# Prepartum and Postpartum Serum Mineral and Steroid Hormone Concentrations in Cows with Dystocia

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## **INTRODUCTION**

Dystocia is defined as any birth that requires assistance and it is one of the most serious complications of pregnancy in cattle. Its incidence is 3–25 % in all pregnancies and the major cause of decline on the total performance and consequently economic loss (Stephan 1986; Oakes and Parkinson 2001). Numerous factors are believed to influence dystocia such as pelvic area of the dam, disproportionate calf birth weight (BWT), age of dam, twin pregnancies, hormonal disorders, nutritional level of dam during gestation and other unknown factors (Abeni et al. 2004).

The minerals have a significant role to play in many aspects of production including successful establishment of pregnancy. Abnormalities in trace element regulation have been reported in various obstetric pathologies both human (Ajayi and Fadiran 1998; Juretic and Frkovic 2005) and animals (Graham et al. 1994; McSporran et al.1997; Naziroglu et al. 1998; Olujohungbe et al. 1998); however, no detailed measurements of serum mineral concentrations during the pregnancy and after parturition in the cows with dystocia have been reported (Zhang et al.1999a). It is expected that nutritional requirements of pregnancy increase markedly during the last trimester (Oakes and Parkinson 2001), especially in dystocial cattle, due to the relative oversize or twin pregnancy mothers suffering from dystocia. For this reason, peripartum alterations in serum concentrations of minerals may contribute to dystocia in dairy cows.

Many of the studies have investigated the relationship between maternal blood hormone concentrations before calving and abnormal pregnancy in cattle. It is suggested that one of the most practical and reliable means for monitoring pathological pregnancy may be determination of eastradiol concentrations in maternal blood (DeGraaf e al. 1982; Echternkamp 1992; Gregory and Echternkamp 1996; Wischral et al. 2001). It is possible that endocrine system affects serum mineral levels just like it does all systems in the body (Graham et al. 1994). Also, dietary factors can affect reproduction by influencing the animal at the level of the endocrin systems.

Additional tools to prevent of dystocia may be researched by monitoring the maternal nutritional state during the late gestation. In this study, Ca, P, Fe, Mg, Na, K, Cl, Cu, Zn, Ca/Mg ratio, estradiol, progesteron, LH and FSH levels of serum were measured both prepartum and postpartum stage of Holstein cows and the role of these parameters on the predisposition effect of the dystocia were investigated. In order to determine whether changes originated from a specific etiopathogenesis cows with dystocia were divided by clinical signs of dystocia into three subgroups: those with absolut birth weight (the high birth weight of calf), twin pregnancy and presentation disposition.

# **MATERIAL and METHODS**

## Animal and sample collection

The study was carried out using aged 3-6 years Holstein cows maintained in Ceylanpınar dairy farm affiliated to Turkish Agriculture Ministry (TIGEM) (Urfa, Turkey). Initially, 200 pregnant multiparous Holstein cows were used as the material of the study. During the study all animals were kept under identical conditions. The samples were taken both on the 7th month of pregnancy and within the first 15 minute after delivery. After parturition animals were divided into two groups according to the type of birth: group 1, dystocia (difficult labour) (n=16) and group 2, normal partum (eutocia) that were randomly selected within the nondystocial ones (n=21). Calving was considered as 'eutocia' if it needed either no assistance or assistance by one person with or without calving ropes for a few minutes, or 'dystocia' if it needed either two or more persons equipped with a mechanical puller or veterinary assistance to perform caesarean section (1, 2). For the determination of the effect of specific ethiopathogenes 1st groups were assigned in three group based on cause of dystocia (1. absolute birth weight, 2. twin pregnancy, 3. presentation disposition).

The calves were weighed within the 1 hour after delivery. Blood samples were obtained by jugular venapuncture, using evacuated tubes (Vacutainer®), and immediately stored in ice and serum were separated from cells by centrifugation at 1800 x g for 10 min, and aliquots were stored in polyethylene tubes at -80 °C until the analysis.

