AND BIOCHEMICAL PARAMETERS IN ROMANIAN BUFFALO

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ABSTRACT

The aim of this study was to investigate the changes of hematic constants related to age in Romanian buffalo, in order to establish references values based on age of the animals. A number of 45 Romanian buffalo animals, clinically healthy, were divided into 3 groups (n=15/group) according to their age, as follows: Group T_1 = un-weaned buffalo calves of 1 to 3 months of age, Group T_2 = young buffalo heifers of 6 to 12 months, and Group T_3 = buffalo adult cows of 3 to 5 years of age. Different hematological parameters and biochemical parameters were investigated in the three groups. Generally, a significant effect of age on both hematological and biochemical indices studied was observed ($P<0.05$). The concentration of total cholesterol and the concentration of inorganic phosphorous were not influenced by age of the animals ($P>0.05$). Significant positive correlations were found between total protein and age of the animals (P=0.0019), while significant negative correlations were observed between hemoglobin (P=0.0227), white blood cells count (P=0.0352), lymphocytes (P=0.0002), platelet (P=0.0022), total cholesterol (P=0.1201), calcium (P=0.0077) and age of the animals. The results of the current pilot study could prove useful in establishing a baseline and health alarm thresholds for hematic parameters in water-buffalo, based on age of the animals and physiological status.

Keywords: *Bubalus bubalis*, buffaloes, animal physiology, biochemical profile, haematological profile, Romanian buffalo

INTRODUCTION

The water buffalo (*Bubalus bubalis*) are valuable animals that contribute to the economies of many countries, representing an important genetic resource (Li *et al*., 2019; Khan *et al*., 2022). According to the FAO database (2019), there are more than 204 million buffaloes in the world. Knowledge of haematological and biochemical profile reference values at different ages is extremely important for assessment and monitoring of health and nutritional status in buffaloes (Islam *et al*., 2019). Therefore, exact validated reference intervals are needed for appropriate interpretation of blood analyses results and comparison. The adoption of

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metabolic profile tests helps in the early detection of some biochemical and haematological disorders and unbalanced feeding diets, which further allows the surveillance of large herds of animals and rapid changes of nutritional management measures (Couch *et al*., 2017). It was proven that feeding level, age, sex, breed, seasonal variation and physiological status of buffaloes (Devi and Kumar, 2012) have a significant influence on the cellular hemodynamic (Patel *et al*., 2016). Reference values of different blood variables have been established for adult buffalo cows up to date in some breeds (Mohan *et al*., 2009; Khan *et al*., 2018), while for un-weaned calves and replacement heifers there is a significant lack of data available. The values of different blood variables in un-weaned buffalo calves are changing significantly with age. As a consequence, the aim of this study was to investigate the changes of hematic constants related to age in Romanian buffalo, in order to establish references values based on age of the animals.

MATERIALS AND METHODS

Ethical statement

The research activities were performed in accordance with the Romanian Law no. 43/2014 and the European Union's Directive 2010/63/EU regarding handling and protection of animals used for scientific purposes.

Animals, diets and protocol design

Forty-five Romanian buffaloes, that were clinically healthy and had no previous disease history (n=15/group), were screened for their metabolic profile, (using haematological and biochemical indicators. Based on the experimental design, the groups were as follows: Group T_1 - un-weaned buffalo calves of 1 to 3 months of age, Group T_2 - young buffalo heifers of 6 to 12 months, and Group T_3 - adult (lactating/non-lactating) buffalo cows of 3 to 5 years of age. The study was carried out at the Research and Development Station for Buffalo in Sercaia, Romania.

The un-weaned calves and young buffaloes' heifers were group-housed (7 to 8 heads/pen), while the adult buffalo cows were housed free-ranging, under natural pasture conditions, with 1 livestock unit per hectare. The feeding system for the un-weaned buffalo calves consisted out of a daily diet of 6 kg of raw milk (2 portions/day), 1.2 kg of concentrates (17% crude protein), and ad libitum access to natural marsh hay. While for the young buffalo heifers the daily diet consisted of 2 kg concentrates (18 % crude protein) and *ad libitum* access to natural marsh hay, and for the buffalo adult cows their diet consisted of *ad libitum* access to natural pasture, mineral blocks and permanent watering availability.

