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Brief communication

Full title: **Biochemical variables from Holstein-Friesian calves older than one week are comparable to those obtained from adult animals of stable metabolic status on the same farm.**

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Short title: **Biochemical analytes in calves' first 3 months**

1 **Abstract**

2 Background: Age-related changes in blood variables have previously been reported for
3 cattle. A few studies have investigated differences in biochemistry analytes over time
4 using repeat measurements.

5 Objectives: The purpose of the study was to investigate the variation in select variables
6 during the first 3 months of life of Holstein-Friesian calves using repeat sampling.

7 Methods: Twenty healthy calves were sampled on the day of birth (0) and at 6, 13, 21,
8 30, 60 and 90 days of life. Analyzed variables included concentration of glucose, total
9 protein, albumin, urea and creatinine, and activity of AST, GGT, CK. Reference
10 intervals (RI) were obtained from 40 adult cows using specific computer software. The
11 variation in each analyte throughout the study period and differences relative to the RI
12 in adult cattle were assessed.

13 Results: With the exception of glucose, the studied variables were affected by the calf's
14 age. However, for most of the analytes studied, the values obtained from calves from
15 one week of age onwards remained within the RI determined in adult cattle. During the
16 calves' first week of life, some variables outside the RI of adult cattle were due to the
17 changes associated with birth and colostrum intake.

18 Conclusions: With the exception of AST and GGT activity, RI for standard biochemistry
19 variables determined for adult cattle are likely to be valid for calves older than one
20 week. However, special consideration is necessary for calves within their first week of
21 life.

22

23 **Keywords:** Analyte, dairy calf, metabolic profile, reference interval, serum parameter

24 Age-related changes in blood variables have been reported in several species¹,
25 including cattle.²⁻⁴ Reference intervals (RI) aim to provide a range of values originating
26 from the natural variation found in healthy individuals that can be compared to the
27 values obtained from an individual for diagnostic purposes. However, in cattle medicine
28 most of the published RIs are based on adult animals^{1,5,6}; which may not be adequate
29 for the assessment of the values obtained from neonatal and young calves. So far,
30 limited information is available regarding RIs in calves at different ages.⁷ Furthermore,
31 only a few reports have investigated the variation in blood analytes during the first 3
32 months of life via repeated sampling and analysis. In addition, these studies were
33 either only focused on hematology variables⁴, or when analyzing biochemistry
34 constituents, employed beef breeds^{2,3}; which might not be directly translatable to dairy
35 calves because of different management practices. Therefore, the aim of this study
36 was to investigate the variation in selected serum variables commonly used in bovine
37 panels during the first 3 months of life of Holstein-Friesian calves, and compare them to
38 the RIs determined for adult animals on the same farm.

39

40 All experiments followed the Spanish standards for the protection of animals
41 used for experimental and other scientific purpose, and all animal use was previously
42 approved by the Bioethical Committee of the University of Santiago de Compostela.

43 Serum samples from 20 Holstein-Friesian calves (11 males, 9 females) from the
44 same farm, recruited for another previous experiment⁸ were used. Blood was collected
45 by jugular venipuncture, into uncoated tubes (BD Vacutainer; Becton, Dickinson and
46 Company, Plymouth, UK). The first sampling was 2 hours after the ingestion of
47 colostrum on the day of birth (day 0), followed by samplings on days 6 (range: ± 1), 13
48 (± 1), 21 (± 1), 29 (± 1), 60 (± 2) and 90 (± 2). Calves received 3 L of first-milked colostrum
49 within the first 3 h of life through a nipple bottle. Thereafter they were fed milk replacer
50 until the age of 6 weeks, when they were subsequently weaned. Grass hay, pelleted
51 concentrate and water were offered ad libitum from birth. From the same farm, serum

52 samples were obtained by coccygeal vessel puncture into 10 mL uncoated vacuum
53 tubes from 40 adult cows during the 4th-5th month of pregnancy, when the metabolic
54 effects of pregnancy and lactation are expected to be minimal.⁹ Collected tubes were
55 rapidly cooled on crushed ice and then centrifuged at 2000 × g for 20 min, and serum
56 was harvested and stored at -20 °C until analysis within 3 months after collection. Both
57 calves and adult cows were managed according to the conventional intensive farming
58 system in Spain; a more detailed description of the animals' nutrition and husbandry
59 practices is available from previous studies.^{8,9}

60 Serum was analyzed for concentrations of glucose, total serum protein (TP),
61 albumin, urea and creatinine, and activities of AST, GGT and CK. Globulin
62 concentration was assessed by subtracting the albumin from TP concentration, and the
63 albumin:globulin ratio (A/G) was also calculated. The analytical methods employed and
64 the inter- and intra-assay coefficients of variation (CVs) are described in Table 1. Intra-
65 assay precision was determined on 20 replications of 3 samples, whereas inter-assay
66 precision was determined for 2 samples during 4 consecutive days with 15 replications
67 on each day, for 2 samples.

68 Measurements were performed using commercially available kits on a
69 biochemistry autoanalyzer (CST-240, DIRUI Industrial Co., Ltd, Changchun, China)
70 calibrated against a commercial multicalibrator (Biocal, RAL Técnica para el laboratorio
71 S.A., Spain) and with 2 commercial human serum controls (Gernorm and Gernpath,
72 RAL Técnica para el laboratorio S.A., Spain) for a 2-level control. The results from
73 adult cows were initially assessed with histograms, obvious outliers were detected
74 visually or identified as values falling outside 3 × the interquartile range and excluded
75 from the analysis. The normal distribution of the data was confirmed using the Shapiro–
76 Wilk test. Subsequently, RIs were calculated using the Reference Value Advisor
77 software¹⁰.