#### Serum analysis

The concentrations of Cu and Zn in serum were determined by flame atomic absorption spectrophotometry (Unicam 925, United Kingdom) equipped with an air-acetylene flame burner and hollowcathode lamps (operated at 20 mA for copper and at 15 mA for zinc). Atomic absorption was measured at 324.8 nm for copper and 213.9 nm for zinc. The spectral bandwidth was 0.5 nm. In order to minimalize physical interference, Zn and Cu have been diluted with a ratio of 1/10 and 1/4 respectively with 6% N-Butanol in flame mode (20). The standard stock solutions for trace elements were obtained from Sigma. Standard solutions and samples were diluted in double-distillate water. Ionized Ca, Na, K, Cl, P and Mg were determined by auto analyzer (Olympus, AU 2700, Germany) with ion selective electrode (ISE) method. Iron concentration was measured colorimetrically with auto analyzer (Olympus, AU 2700, Germany) used commercially available kits. coefficients of variation for all variables were < 5%.

Serum concentrations of estradiol and progesterone were determined radio immunoassay (Diagnostic Systems Laboratories, Inc. Texas, USA), with intra-assay coefficients of variation (CV) of 2.01 and 8%, the interassay CV 7.37 and 13.1 respectively. The assays sensitivity was of 0.01 pg/ml and 0.01 ng/ml respectively, for estradiol and progesterone. Analytical sensitivity was as follows: estradiol, 11 pg/ml; progesterone 0.1 ng/ml. The serum FSH and LH concentrations were determined by Immunoradiometric assay (Diagnostic Systems Laboratories, Inc. Texas, USA). The intra-assay CV was 2.6 and 4.8% and inter-assay CV 7.7 and 6.8%, respectively, for FSH and LH.

## Statistical analysis

The following statistical procedures were used; analysis of differences between prepartum and postpartum stages for each group, paired-samples t test. The independent-samples t test was performed in order to compare two groups for each evaluated parameters. Sub-groups values of dystocia were compared by Kruskal-Wallis analysis H followed by Mann-Whitney U-test. To determine the significance of interactions between various variables in each group, Pearson's and Spearman's correlation analysis was performed. Distribution of the sex of calves according to sort of labour were analyzed with the Pearson chi-square test. A difference with p<0.05 was considered to be significant. All statistical analyses were performed with statistics package SPSS version 13.0 (SPSS Inc., Illinois, USA).

## RESULTS

The measured variables in dystocia and in normal parturition (control) at 7<sup>th</sup> month of pregnancy and after delivery are summarized in Table–1. The concentration of the all measured variables according to dystocia subgroups and the normal parturition are given in Table–2. In addition, distribution of the sex of calves according to sort of labour are summarized in Table–3. The results of the analysis are expressed as mean ± standard deviation. Determined differences are shown according to groups and periods in the same tables.

