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Blood cell count and morphology, and vitamin B12 concentration in pre- and post-weaned calves

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Abstract: Haematological indicators may resent physiological variation by age. Vitamin B12 promotes haematopoiesis. The aims of this study were: 1) to compare the values of the haematological variables and the concentration of vitamin B12 in pre- or post-weaned veal calves and 2) to identify the possible association between the values of the haematological variables and the concentration of B12 in the blood of veal calves. Blood was collected on the same day from 31 pre-weaned and 31 weaned calves of the Limousine breed from the same farm. The complete blood count, including the blood cell morphology evaluation, was performed and the serum B12, total protein and albumin concentrations were determined. The serum concentration of vitamin B12, the haematocrit (HCT), the haemoglobin concentration (HGB), the platelet count and the lymphocyte count were significantly higher in the weaned calves. A very strong positive correlation was found between the concentration of the vitamin B12 and HCT and HGB before weaning, while these correlations were moderately positive following weaning and in the total population tested as well. The observed variation in the blood cell count and morphology, such as poikilocytosis and the presence of macrocytes and hypersegmented neutrophils, along with the age of the animal seem to be related to the vitamin B12 concentration.

Keywords: age; cobalamin; haematological variables; macrocytes; poikilocytosis; weaning

Age has been reported as being a source of physiological variation in the concentration of vitamin B12 (Andres and Federici 2009; Gonzalez-Gross et al. 2012) as well as in the haematological profile of humans and cattle (Mohri et al. 2007; Panousis et al. 2018). In calves, a decrease in the packed cell volume (PCV) and haemoglobin (HGB) concentration was observed from birth to the end of the first

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month of age followed by an increase of these values with the age (Mohri et al. 2007). However, it still remains uncertain whether these alterations in the erythrocyte and haemoglobin profile in calves are associated with the expected variation in the blood serum vitamin B12 concentration.

Vitamin B12, also known as cobalamin, is a water-soluble vitamin that plays a significant role in principle functions of the organism, including haematopoiesis (McDowell 2000). Along with folate, vitamin B12 also plays a significant role as a maturation factor contributing to the DNA synthesis; this is extremely important especially for the erythrocyte precursors, because of their high rate of multiplication. Therefore, a reduced vitamin B12 concentration results in maturation asynchrony between the nucleus and the cytoplasm which cause a condition known as megaloblastic anaemia and is reflected in the blood smear by the presence of normochromic macrocytes (Koury and Ponka 2004; Federici et al. 2007).

Vitamin B12 is synthesised only by some bacteria, blue-green algae and yeast, but not by animal cells (Martens et al. 2002). Given that this vitamin is not present in plants and in the feedstuff of plant origin, the main source of B12 in ruminants is the rumen flora (Watanabe and Bito 2018). In common practice, the rumen synthesis of vitamin B12 gradually increases and becomes adequate for the requirement for vitamin B12 in ruminants by 6 to 8 weeks of age, depending on the cobalt intake (Stangl et al. 2000). However, cobalt supplementation needs in ruminants are highly variable because of the influence of the season and the soil in the concentration of the cobalt in the herbage as well as the bioavailability of cobalt provided by different sources (Tiffany et al. 2003). On the other hand, newborn calves rely on the milk content to meet their B12 requirements for this vitamin (Watanabe and Bito 2018). Therefore, despite the lack of such evidence in the available literature, it could be hypothesised that newborn calves might have different blood serum vitamin B12 concentrations compared to weaned calves.

The aims of this study were: 1) to compare the values of the haematological variables and the vitamin B12 concentration in pre-weaned and post-weaned veal calves and 2) to identify any possible association between the variation in the morphology and blood cell count and the B12 concentration in the blood of veal calves.

MATERIAL AND METHODS

All the procedures were performed according to the ethical standards in the Helsinki Declaration of 1975, as revised in 2000, as well as the national law and after receiving approval from our Institutional Animal Use Ethics Committee (No. 79/ 24-9-2019).

Prior to the onset of the study, the minimum required sample size was calculated using the General Linear Multivariate Model with the Wilks Likelihood Ratio procedure with the GLIMMPSE software v3.0.0 (http://glimmpse.samplesizeshop.org/). The desired power was set at 0.8, the type I error rate was set at 0.05, and the desired detectable difference for the serum B12 concentration between the groups was set at 50 with a standard deviation of 50. The mean scale and variability factors were set at 1. The results of the analysis revealed that a minimum total sample size of 34 animals (17 per group) was required (power = 0.807).