Blood sampling

Blood samples were collected aseptically from the jugular vein of each animal, 2 to 4 h after the morning feeding. The amount of blood was of 2 ml for each sample in vacutainer tubes with dipotassium-ethylenediaminetetraacetic acid (K_2-EDTA) as an anticoagulant, for hematological analysis. After harvesting, the samples were chilled to +4^{o}C and were analyzed after maximum 6 h from collection of samples. For the biochemical examination of blood serum, the samples (9 ml) were collected in dedicated vacutainer tubes under sterile conditions. The blood samples were kept at room temperature (22 to 24^{o}C) until the serum was expressed. The serum was separated by centrifugation at 3000 rpm for 15 minutes and stored in aliquots at 20^{o}C until further analysis. All blood samples were collected during the summer season.

Haematological and biochemical analysis

All haematological parameters: red blood cells count (RBC), haemoglobin concentration (HGB), hematocrit percentage (HTC), red blood cells distribution width (RDW), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count (PLT), mean platelets volume (MPV), platelets distribution width (PDW), total white blood cells count (WBC), lymphocytes percentage (LY), monocytes percentage (MO), neutrophil percentage (NE) were determined using automated hematology analyzer Abacus Junior Vet 5 (Diatron, Hungary). While the blood biochemical related parameters: total protein, total cholesterol, aspartate transaminase (AST), alanine aminotransferase (ALT), alkaline phosphatase (PAL), calcium (Ca), inorganic phosphorus (P) and magnesium (Mg) were determined using the StarDust MC 15 semiautomated biochemical analyzer (DiaSys Diagnostics Systems Gmbh, Germany) and DiaSys reagents kits, following the producers instructions. The analyses were carried out in the Animal Physiology Laboratory of the Research and Development Institute for Bovine Balotesti, Romania.

Statistical approaches

Statistical analyses were carried out using descriptive statistical tool (Statistica 64-StatSoft and Microsoft Excel). Results were expressed as means ± standard deviations. One-Way ANOVA was used to test the influence of the age on the blood parameters studied and the Tukey's test was applied for post hoc comparisons. The relationship between blood parameters and age was computed using multiple regression analysis. Significance was declared when $P \leq 0.05$ and trends when $P \leq 0.10$.

RESULTS AND DISCUSSIONS

The means and standard deviations of RBC, HGB, HTC, RDW, MCV, MCH and MCHC are presented in Table 1. A significant effect between the three different age groups for RBC ($F_{(2;42)}=15.46$; P=0.0000), HGB ($F_{(2;42)}=7.47$; P=0.0016), HTC ($F_{(2;42)}=1.31$; P=0.2788), and RDW ($F_{(2;42)}=10.63$; P=0.0000) was found. The mean value for red blood cell count (RBC) was $9.72 \pm 1.91 \times 10^6$/µl in the first 3 months of life (T_1), of $9.76 \pm 2.15 \times 10^6$/µl at 6 to 12 months of life (T_2) and decreased to $6.72 \pm 0.76 \times 10^6$/µl in adult buffalo cows (T_3).

The HGB concentration ranged from 12.68±2.46 g/dl at 3 months of life (T_1) to 14.42±1.37 g/dl at 6 to 12 months of life (T_2), and 11.90±1.46 g/dl at 3 to 5 years of life (T_3). The HCT percentage ranged from 35.69±8.26% at 3 months of life (T_1), to 39.52±8.94% at 6 to 12 months of life (T_2) and 39.67±5.06% at adult age (T_3), respectively. RDW decreased with age of the animals, from 24.23±3.70% in un-weaned calves (T_1) to 22.16±4.07% in 6 to 12 month of age heifers (T_2), to 18.88±0.71% in adult buffalo cows (T_3). The obtained mean values for MCV, MCH, and, MCHC (Table 2) were different between the three studied age groups (MCV: $F_{(2;42)}=51.02$; P=0.0001; MCH: $F_{(2;42)}=13.88$; P=0.0000; MCHC:$F_{(2;42)}=13.90$; P=0.0000).