Variables	_	Dystocia (Gro	oup 1) (n=16)	Normal Partum (Group 2) (n=21)				
Variables		Prepartum	Postpartum	Prepartum	Postpartum			
Са	(mg/dl)	9.06 ± 1.02	9.21±1.66	8.7±0.84	9.04±1.25			
Р	(mg/dl)	5.86 ± 1.91 <sup>2</sup>	8.2±1.48 <sup>2</sup>	6.2±1.18 <sup>3</sup>	7.95±1.36 <sup>3</sup>			
Ca/Mg Ratio		3.77 ± 2.2 <sup>a,2</sup>	$1.37 \pm 0.32$ <sup>2</sup>	2.59 ± 1.1 <sup>a,3</sup>	$1.32 \pm 0.29$ <sup>3</sup>			
Fe	(µd/dl)	89.62 ± 37.1 <sup>2</sup>	138 ± 27.3 <sup>2</sup>	86.67 ± 40.69 <sup>2</sup>	$164.09 \pm 104.23^2$			
Mg	(mg/dl)	3.33 ± 1.95 <sup>b, 3</sup>	6.89 ± 1.26 <sup>3</sup>	4.1 ± 1.92 b, 3	$6.98 \pm 1.08$ <sup>3</sup>			
Na	(mmol/l)	139.44 ± 8.36	$142.06 \pm 4.64$	$138.38 \pm 4.38$	145.47 ± 6.87			
К	(mmol/l)	5.46 ± 0.53	$5.59 \pm 0.75$	$5.57 \pm 0.43$	5.6 ± 0.79			
Cl	(mmol/l)	108.94 ± 7.53	108.06 ± 2.43	$108.09 \pm 5.14$	110.38 ± 6.41			
Cu	(mg/l)	$0.87 \pm 0.11$ <sup>3</sup>	$0.68 \pm 0.1$ <sup>3</sup>	$0.82 \pm 0.16$ <sup>2</sup>	$0.7 \pm 0.13$ <sup>2</sup>			
Zn	(mg/l)	$0.56 \pm 0.16$ <sup>2</sup>	$0.84 \pm 0.31$ <sup>2</sup>	$0.6 \pm 0.25$ <sup>1</sup>	$0.86 \pm 0.42$ <sup>1</sup>			
Estradiol	(pg/ml)	581.71 ± 971.37	450.49 ± 401.81	292.62 ± 194.1	444.69 ± 477.11			
Progesteron	(ng/ml)	2.04 ± 1.58 <sup>1</sup>	$0.85 \pm 0.85$ <sup>1</sup>	8.04 ± 18.51	$1.62 \pm 4.37$			
LH	(mIU/ml)	$0.26 \pm 0.35$	$0.08 \pm 0.03$	$0.23 \pm 0.34$	$0.15 \pm 0.25$			
FSH	(mIU/ml)	$0.09 \pm 0.09$	$0.045 \pm 0.036$	$0.1 \pm 0.11$	$0.097 \pm 0.14$			
Birth weight	(kg)	43.81 ± 6.22		39.91 ± 5.59				
Age of cows	(year)	$4.19 \pm 1.17$		$4.86 \pm 1.04$				

**Table 1**. The comparison of the values in Dystocia and Normal parturition groups at prepartum and postpartum.

 **Tablo 1**. Prepartum ve postpartum dönemde normal ve güc doğum sekillenen grupların kan değerlerinin karsılaştırılmaşı

*Note:* All values are given as mean ± SD. The measured variables were compared with both between the groups and according to the periods in same group.

a, b: when this period was compared with corresponding period in the other group, differences is significant (Independent Sample t test) (p<0.05, p<0.01 respectively).

 $^{1,2,3}$ : The differences between mean values having same number in each group is significant (Paired Sample t test) (p<0.05, p<0.01, p<0.001 respectively).

<b>Table 2.</b> The concentration of the all variables according to dystocia sub-groups and normal parturition group in Pre-partum.
Tablo 2. Doğum şekillerine gore prepartum dönemdeki tüm kan parametrelerinin konsantrasyonları