78 Generalized Linear Mixed Models (GLMM) with repeated measures were built
79 with calf as the experimental unit for the outcome variables glucose, AST, GGT, CK,

80 TP, albumin, globulins, A/G, urea and creatinine with calf's age as the fixed effect and
81 the sex of the calf as a random effect. The variance-covariance structure with time was
82 assumed to have a first-order autoregressive correlation (AR-1). Bonferroni corrections
83 were included for post-hoc analysis. All statistical analyses were performed using
84 SPSS v.20 for Windows (IBM) and the criterion for statistical significance was $P < .05$.

85

86 A significant effect of the age of the calf was found for all the studied analytes
87 with the exception of glucose concentration (Table 2), further supporting the hypothesis
88 that age-related changes in the concentration of serum analytes exist. However, the
89 validity of using adult RIs for calves is also of practical importance. Several authors
90 have published RIs for cattle using adult animals.^{1,5,6} However, due to differences in
91 analytical methods and geographically distinct populations of animals, RIs should be
92 established by each laboratory; therefore, intervals from 40 healthy lactating cows with
93 a stable metabolism from the same herd as the calves were obtained using specific
94 software¹⁰ (Table 3).

95 Glycemia in calves was always close to the upper limit of the reference value,
96 and at the age of 1 and 2 weeks it was slightly above this value (Figure 1).
97 Nevertheless, this was an expected outcome, as a consequence of the differences in
98 the glucose metabolism documented in adult and suckling ruminants, with the former
99 having to synthesize their own glucose while the latter are able to absorb it directly
100 from the milk. However, glycemia is under tight endocrine control¹¹, which may explain
101 the absence of a significant age effect on serum glucose concentration.

102 AST activity was below the RI in adult animals until the second and third months
103 of age (Figure 2). In ruminants the liver is responsible for the synthesis of blood
104 glucose, as this is not directly absorbed from the diet. However, unweaned calves,
105 essentially monogastric animals, take up glucose and other substances from the milk
106 or milk replacer. Because AST is a key enzyme in gluconeogenesis⁵ and these animals
107 relied to a lower extent on this metabolic pathway to maintain glycemia. variations in

108 AST activity could be attributable to a lower liver activity. It can be hypothesized that
109 the AST activity of these animals was lower while they were receiving milk replacer
110 than once they were weaned or in comparison to adult animals, GGT activity
111 decreased progressively from birth to 3 weeks of age with significantly higher activity
112 than in the adult group (Figure 3). GGT is produced in the mammary gland¹² and
113 therefore is also found in colostrum; so in calves that have ingested colostrum, serum
114 GGT activity is usually higher than in adult animals.¹³ Indeed it is commonly used as an
115 indicator of colostrum intake when assessing failure in passive immune transfer
116 (FPT).¹⁴

117 Newborn calves depend upon colostrum intake for the acquisition of
118 immunoglobulins, as they are agammaglobulinemic at birth. However, the maximal
119 concentration of these is usually not achieved until 12-24 h after birth¹⁵, and this may
120 explain why the globulin concentration was below the adult animals' RI 2 h after
121 colostrum ingestion (Figure 4). Likewise, this may also explain the lower TP
122 concentration in calves at the same time point (Figure 5). Indeed, TP quantification by
123 refractometry is a common method used to assess FPT in the field, as it is directly
124 correlated with the concentration of immunoglobulins.¹⁴ In addition, as albumin
125 remained within the RI for adult cattle throughout the study period, being stable from
126 the first week onwards (Figure 6), the changes in the A/G ratio (Figure 7) are a
127 reflection of the calves' own globulin production. Urea also remained within the adult
128 cattle RI during the first 3 months of life.

129 CK is commonly used as an indicator of muscle injury.⁵ In this study, the CK
130 activity in calves was higher than in adult cattle at day 0, remaining stable within the RI
131 thereafter (Figure 8). This increase was probably due to the tissue damage associated
132 with the passage of the calf through the birth canal. Similarly, a high creatinine
133 concentration was found at day 0 (Figure 9), as previously reported for calves³ and
134 infants¹⁶, among other species. In people it has been proven that these high levels are
135 not a consequence of immature renal function, but rather are derived from (i)

136 maternofetal transfer of creatinine through the placenta and (ii) endogenous
137 degradation of creatine and phosphocreatine in muscle.¹⁶ However, given the
138 particularities of the ruminant placenta, maternofetal transfer via the placenta cannot be
139 assumed to occur in cattle without further investigation, whereas muscle damage at
140 calving may play a role in this temporarily high concentration of creatinine, since a
141 correlation between muscle mass and creatinine exists¹.

142

143 Our results show that with the exception of AST and GGT activities, the RI for adult
144 cattle can be used to assess the clinical biochemistry results of calves older than one
145 week. At younger ages, the changes associated with birth and colostrum intake must
146 be taken into consideration, as they might cause a physiological increase or decrease
147 in the concentration of some variables.

148

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158

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160

competition with, the subject matter or materials discussed in this article.

161

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214 the measurement of catalytic activity concentrations of enzymes at 37 degrees C. Part
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216 kinase. *Clin Chem Lab Med* 2002;40:635-642.