The study was conducted in a semi-extensive beef cattle herd. According to the farm routine, the animals grazed from early March to late October and were kept indoors the rest of the months. Calvings were scheduled to occur during the housing season. Blood samples were collected from 31 calves before weaning (aged 2 to 3 weeks) and from 31 weaned calves (aged 15 to 16 weeks) of the Limousine breed that were reared at the same farm. Before weaning, all the calves were housed with their mothers in a free stall barn, and were nursing freely from their dams and had *ad libitum* access to water, to a commercial calf starter and to good quality alfalfa hay for *ad libitum* consumption. According to the farm schedule, the calves were weaned at the age of 12 weeks. After weaning and during the housing period, they are housed in groups of six calves in concrete floor pens with straw bedding. They consumed a ration consisting of 50% straw and 50% of a commercial concentrate diet on dry matter basis *ad libitum*. The concentrate feed contained 0.4 mg of cobalt carbonate per kg.

Two days before the blood sampling, faecal samples were obtained from ten calves aged between 15–16 weeks for a parasitological examination; the animals were found free from internal parasites. On the day of blood sampling, the calves were thoroughly clinically examined and inspected for the presence of ectoparasites (ticks). All 62 calves included in the study were judged as being healthy,

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without having diarrhoea or any signs of respiratory disease and without any visible ectoparasite infection.

The blood of each animal was collected by jugular venepuncture with a 21-gauge needle into two vacutainers (Venoject; Terumo Europe, Leuven, Belgium), one with no anticoagulant for the serum and one containing ethylenediaminetetraacetic acid (EDTA) for the whole blood. The EDTA containing vacutainer tubes were mixed gently immediately after collection to avoid coagulation as well as every time before analysing.

The samples were transferred to the diagnostic laboratory placed in a cooler with icepacks avoiding direct contact with the tubes. The haematological tests were performed within 2 h after collection. The samples were analysed using an IDEXX VetAutoread® Hematology Analyzer (IDEXX Laboratories, Westbrook, ME, USA), according to the specific methodology proposed by the manufacturer for this animal species. Blood smears were prepared to determine the white blood cell (WBC) differential count and blood cell morphology, were left to dry at room temperature and then Giemsa stained. The morphology of the erythrocytes was evaluated and scored as previously described (Harvey 2012).

After centrifugation at 1 600 *g* for 10 min and serum separation, the serum samples were frozen at –20 °C and analysed within two days after collection based on previously demonstrated stability specifications under similar storage conditions (Drammeh et al. 2008).

The serum B12 concentrations were determined using an automated chemiluminescence immunoassay (Immulite 1000; Siemens Healthcare Diagnostics, Deerfield, USA). The serum total protein (STP) concentration was measured refractometrically with a temperature compensated refractometer (Reichert TS Meter refractometer, Model 1310400A; Reichert Scientific Instruments Buffalo, NY, USA) as previously described and validated (Katsoulos et al. 2017). The serum albumin (SALB) concentration was determined using the bromocresol green method, in an automatic biochemical analyser (Vitalab Flexor E; Vital Scientific N.V, Spankeren, The Netherlands) using a commercially available diagnostic kit (ThermoFisher Scientific Inc. VA, Waltham, USA).

The data analysis was performed using the statistical software IBM SPSS Statistics v25 (IBM, USA). The normality of the data was tested with the Kolmogorov-Smirnov test. Independent samples *t*-test were used in order to detect statistically significant differences between the pre-weaned and post-weaned calves for each parameter for normally distributed values, whereas the Mann Whitney *U* test was used for the non-normally distributed parameters.

Also, based on the normality results, the linear association between B12 and the other evaluated parameters was tested with the Pearson and Spearman rho correlation coefficient in the cases of the normal and non-normal distribution of the data, respectively, and the strength of the correlation is interpreted as previously described (Bland and Altman 1986; Akoglu 2018).

A significance level of $P \leq 0.05$ was used in all the comparisons.

RESULTS

The data analysis revealed that the serum concentration of vitamin B12, the haematocrit (HCT), the haemoglobin concentration (HGB), the platelet count and the lymphocyte count were significantly higher in the post-weaned compared to the pre-weaned calves (Table 1).

In contrast, the WBC count, neutrophil count (both segmented and band neutrophils) as well as the eosinophil and monocyte counts were significantly lower (Table 1).

A very strong positive correlation was found between the concentration of vitamin B12 and the HCT and HGB before weaning while these correlations were moderately positive post-weaning and in the total population tested as well. This result could suggest that the possible enhancement of the dietary B12 intake might increase the haematocrit in newborn calves, but this needs further investigation. Regarding the WBC and the lymphocyte count, a moderate positive correlation was observed post-weaning and in the total population, respectively. Concerning all the other blood cell populations, either there was no correlation with the vitamin B12 concentration or this correlation was fair/poor (Table 2).