			Normal Partum		
Variables	Absolute Birth Weight (n=8)		Twin Pregnancy (n=4)	Presentation Disposition (n=4)	(Group2, n=21)
Са	(mg/dl)	8.89 ± 0.73	9.09 ± 1.58	9.35 ± 1.12	$8.7 \pm 0.84$
Р	(mg/dl)	6.11 ± 2.24	$6.62 \pm 1.35$	4.57 ± 1.28	$6.2 \pm 1.18$
Ca/Mg Ratio		4.31 ± 2.81	$3.13 \pm 1.29$	3.31 ± 1.6	2.59 ± 1.1
Fe	(µd/dl)	105.12 ± 40.17	82.75 ± 34.95	65.5 ± 20.4	86.67 ± 40.69
Mg	(mg/dl)	3.19 ± 2.12 1	3.35 ± 1.79 <sup>1</sup>	$3.6 \pm 2.25$	4.1 ± 1.92 1
Na	(mmol/l)	$138 \pm 3.66$	135.5 ± 1.29	146.25 ± 15.1	138.38 ± 4.38
K	(mmol/l)	$5.4 \pm 0.51$	$5.15 \pm 0.51$	$5.87 \pm 0.4$	$5.57 \pm 0.43$
Cl	(mmol/l)	106.75 ± 2.87 ª	104.25 ± 2.87 <sup>b</sup>	118 ± 10.23 a, b, 1	$108.09 \pm 5.14^{1}$
Cu	(mg/l)	$0.86 \pm 0.11$	$0.87 \pm 0.12$	$0.87 \pm 0.13$	$0.82 \pm 0.16$
Zn	(mg/l)	$0.61 \pm 0.17$	$0.55 \pm 0.08$	$0.48 \pm 0.2$	$0.6 \pm 0.25$
Estradiol	(pg/ml)	$273.46 \pm 176.04^{a}$	1448.13 ± 1803 <sup>a,2</sup>	331.77 ± 234.63 ª	292.62 ± 194.1 <sup>2</sup>
Progesteron	(ng/ml)	$2.36 \pm 1.62$	$1.59 \pm 2.1$	$1.84 \pm 1.16$	$8.04 \pm 18.51$
LH	(mIU/ml)	$0.37 \pm 0.44$	$0.05 \pm 0.03$	$0.23 \pm 0.25$	$0.23 \pm 0.34$
FSH	(mIU/ml)	$0.06 \pm 0.06$	$0.07 \pm 0.03$	$0.18 \pm 0.14$	$0.1 \pm 0.11$
Birth weight	(kg)	46.75 ± 6.23 a, 1	$37 \pm 2$ a, b, 2	44.75 ± 3.6 <sup>b</sup>	39.91 ± 5.59 <sup>1,2</sup>
Age of cows	(year)	$4.0 \pm 1.19$	$5.25 \pm 0.96$	3.5 ± 0.58 1	$4.86 \pm 1.04$ <sup>1</sup>

Note: All values are given as mean  $\pm$  SD. The measured variables were compared with both among the sub-groups and the other group (Kruskal Wallis and Man Whitney U).

a, b: The differences between mean values having same superscripts in dystocia group is significant (p<0.05, p<0.01, p<0.001 respectively). (Kruskal Wallis and Man Whitney U).

 $^{1,2,:}$  When the sub-groups of dystocia was compared with normal partum (Group 2) this difference was significant (p<0.05, p<0.01 respectively). (Man Whitney U)

The cows with dystocia had a tendency non significant higher serum estradiol and lower progesteron concentration compared with cows without dystocia in both periods. However, there was a significant increase in serum estradiol levels in 7<sup>th</sup> month of gestation in twin pregnancy as compared to normal pregnancy (p<0.01). All of the other subgroups (absolute birth weight and presentation anomalies) in dystocia found that estradiol levels were not changed compared to normal pregnancy in prepartum. LH and FSH levels did not show significant difference before and after parturition in both groups. There is no correlation between the circulating steroid hormon concentrations and mineral levels.

Cows with dystocia had significantly lower serum concentration of Mg (p<0.01) and higher ratio of Ca/Mg (p<0.05), compared with cows without dystocia in prepartum. Except for presentation disposition, lower serum Mg concentrations was also observed in other sub groups in the 7<sup>th</sup> month of pregnancy as compared with the normal pregnancy (p<0.05). The serum Ca concentrations showed a nonsignificant increase in the dystocia in all subgroups, compared with cows control in prepartum.

However, the concentrations of Cu were higher and the concentration of Zn lower in dystocial cattles at 7th month of pregnancy, but these alterations were not significant. In addition, cows with dystocia had higher serum Cu concentration in all subgroups, compared with cows control in prepartum, but it was not found statistically signification.

No differences were seen in respect of Na, Cl and K levels between the dystocia and control groups depending on the parturition type. Significant increases were found only for serum Cl concentration in presentation disposition when compared with the other subgroups (p<0.05) and normal pregnancies (p<0.05) in prepartum. The serum Fe and P concentrations were not changed depending on the parturition type in both periods. When the periods were compared, there was a statistically decrease in P, Fe, Mg and Zn levels and increase in Ca/Mg ratio in prepartum in both groups. Ionized Ca, Na, Cl, K, LH and FSH concentrations did not show significant difference before and after parturition. Also, no difference were found between the sex of calves and sort of labour ( $X^2$ =2.538, p>0.05).