218 **Table 1.** Intra- and interassay precision of biochemical variables determined in calves between birth and 90 days of age

Analyte	Method	Precision [†]														
		Intra-assay									Inter-assay					
		Sample 1			Sample 2			Sample 3			Sample 1			Sample 2		
Mean	SD	CV (%)	Mean	SD	CV (%)	Mean	SD	CV (%)	Mean	SD	CV (%)	Mean	SD	CV (%)		
Glucose (mg/dL)	Oxidase [†]	54.6	1.17	2.15	77.3	1.55	2.01	88.7	1.84	2.07	55.2	1.69	3.06	77.9	2.32	2.98
Creatinine (mg/dL)	Jaffé [†]	1.52	0.025	1.63	0.98	0.020	2.07	2.30	0.040	1.73	1.67	0.069	4.12	0.98	0.038	3.86
Urea (mg/dL)	Urease [†]	14.7	0.47	3.21	21.6	0.64	2.97	40.8	1.20	2.94	15.1	0.71	4.68	23.1	1.16	5.04
AST (U/L)	Schumann, et al. ^{17†}	38.2	0.42	1.11	122.1	1.61	1.32	58.4	0.74	1.26	39.6	0.58	1.46	123.5	2.22	1.8
GGT (U/L)	Schumann, et al. ^{18‡}	48.3	0.91	1.89	31.2	0.68	2.17	326.9	7.62	2.33	46.9	1.82	3.87	32.7	1.12	3.43
CK (U/L)	Schumann, et al. ^{19†}	223.5	6.32	2.83	167.3	4.00	2.39	83.8	2.31	2.76	221.6	6.85	3.09	164.2	5.47	3.33
TP (g/dL)	Biuret [†]	6.28	0.054	0.86	7.35	0.071	0.97	7.42	0.069	0.93	6.33	0.11	1.69	7.39	0.098	1.33
Albumin (g/dL)	Bromocresol Green [§]	3.76	0.066	1.76	4.24	0.081	1.91	4.16	0.075	1.82	3.83	0.093	2.44	4.21	0.097	2.3

219 [†] Intra-assay precision was determined on 20 replications of 3 samples; inter-assay precision was determined for 4 consecutive days with 15 replications on
 220 each day, for 2 samples.

221 Commercial kits supplied by: [†] Gernon, RAL Técnica para el laboratorio, S.A. (Barcelona, Spain). [‡] Spinreact S.A. (Girona, Spain). [§] Human Gesellschaft für
 222 Biochemica und Diagnostica GmbH (Wiesbaden, Germany)

223 **Table 2.** Effect of calf age on the clinical biochemistry variables determined between
224 day 0 and 90.

Analyte	Calf age effect (<i>P</i> value)
Glucose	.278
AST	< .001
GGT	< .001
Globulins	< .001
TP	< .001
Albumin	.001
A/G	< .001
Urea	.001
CK	.014
Creatinine	< .001

225

226 **Table 3.** Reference intervals* for select biochemistry variables in 40 clinically healthy cows between the 4th and 5th months of pregnancy.

227

Analyte	Units	n	Mean	SD	Median	Min	Max	Reference interval [†]	Lower limit 90% CI	Upper limit 90% CI	Distribution ^a	Method ^b
Glucose	mg/dL	40	61.0	6.4	59.1	48.6	86.3	48.6 – 86.0	48.6 – 52.4	73.9 – 86.3	NG	NP
Creatinine	mg/dL	40	1.1	0.1	1.1	0.92	1.35	0.9 - 1.4	0.9 – 0.9	1.3 – 1.4	G	R
Urea	mg/dL	39	26.4	5.9	26.3	13.0	39.4	14.4 - 38.5	11.9 – 17.0	35.8 – 41.1	G	P
AST	U/L	39	93.6	22.8	94.5	51.5	153.1	46.9 – 140.3	37.5 – 56.9	129.8 – 150.3	G	P
GGT	U/L	40	40.5	10.6	41.9	23.0	65.0	18.9 - 62.1	14.5 – 23.5	57.3 – 66.8	G	P
CK	U/L	40	166.5	67.1	142.4	79.8	338.4	80.1 – 259.8	79.8 – 90.9	231.6 – 260.0	NG	NP
TP	g/dL	40	7.6	0.9	7.7	5.7	9.4	5.8 - 9.3	5.4 – 6.2	8.9 – 9.7	G	P
Albumin	g/dL	39	4.0	0.4	3.9	3.4	4.9	3.2 – 4.7	3.1 – 3.4	4.6 – 4.9	G	P
Globulins	g/dL	40	3.6	0.8	3.7	1.6	5.1	2.0 – 5.2	1.6 – 2.3	4.8 – 5.5	G	P
A/G	--	39	1.1	0.3	1.1	0.77	1.7	0.7 – 2.2	0.7 – 0.8	1.8 – 2.5	G	R

228

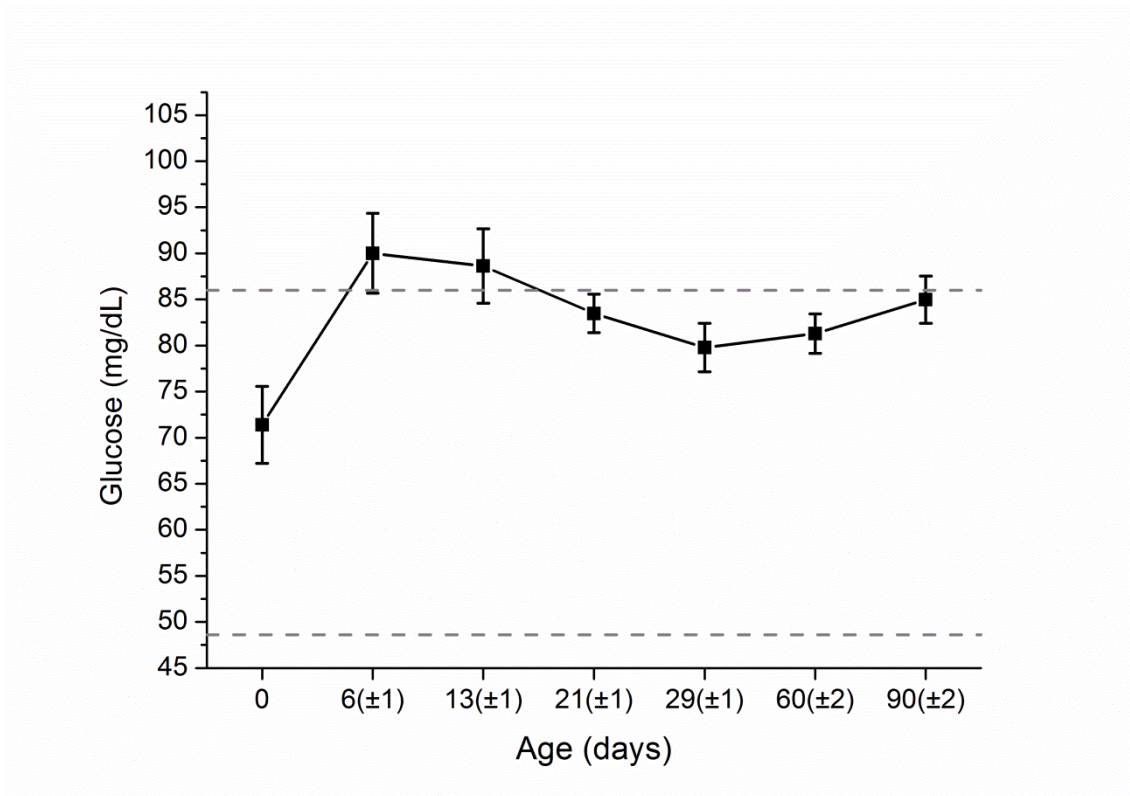
229 [†]Calculated using the Reference Value Advisor software.¹⁰