Similarly, in a comparative study on healthy and ill buffalo calves, Sharma *et al.* (2019) reported values of $6.50 \pm 0.05 \times 10^6$/µl for RBC, 13.23±0.24 g/dl for HGB and 40±0.20 % for HTC in healthy animals. The slight increase in HTC levels in our study could be attributed to the hemoconcentration and dehydration of plasma, considering the season for our sample collection and that under severe heat stress, the buffaloes try to maintain their body temperature through evaporative water losses. The increase might also be attributed to the fact that an animal requires more oxygen under stressful conditions such as handling for blood sampling, and as a consequence, hemoglobin concentration rise (Haque *et al*., 2013).

The MCV and RDW ratio is an important tool for anemia differential diagnosis. The increase of RDW is the first early sign of iron deficiency, which is associated with anemia in ruminants, while the MCV is lower than RDW (Brun Hansen, 2006; Brockus, 2011). In healthy calves, MCV is decreased, resulting in wider RDW levels (Roland *et al*., 2014).

Inconsistent results to current data were found in Colombian buffalo by Jaramillo *et al.* (2022), which reported lower RDW levels in buffalo calves and replacement heifers, and higher levels for adult buffalo cows. In un-weaned calves, HGB, MCH and MCHC tend to decrease during the first month of life and then start to increase during the 3rd months of life (Mohri *et al*., 2007), authors concluding that this coincides with the replacement of foetal HGB with adult HGB.

Opporist to our results, Siddhapara *et al.* (2022) reported higher MCV and MCH values in healthy Surti buffalo calves, while the MCHC values in their study were significantly lower.

The PLT ($F_{(2;42)}$=33.23, P=0000), MPV ($F_{(2;42)}$=33.15, P=0.0000), and PDW ($F_{(2;42)}$=9.84, P=0.0003) levels showed in Table 2, presented significant differences between age groups. The means values recorded for PLT and MPV were situated in physiological limits (233.06±127.27x10^3/µl to 556.93±150.40x10^3/µl for PLT; 6.5±0.49 fl to 9.25±0.87 fl for MPV), while the means values for PDW were higher than physiological limits (34.07±2.79% to 39.63±2.21%).

In accordance with our results, Dhillon *et al.* (2020) reported a mean value of 233±67.3x10^3/µl for PLT, in adult healthy buffalo-cows.

Conversely, Khan *et al.* (2018) reported significantly higher values for PLT in buffalo calves, of 638±33.1x10^3/µl at 3 months of age, and of 568.93±45.8x10^3/µl at 6 months of age.

The WBC count ($F_{(2;42)}$=4.21, P=0.0214), LY percentage ($F_{(2;42)}$=8.78, P=0.0006), MO percentage ($F_{(2;42)}$=5.42, P=0.0080), and NE percentage ($F_{(2;42)}$=9.25, P=0.0004), showed statistical differences between Age groups (Table 3). The count of WBC was highest at 3 months of age (14.58±4.97x10^3/µl) and decreased to adult age (11.14±1.59x10^3/µl), with all three means being higher than the reference interval for the species. The LY, MO and NE mean values were situated in physiological limits for the un-weaned calves, whereas the MO for T_2 group and the NE for T_3 group where higher than reference limits. Kuralkar *et al.* (2018) reported values of 9.91±0.29x10^3/µl for WBC, 70.49±0.92 for LY, 2.77±0.16% for MO and 25.91±0.85% for NE in young Purnathadi buffalo, and values of 12.64±0.38x10^3/µl for WBC, 65.98±0.97 for LY, 2.39±0.15% for MO and 30.33±0.92% for NE in adult Purnathadi buffalo which are conversely with our obtained results.