**Table 3.** Distribution of the sex of calves according to sortof labour (Pearson's chi-square test)

Tablo 3. Cinsiyet ve doğum şekli arasındaki ilişki

	Dyst	tocia	Normal Partum	
	Male	Female	Female	Male
Number of calves	10	6	8	13
Within the pregnancy (%)	62.5%	37.5%	42.1%	57.9%

### DISCUSSION

Many of the studies recognized the alterations of the mineral concentrations in pathological pregnancy in humans and various mammalian species. And most of these targeted serum Ca, Mg, Cu and Zn concentrations. Human studies have shown serum Mg, Ca and Zn levels to be lower in preeclampsia (Atamer et al. 2005) and there is an imbalance between Ca and Mg ratio in abortus (Borella et al. 1990). Likewise, plasma Mg was found decrease during abnormal gestation and teratogenicity (Ajayi and Fadiran 1998). On the other hand, Cu levels were found to

be significantly higher in women with abortus (Juretic and Frkovic 2005), preeclampsia (Atamer et al. 2005) and fetal retardation (Borella et al. 1990).

Animal studies have also shown that Mg deficiencies can cause aborted ewes (Naziroglu et al. 1998), and goats (Unanian and Feliciano-Silva 1984). Furthermore, Naziroglu et al. (1998) indicated that premature delivery may be the most likely complication of maternal Zn deficiency in the ewe. Similarly with various obstetric pathologies in the other species, it was reported that decreased serum Zn and increased Cu concentrations is related to abortion in mares (Graham et al. 1994; 1995) mainly retained fetal membranes (RFM), which usually occurred after dystocia (Olujohungbe et al. 1998), and abortus were investigated. These investigators observed lower Ca (Akar et al. 2002; Melendez et al. 2004) concentrations soon after calving in RFM cases than in control animals. In addition, it has been reported that low serum levels of Mg and Zn might cause to RFM in cows (Graham et al. 1995; Akar and Yildiz 2005). Lower levels of serum Zn and higher levels of Cu in aborted cows were also observed by Graham et al (1994)

It is attracted that all of the above mentioned studies indicate that decreased Mg, Ca, Zn and increased Cu concentrations, although they are different obstetric pathologies in different mammalian species.

In bovine dystocia, a few reports have been published concerning involvement of different minerals which are inconsistent, Akar and Yildiz (2005) indicated that the blood serum Na, K, Mg, Cu and Zn concentrations did not differ between normal cows and those with dystocia. McSporran et al (1997) also reported that sheep with dystocia did not exhibit a fall in plasma Zn similar to that recorded in cattle. Differently, Paterson and Terry (2005) informed that the main effects of Zn and Cu deficiencies are increased incidence of dystocia. Likewise, it is determined that supplemented ration with bone meal for the last trimester of pregnancy, the incidence of dystocia was reduced 75% to 10% (Carson et al. 1978). The cause of controversial results in different studies may be influence of dystocia from different factors and the difference in the time of sample collection. Indeed, the time trend of serum mineral concentrations can be affected by the stage of pregnancy (Yokus and Cakir 2006); however, all of studies were evaluated the serum mineral concentrations after parturition in dystocia.

In this research, the tendency of the higher Ca level in dystocial cattle than non-dystocial ones (p>0.05) probably is the increase of Ca in bone resorbtion because of increased parathyroid hormone (PTH) caused by estradiole, which is increased in dystocia (Yokus et al. 2004). Also, all subgroups in the dystocia showed a tendency to higher levels of serum Ca, although this difference did not rich statistical significance.