230 ^a G, Gaussian; NG, non-Gaussian.

231 ^b Method for establishing reference interval: P, parametric; NP, nonparametric; R, robust.

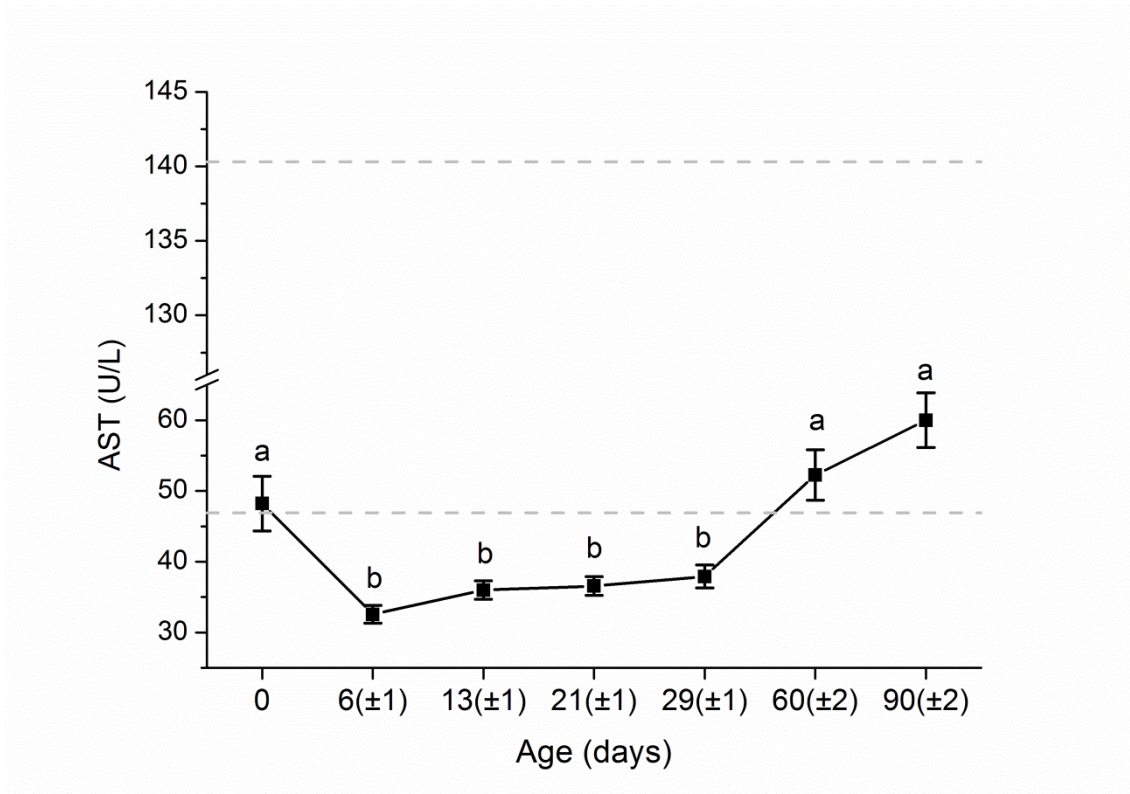
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233 **Figure captions**