The correlation between the vitamin B12 and STP concentration was fair before weaning. On the other hand, the correlation between the vitamin B12 and SALB concentration was fair post-weaning.

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Table 1. Mean ± standard error of the mean of the vitamin B12 and haematological variables in the pre-weaned (age 2 to 3 weeks) and weaned calves (aged 15 to 16 weeks)

Parameter	Pre-weaned (mean ± SE)	Post-weaned (mean ± SE)	Laboratory upper and lower limit
B12 (pmol/l)	123.68 ± 3.07[a]	304.64 ± 21.38[b]	100–500
STP (g/l)	58.29 ± 1.77[a]	60.90 ± 2.40[a]	58–80
SALB (g/l)	28.4 ± 0.95[a]	25.82 ± 0.55[a]	25–36
HCT (%)	28.23 ± 0.77[a]	33.43 ± 0.95[b]	28–38
HGB (mmol/l)	5.82 ± 0.16[a]	6.9 ± 0.2[b]	5.59–8.69
PLT (/µl)	625 727.96 ± 61 152.57[a]	845 191.48 ± 48 699.12[b]	300 000–800 000
WBC (/µl)	10 754.84 ± 682.47[a]	8 000.00 ± 385.68[b]	4 000–12 000
NEU (/µl)	5 912.35 ± 470.54[a]	1 968.55 ± 117.05[b]	1 600–7 100
BAND (/µl)	157.65 ± 18.82[a]	77.48 ± 12.38[b]	0–200
LYMPH (/µl)	3 995.45 ± 164.03[a]	5 572.35 ± 286.39[b]	2 500–6 500
MONO (/µl)	397.06 ± 38.63[a]	233.80 ± 23.02[b]	100–700
EOS (/µl)	246.96 ± 29.68[a]	103.00 ± 10.44[b]	100–250
BASO (/µl)	45.35 ± 10.86[a]	44.81 ± 9.26[a]	0–200

B12 = vitamin B12; BAND = band neutrophil count; BASO = basophil count; EOS = eosinophil count; HCT = haematocrit; HGB = haemoglobin; LYMPH = lymphocyte count; MONO = monocyte count; NEU = neutrophil count; PLT = platelet count; SALB = serum albumin; STP = serum total protein; WBC = white blood cell count

[a,b]Figures with a different superscript are indicative of a statistically significant difference ($P < 0.05$)

Table 2. Correlation coefficients (R) between the vitamin B12 and haematological variables in all the tested calves, in the pre-weaning and post-weaning calves

Parameter	Vitamin B12		
	total	pre-weaning	post-weaning
STP	0.16	0.44*	0.10
SALB	–0.05	0.33	0.45*
HCT	0.65**	0.81**	0.55**
HGB	0.65**	0.81**	0.55**
PLT	0.07	0.12	–0.49**
WBC	–0.21	0.02	0.55**
NEU	–0.67**	0.02	0.49**
BAND	–0.41**	–0.27	0.07
LYMPH	0.55**	0.10	0.39*
MONO	–0.41**	–0.10	–0.19
EOS	–0.53**	–0.13	–0.07
BASO	0.17	0.47**	0.13

BAND = band neutrophil count; BASO = basophil count; EOS = eosinophil count; HCT = haematocrit; HGB = haemoglobin; LYMPH = lymphocyte count; MONO = monocyte count; NEU = neutrophil count; PLT = platelet count; SALB = serum albumin; STP = serum total protein; WBC = white blood cell count

*$P < 0.05$, **$P < 0.01$

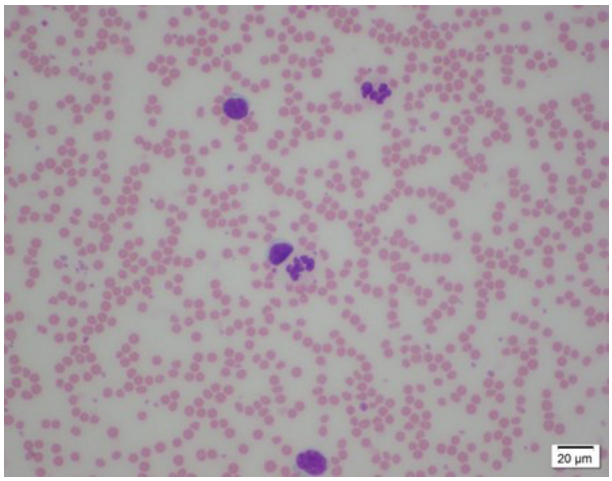

Figure 1. Blood smear from calf with mild poikilocytosis and the presence of moderate numbers of hypersegmented neutrophils mixed with a lower number of lymphocytes (modified Wright-Giemsa). Objective × 40

Regarding the blood cell morphology, the microscopic evaluation of the blood smears revealed the presence of poikilocytosis in eight pre-weaned calves. The score of the poikilocytosis was from 1+ up to 3+.