The obtained mean values for serum biochemical indicators are presented in Table 4. The concentration of TPr ($F_{(2;42)}$=8.58, P = 0.0006), AST ($F_{(2;42)}$=21.56, P = 0.0000), ALT ($F_{(2;42)}$=47.11, P=0.0000), ALP ($F_{(2;42)}$=810.48, P=0.0000), Ca ($F_{(2;42)}$=3.60, P=0.0357) and Mg ($F_{(2;42)}$=13.84, P=0.0000) showed statistical differences between age groups, unlike concentrations of

TCho ($F_{(2;42)}$=2.69, P=0.0792) and inorganic P ($F_{(2;42)}$=0.10, P=0.8973). The levels of AST, ALT, and ALP increased with age group of the animals, being significantly higher in replacement heifers and adult water buffalo-cows. The tissue growth, immunity maturing and complete functionality of the organs (e.g. rumen), could be responsible for the progressive increase of the serum ALT and AST levels registered in the current study, this hypothesis being in accordance with results published by Campanile *et al.* (1993).

It was previously shown that ruminants neonates have different biochemical parameters compared to adults, this being related to bone growth, immature immune systems, and distinct metabolism of nutrients (Borjesson, *et al*., 2009), data which supports our current findings in water-buffalo. The un-weaned buffalo calves presented significantly higher levels, compared to adult animals. Current findings are in accordance with those of Husakova *et al.* (2014), which reported that due to bone growth during the first year of life, neonates typically have higher phosphorous and ALP concentrations, when compared to adult animals. Moreover, it was shown that free-ranging and confined animals often have dramatically different diets, which reflect in serum levels of essential minerals such as calcium and phosphorus (Miller *et al*., 2010).

In growing un-weaned calves and replacement heifers, feeding (feeding effects become more apparent after the 1^{st} month of life, when calves start to consume dry feed), age and rearing system influence the values of different blood indicators (Klinkon and Jezek, 2012), findings which supports our data. However, identification of these factors mechanisms and their interactions is necesary for the correct interpretation of the blood parameters data. Multiple regression of blood parameters and age are presented in Table 5. Age group of the animals had a statistically significant negative correlation for HGB (r=-0.3389, P=0.0227), WBC (r=-0.3146, P=0.0352), LY (r=-0.5263, P=0.0002), PLT (r=-0.4444, P=0.0022), TCho (r=-0.3466, P=0.1201) and Ca (r=-0.3922, P=0.0077). Current results show that with increasing of age, the level of these parameters decrease. Very low R square values were obtained for P (0.0014), MO (0.0182), HCT (0.024), WBC (0.0989), TCho (0.1201), Ca (0.1538) and PLT (0.1975). The level of serum TPr was found to have a statisticaly significant and positive correlation with age (r=0.4489, R^2=0.2015, P=0.0019), the level of this parameter increases with increasing of age. Multiple regression analysis of blood parameters and age confirms that age can be an indicator used in the evaluation of the blood parameters in buffaloes.

CONCLUSIONS

A significant effect of age on haematological and biochemical indices was observed. These results could help in future with the design of nutritional requirements in water buffalo, especially in un-weaned calves and replacement heifers, to avoid technopathies such as rickets and stunned growth. Age of the animal had a significant positive correlation with total protein and a significant negative correlation with hemoglobin, white blood cells, lymphocytes, platelet, total cholesterol, calcium. The information from the current pilot study on hematic constants could prove helpful in evaluation of the feeding management practices and animal health. Current data builds up to a growing body of literature available, with the ultimate purpose as to establish

Table 1. Mean values (±SD) for RBC, HGB, HTC, RDW, MCV, MCH and MCHC in water-buffalo at different ages.