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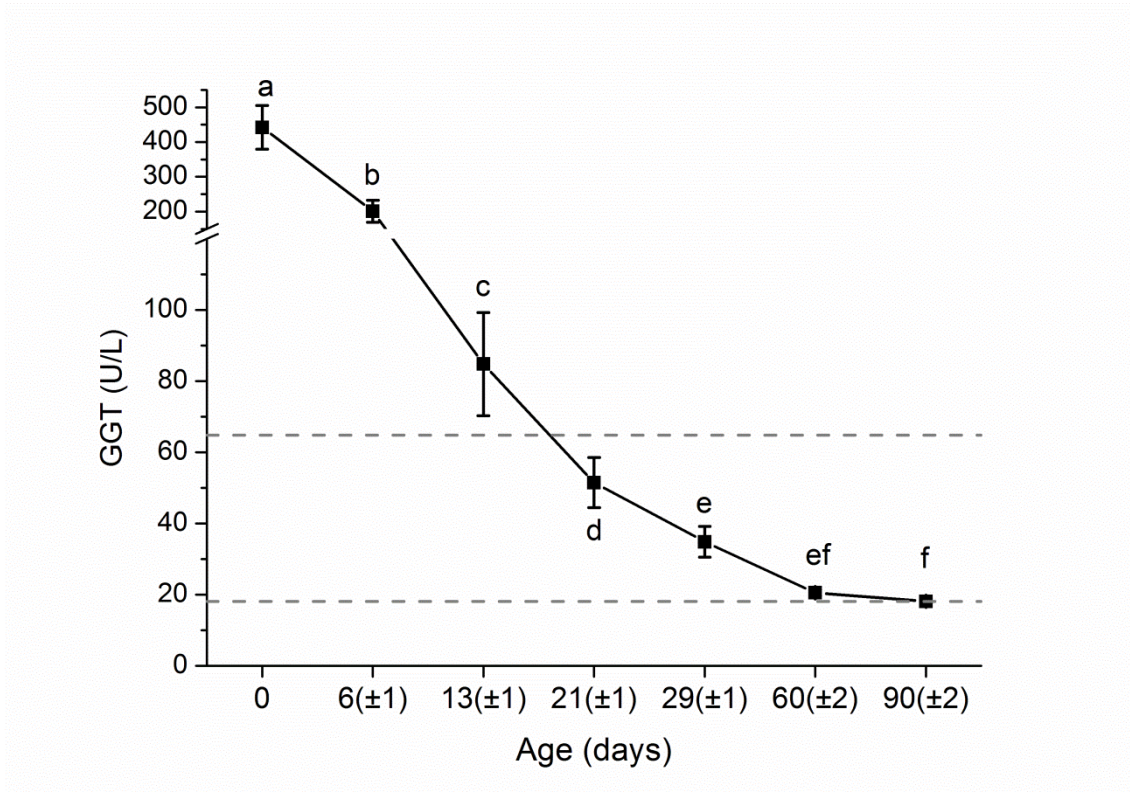
235 **Figure 1.** Mean (\pm SEM) serum glucose concentration in calves between day 0
236 and 90. Dashed lines represent the lower and upper limits of the reference interval,
237 respectively, determined for the adult animals. Day 0 samples were obtained 2 h after colostrum
238 ingestion.



239

240 **Figure 2.** Mean (\pm SEM) values of serum AST activity in calves between day 0 and
241 90. Dashed lines represent respectively the lower and upper limits of the reference interval de
242 reference interval determined for the adult animals. Day 0 samples were obtained 2 h after
243 colostrum ingestion.

244 ^{a, b} Values without a common superscript letter are significantly different ($P < .05$).

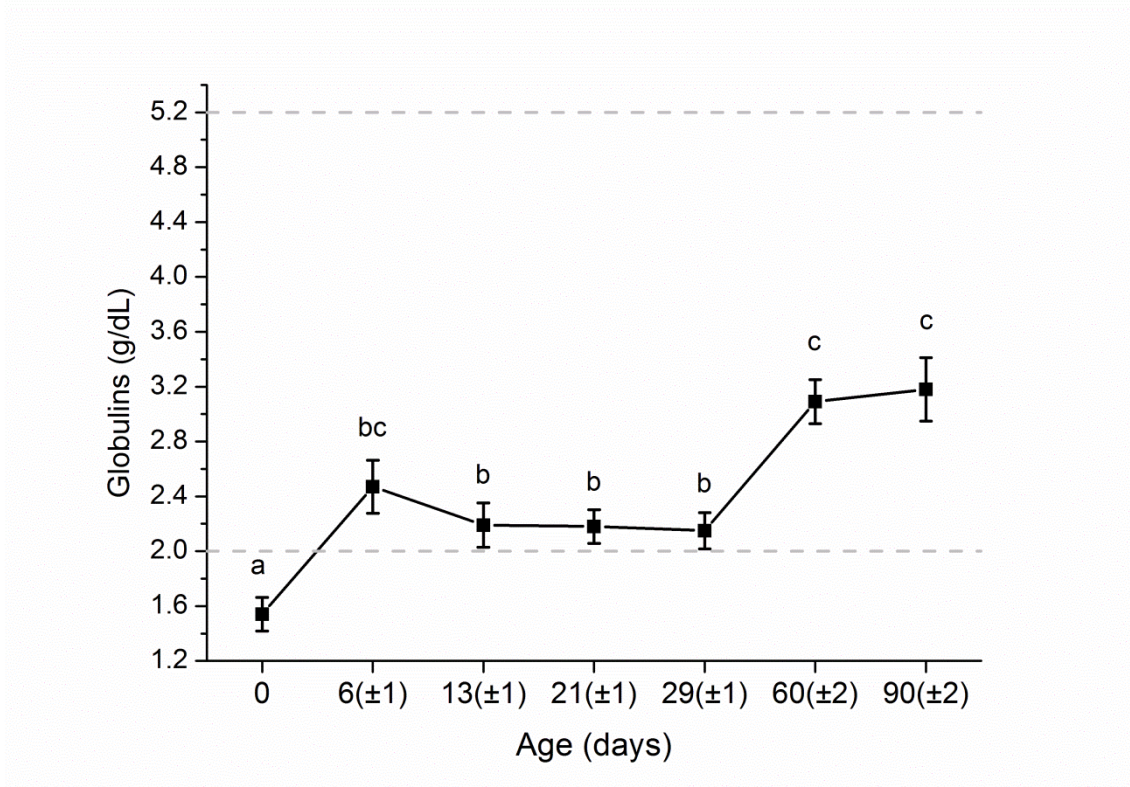


245

246 **Figure 3.** Mean (\pm SEM) serum GGT activity in calves between day 0 and 90.
247 Dashed lines represent respectively the lower and upper limits of the reference interval
248 determined for the adult animals. Day 0 samples were obtained 2 h after colostrum ingestion.