Macrocytes were also observed in three, as well as hypersegmented neutrophils in two out of the eight pre-weaned calves (Figure 1).

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DISCUSSION

The study was conducted in a beef cattle herd with the housing period lasting for only 4 months per year. We selected to take the blood samples at the end of the housing period in order to assure that: 1) the cows were adequately fed during the last 2–3 months of the dry and at the lactating period and 2) the weaned calves had no access to a pasture from birth until sampling.

These limitations were considered necessary in order to avoid biases that could arise from the possible improper nutrition during grazing.

Thus, the authors decided to sample, on the same day, an equal number of animals of two different populations, nursing and weaned, instead of repeatedly sampling the same animals 12–14 weeks apart.

The observed difference between the vitamin B12 blood serum concentrations pre- and post-weaning could be attributed to the different sources of vitamin supply among these ages; weaned calves have a fully developed and functional reticulorumen and the rumen flora synthetises the required amount of vitamin B12, whereas newborn calves rely on the ingested milk. Evidence in the literature suggests that the milk's vitamin B12 concentration was notably low in both ovine and bovine milk from the first days after lambing or calving, respectively (Anthony et al. 1951; Gruner et al. 2004). Moreover, in cases where a milk replacer was used in calves, the vitamin B12 intake was suboptimal to maintain the B12 vitamin stores (Grace et al. 2014). In contrast to the findings of the present study, as well as the studies mentioned above, no differences between the before and after weaning period have been reported in lambs. The authors attributed these results to the augmented liver stores of vitamin B12 that could have buffered the effect of the vitamin intake with the milk and reticulorumen synthesis on the vitamin B12 concentration, pre- and post-weaning, respectively (Knowles and Grace 2017).

The differences in the erythrocyte variables in the present study before and after weaning are in accordance with previous reports. Compared to the hypoxia provoked elevation of HCT in neonatal calves (Steinhardt et al. 1993), there is a decrease in the erythrocytes containing foetal haemoglobin during the first weeks after calving, due to their replacement by erythrocytes containing adult haemoglobin (Scheidegger 1973). Moreover, the increase in the HCT and HGB concentration in the present study after weaning has been previously reported and changes in the haematological variables with the age are considered to be influenced by the feeding and rearing systems (Reece and Hotchkiss 1987).

Regarding the WBC count, similar to our findings, an elevated upper reference limit in neonatal calves compared to cattles has been reported (Panousis et al. 2018). The simultaneous increase in the lymphocyte count and decrease in the neutrophil count resulting in a decrease in the neutrophil/lymphocyte ratio, in contrast with other species such as dogs, cats, and horses, where neutrophils are the dominant WBC subsets, has also been well documented (Wood and Quiroz-Rocha 2010; Panousis et al. 2018). The decrease in the eosinophil and monocyte count with the age is in accordance with previous reports (Wood and Quiroz-Rocha 2010). Moreover, the platelet count has been documented to be higher in neonatal calves up to 9 days of age when compared to adults (Panousis et al. 2018). An increase in the platelet count was also observed in our study, although a comparison was not made between neonatal and adult animals, but in calves of two different ages.

The protein and albumin concentrations were determined in this study due to a reported association between the total protein and globulin concentration with the vitamin B12 concentration in lambs (Fisher 1991) and humans (Bowen et al. 2006).

Poikilocytosis can be observed in clinically healthy calves (Harvey 2012) and in calves up to 2 months old, probably because of abnormalities in the haemoglobin composition and a protein in the membrane of the erythrocytes (Okabe et al. 1996). In this study, except from the poikilocytes, enlarged RBCs were observed in the blood smear of the calves with lower PCV values. The simultaneous presence of poikilocytosis, macrocytosis and hypersegmented neutrophils could be the result of the low concentration of vitamin B12, as can be observed in humans (Green 2017). Even though, there is a strong indication that low concentrations of vitamin B12 could account for the differences in the morphology of the erythrocytes and neutrophils, B12 supplementation studies are required to further support this observation.

Nevertheless, the blood cell morphology has been first reported in the present study including the presence of poikilocytosis, macrocytosis and hypersegmented neutrophils only in pre-weaned calves with a lower vitamin B12 concentration and not in the weaned calves.

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Conflict of interest

The authors declare no conflict of interest.

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