Blood indicators	Groups			Reference values
	T_1 (1-3 months)	T_2 (6-12 months)	T_3 (3-5 years)	
	$\bar{x}$±SD	$\bar{x}$±SD	$\bar{x}$±SD	
RBC, 10^6/µl	9.72±1.91	9.76±2.15	6.72±0.76	5-8
Pairwise comparisons, p	T_1 vs. T_2: P = 0.9977; T_1 vs. T_3: P = 0.0000; T_2 vs. T_3: P = 0.0000			
HGB, g/dl	12.68±2.46	14.42±1.37	11.9±1.46	9-11
Pairwise comparisons, p	T_1 vs. T_2: P = 0.0326; T_1 vs. T_3: P = 0.4894; T_2 vs. T_3: P = 0.0014			
HTC, %	35.69±8.26	39.52±8.94	39.67±5.06	32-38
Pairwise comparisons, p	T_1 vs. T_2: P = 0.3608; T_1 vs. T_3: P = 0.0334; T_2 vs. T_3: P = 0.9985			
RDW, %	24.23±3.70	22.16±4.07	18.88±0.71	16-20
Pairwise comparisons, p	T_1 vs. T_2: P = 0.1937; T_1 vs. T_3: P = 0.0001; T_2 vs. T_3: P = 0.0202			
MCV, fl	36.73±4.30	44.06±8.81	59.00±4.17	40-60
Pairwise comparisons, p	T_1 vs. T_2: P = 0.0060; T_1 vs. T_3: P = 0.0000; T_2 vs. T_3: P = 0.0000			
MCH, pg	13.31±2.75	15.25±2.58	17.70±1.19	11-17
Pairwise comparisons, p	T_1 vs. T_2: P = 0.0637; T_1vs.T_3: P = 0.0000; T_2vs.T_3: P = 0.0145			
MCHC, g/dl	36.12±5.06	34.80±2.05	30.03±1.82	30-36
Pairwise comparisons, p	T_1 vs. T_2: P = 0.5244; T_1 vs. T_3: P = 0.0000; T_2 vs. T_3: P = 0.0000			

RBC = red blood cells count; HGB = hemoglobin concentration; HTC = hematocrit percentage; RDW = red blood cells distribution width; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin, MCHC=mean corpuscular hemoglobin concentration.

Table 2. Mean values (±SD) for PLT, MPV and PDW in water-buffalo at different ages.

Blood indicators	Groups			Reference values
	T_1 (1-3 months)	T_2 (6-12 months)	T_3 (3-5years)	
	x̄±SD	x̄±SD	x̄±SD	
PLT, 10^3/µl	556.93±150.40	233.06±127.27	236.42±89.56	100-800
Pairwise comparisons, p	T_1 vs. T_2: P = 0.0000; T_1 vs. T_3: P = 0.0000; T_2 vs. T_3: P = 0.9968			
MPV, fl	6.5±0.49	8.14±1.26	9.25±0.87	7-13
Pairwise comparisons, p	T_1 vs. T_2: P = 0.0000; T_1 vs. T_3: P = 0.0000; T_2 vs. T_3: P = 0.0062			
PDW, %	34.07±2.79	36.44±4.78	39.63±2.21	8-16
Pairwise comparisons, p	T_1 vs. T_2: P = 0.1564; T_1 vs. T_3: P = 0.0002; T_2 vs. T_3: P = 0.0387			

PLT=platelet count, MPV=mean platelets volume, PDW=platelets distribution width.

Table 3. Mean values (±SD) for WBC, LY, MO and NE in water-buffalo at different ages.

Blood indicators	Groups			Reference values
	T_1 (1-3 months)	T_2 (6-12 months)	T_3 (3-5years)	
	x̄±SD	x̄±SD	x̄±SD	
WBC, 10^3/µl	14.58±4.97	12.12±2.51	11.14±1.59	6.5-9.5
Pairwise comparisons, p	T_1 vs. T_2: P = 0.1216; T_1 vs. T_3: P = 0.0196; T_2 vs. T_3: P = 0.7015			
LY, %	61.39±9.77	56.07±14.37	45.17±6.93	45-61
Pairwise comparisons, p	T_1 vs. T_2: P = 0.3770; T_1 vs. T_3: P = 0.0005; T_2 vs. T_3: P = 0.0226			
MO, %	2.87±2.21	5.79±2.94	3.30±2.64	0-4
Pairwise comparisons, p	T_1 vs. T_2: P = 0.0100; T_1 vs. T_3: P = 0.8965; T_2 vs. T_3: P = 0.0332			
NE, %	35.69±11.16	38.12±13.70	51.54±6.46	15-41
Pairwise comparisons, p	T_1 vs. T_2: P = 0.8136; T_1 vs. T_3: P = 0.0007; T_2 vs. T_3: P = 0.0043			

WBC = total white blood cells count; LY = lymphocytes percentage; MO = monocytes percentage; NE = neutrophil percentage.