249 a, b, c, d, e, f Values without a common superscript letter are significantly different ($P < .05$).

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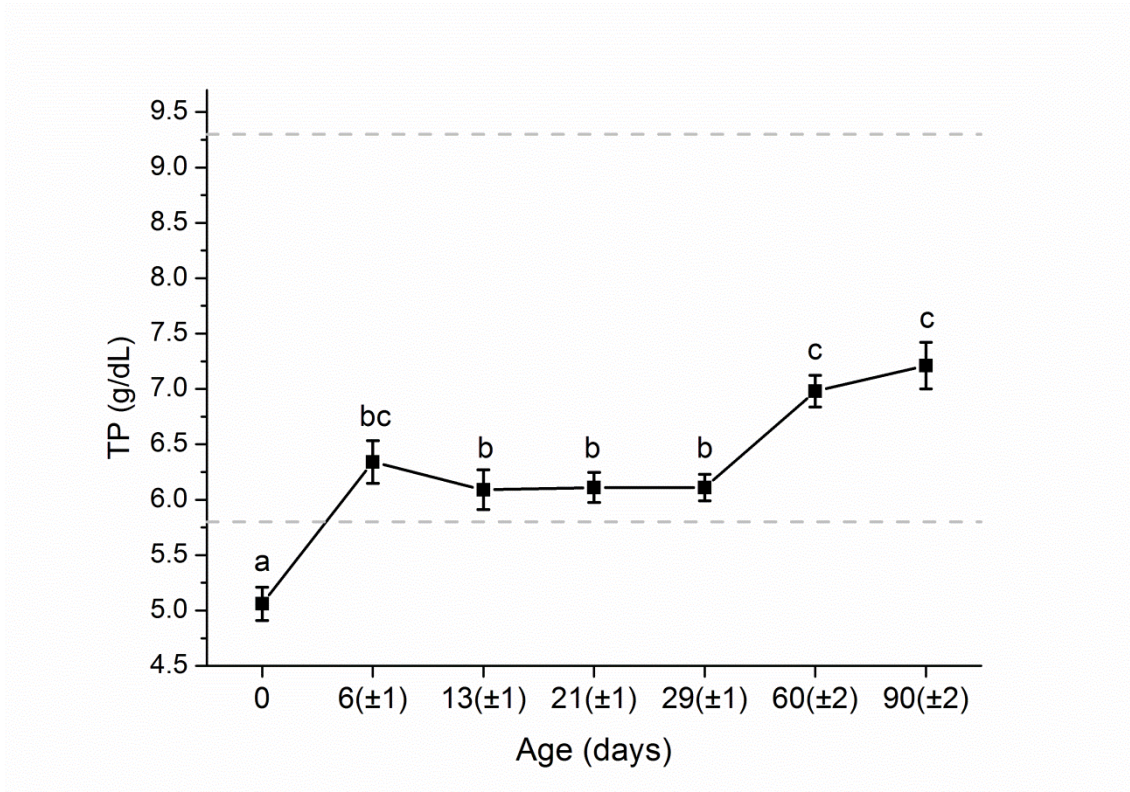


251

252 **Figure 4.** Mean (\pm SEM) serum globulin concentration in calves between day 0 and
253 90. Dashed lines represent respectively the lower and upper limits of the reference interval
254 determined for the adult animals. Day 0 samples were obtained 2 h after colostrum ingestion.

255 ^{a, b, c} Values without a common superscript letter are significantly different ($P < .05$).

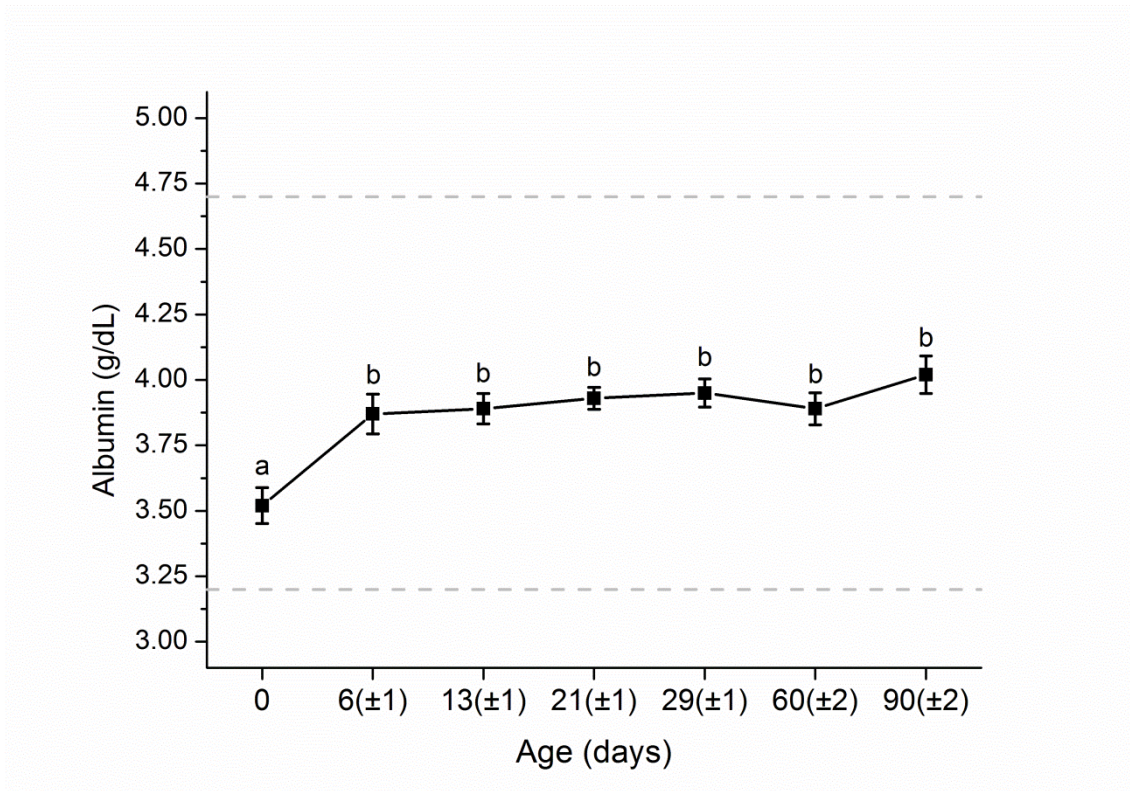
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257

258 **Figure 5.** Mean (\pm SEM) total serum protein concentration in calves between
259 day 0 and 90. Dashed lines represent respectively the lower and upper limits of the reference
260 interval determined for the adult animals. Day 0 samples were obtained 2 h after colostrum
261 ingestion.

262 ^{a, b, c} Values without a common superscript letter are significantly different ($P < .05$).

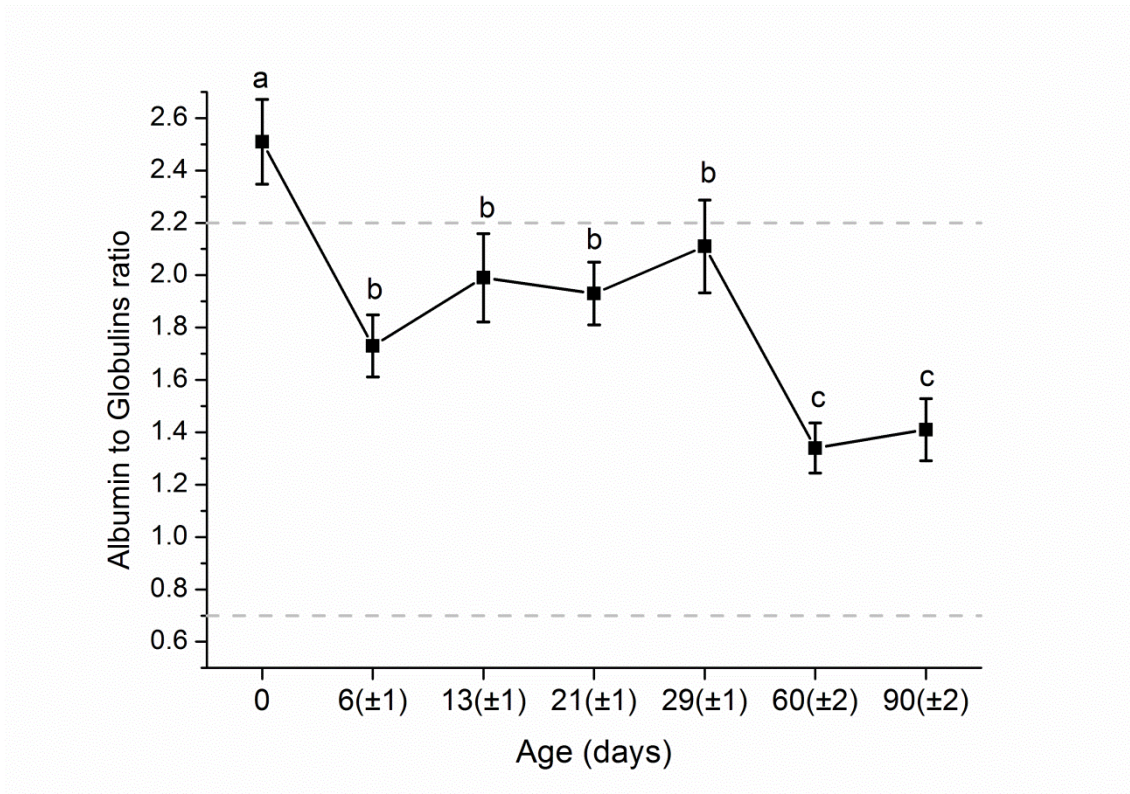


263

264 **Figure 6.** Mean (\pm SEM) albumin concentration in calves between day 0 and 90.
265 Dashed lines represent respectively the lower and upper limits of the reference interval
266 determined for the adult animals. Day 0 samples were obtained 2 h after colostrum ingestion.

267 ^{a, b} Values without a common superscript letter are significantly different ($P < .05$).