Table 4. Mean values (±SD) for some biochemical indicators in water-buffalo at different ages.

Blood indicators	Groups			Reference values
	T_1 (1-3 months)	T_2 (6-12 months)	T_3 (3-5years)	
	x̄±SD	x̄±SD	x̄±SD	
TPr, mg/dl	7.64±0.43	6.90±1.00	8.20±0.98	6.8-8.4
Pairwise comparisons, p	T_1 vs. T_2: P=0.0549; T_1 vs. T_3: P=0.1790; T_2 vs. T_3: P=0.0004			
TCho, mg/dl	157.4±37.69	156.26±50.50	128.4±23.08	124-224
Pairwise comparisons, p	T_1 vs. T_2: P=0.9964; T_1 vs. T_3: P=0.1130; T_2 vs. T_3: P=0.1324			
AST, U/L	28.4±5.31	44.13±8.48	44.23±8.48	21-24
Pairwise comparisons, p	T_1 vs. T_2: P=0.0000; T_1 vs. T_3: P=0.0000; T_2 vs. T_3: P=0.0000			
ALT, U/L	25.2±4.22	53.2±15.63	73.2±17.10	<60
Pairwise comparisons, p	T_1 vs. T_2: P=0.0000; T_1 vs. T_3: P=0.0000; T_2 vs. T_3: P=0.0006			
ALP, U/L	29.60±4.82	40.20±7.88	192.60±19.40	10-36
Pairwise comparisons, p	T_1 vs. T_2: P=0.0611; T_1 vs. T_3: P=0.0000; T_2 vs. T_3: P=0.0000			
Ca, mg/dl	10.02±1.08	9.86±1.15	9.02±1.05	8-11
Pairwise comparisons, p	T_1 vs. T_2: P=0.9227; T_1 vs. T_3: P=0.0430; T_2 vs. T_3: P=0.0995			
P, mg/dl	5.20±0.85	5.06±0.69	5.14±0.91	4.62-7
Pairwise comparisons, p	T_1 vs. T_2: P=0.8882; T_1 vs. T_3: P=0.9784; T_2 vs. T_3: P=0.9619			
Mg, mg/dl	2.40±0.31	2.69±0.45	3.26±0.56	2.1-2.8
Pairwise comparisons, p	T_1 vs. T_2: P=0.1820; T_1 vs. T_3: P=0.0000; T_2 vs. T_3: P=0.0043			

AST = aspartate transaminase; ALT = alanine aminotransferase; ALP = alkaline phosphatase; Ca = calcium, P = inorganic phosphorous; Mg = magnesium.

Table 5. Multiple regression analysis of blood parameters and age.

Blood indicators	Age		
	Multiple R	**R square**	**P**
RBC	-0.6455	0.4166	ns
HGB	-0.3389	0.1148	0.0227
HCT	0.1580	0.024	ns
MCV	0.8183	0.6696	ns
MCH	0.5838	0.3408	ns
MCHC	-0.6357	0.4041	ns
RDW	-0.5668	0.3213	ns
WBC	-0.3146	0.0989	0.0352
LY	-0.5263	0.2770	0.0002
MO	-0.1352	0.0182	ns
NE	0.5513	0.3040	ns
PLT	-0.4444	0.1975	0.0022
MPV	0.7059	0.4982	ns
PDW	0.5713	0.3264	ns
TPr	0.4489	0.2015	0.0019
TCho	-0.3466	0.1201	0.0186
AST	0.8799	0.7743	ns
ALT	0.7483	0.5599	ns
PAL	0.9198	0.8460	ns
Ca	-0.3922	0.1538	0.0077
P	0.0376	0.0014	ns
Mg	0.5852	0.3425	ns

P = significance levels ($P<0.05$); ns = not significant.

a baseline and reference values for hematological and biochemical blood indicators in water- buffalo.

For future studies, we intend to include a greater number of animals, with a more diverse physiological categories (lactating/non-lactating, pregnant/non/pregnant), while also looking at the health status of each animal (e.g., metabolic diseases incidence and hematic profiles), considering a greater number of environmental and genetic related factors.

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