268

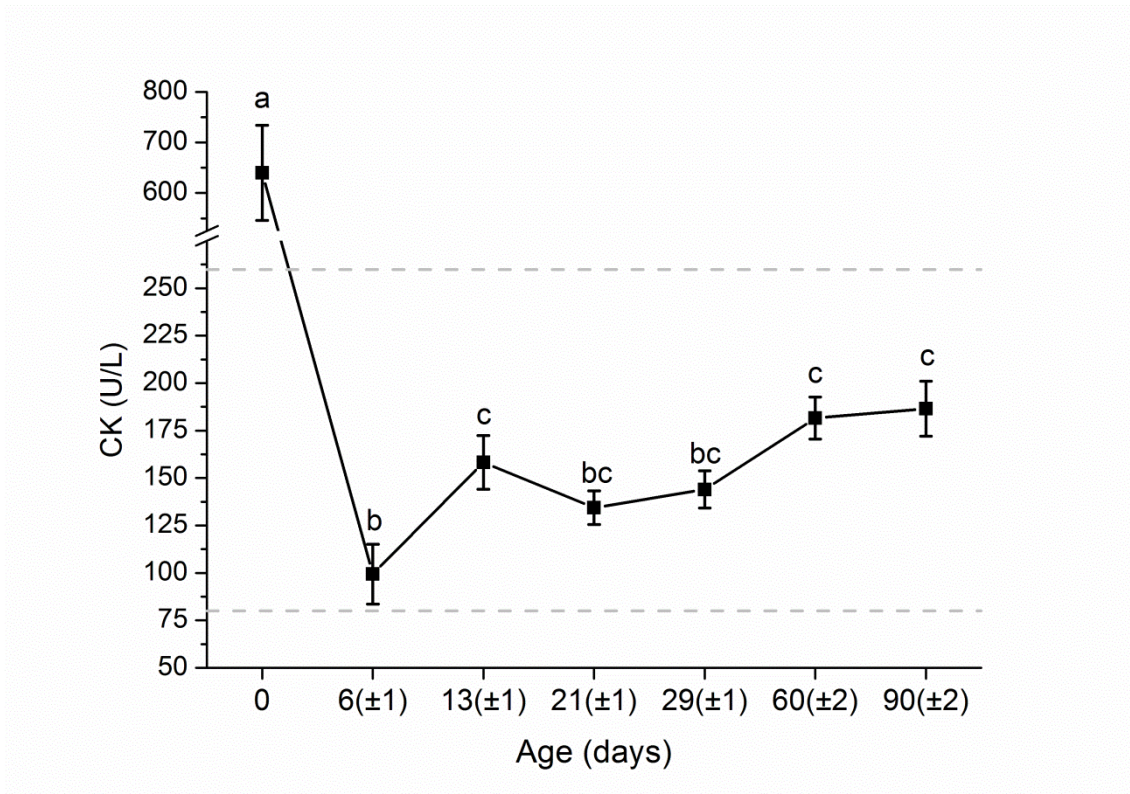


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270 **Figure 7.** Mean (\pm SEM) albumin:globulin ratio in calves between day 0 and 90.
271 Dashed lines represent respectively the lower and upper limits of the reference interval
272 determined for the adult animals. Day 0 samples were obtained 2 h after colostrum ingestion.

273 ^{a, b, c} Values without a common superscript letter are significantly different ($P < .05$).

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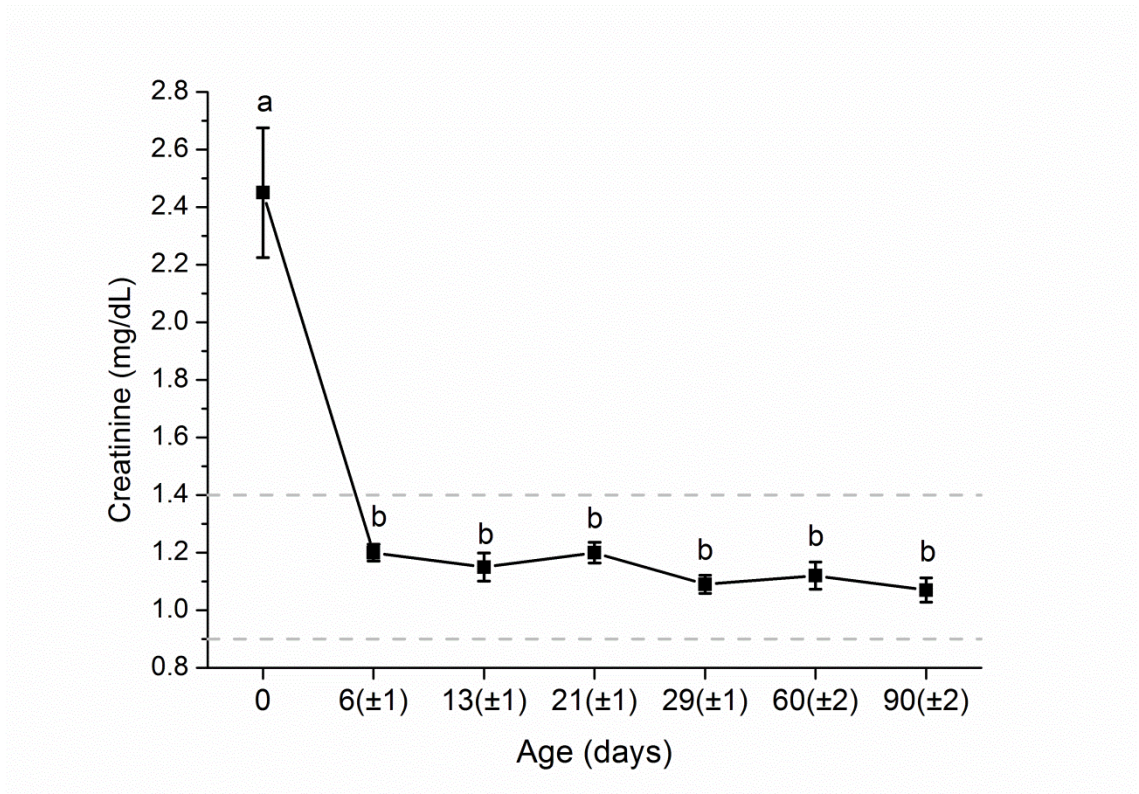


275

276 **Figure 8.** Mean (\pm SEM) serum CK activity in calves between day 0 and 90.
277 Dashed lines represent respectively the lower and upper limits of the reference interval
278 determined for the adult animals. Day 0 samples were obtained 2 h after colostrum ingestion.

279 ^{a, b, c} Values without a common superscript letter are significantly different ($P < .05$).

280



281

282 **Figure 9.** Mean (\pm SEM) serum creatinine concentration in calves between day
283 0 and 90. Dashed lines represent respectively the lower and upper limits of the reference
284 interval determined for the adult animals. Day 0 samples were obtained 2 h after colostrum
285 ingestion.

286

^{a, b} Values without a common superscript letter are significantly different ($P < .05$).