Mg status in dystocial cattle were significantly lower (p<0.01) and high incidence of dystocia could be associated with deficiency in Mg. Lower concentrations of serum Mg was also found in the case of absolute birth weight and twin pregnancy (p<0.05), which may be higher demand of fetus due to relative fetal oversize. In addition, the average serum Ca/Mg ratio in cows with dystocia were higher than the values of those without dystocia (p<0.05). Interestingly, the relationship between increased Ca/Mg ratio and other pathological pregnancy has been observed in humans (Borella et al. 1990).

These findings support the hypothesis that hypomagnesemia are possible etiologies of obstetric

pathologies. Although the explanation for this hypothesis is not clear, they propose that magnesium promotes vasodilatation and uterine relaxation (Melendez et al. 2004). A high Ca/Mg ratio has been experimentally shown to provoke vasospasm in certain blood vessels in vitro, and Borella et al. (1990) suggested that this is responsible for the spasm of umbilical and placental vasculature at the end of pregnancy. It is likely that the intake of magnesium supplement may help to reduce the incidence of dystocia.

Our findings demonstrated that, in dystocia cases had higher mean serum concentration of Cu and lower Zn levels as compared with control; however, these differences were not significant. This data in partial agreement with the reports of Paterson and Terry (2005) who indicated that decreased Zn levels in dystocia. Furthermore, all pathological conditions in the prepartum subgroups in dystocia showed a tendency to higher levels of serum Cu levels as compared to normal pregnancy; however, these differences were not significant. It was noticed that the Zn levels had a tendency to decrease, when Cu levels are highest in studying groups. This might be the result of copper inhibition of Zn intestinal absorption.

Although, we have not been able to see a published value about the possible influence of mineral levels on the dystocia in prepartum period, and it is not proper to compare our results with the other obstetric pathologies due to the its different ethiologia, our findings bear the resemblances with some of the other studies that found higher Cu, Ca/Mg ratio and lower Zn and Mg concentrations in these pathologies. The reason for this relationship is unclear, and its confirmation and interpretation requires further investigation.

The observation of the nonsignificant difference on the Na, Cl and K concentrations in both groups was consistent with Akar and Yildiz (2005). P and Fe status of cows didn't statistically differ between two groups. Lower concentration of serum P and Fe in both groups in the prepartum confirmed our previous observation in normal pregnant cattle (Yokus and Cakir 2006).

During the pregnancy some pivotal physiological process takes place in the hormonal system. Also it is indicated that changes in hormonal system can be affect of the serum mineral levels (Boland 2003; Graham et al. 1995). Contradictory, there is no correlation between the circulating steroid hormone concentrations and mineral levels in this study. The trends of the increasing serum estradiol and decreasing progesteron levels in normal pregnancy are in agreement with the results reported by researchers in the other breeds (Wischral et al. 2001; Zhang et al. 1999b).

However, the concentrations of estradiol were higher and the concentrations of progesteron were lower in dystocial animals in both periods as compared to controls, these alterations were not significant. Similarly, DeGraaf et al. (1982) found that differences in plasma progesteron or estradiol concentrations between heifers with and without dystocia were not significant. Olujohungbe et al. (1998) reported that increases of estradiol concentration on the day after calving, but not before parturition, decrease in difficult calving. Zhang et al. (1999a) also suggested that, no significant difference in progesteron or estradiol concentrations were found between eutocial and dystocial cattle until the last two weeks before parturition.

Dystocia is more common in twins cattle than in singletons cows (Gregory et al. 1996) and the dams of bearing a multiple calf twins are normally showing higher levels in estradiol as compared to singletons (Echternkamp 1992). The present study likewise observed increased incidence of dystocia in twins than in singletons, and estradiol concentrations increased with twins (p<0.01) but progesteron were not changed. Regardless of whether cattle were bearing single or twin fetuses, there is a tendency of the increased estradiol concentrations in 7<sup>th</sup> month of pregnancy. In fact, increased estradiol levels in dystocia at prepartum period must be strongly linked a consequence of increased estradiol levels in twin pregnancy presumably due to the increased placental activity.

Contrary to Echternkamp et al. (1992) and Zhang et al. (1999b)'s studies in which a positive relationship between maternal estrogen sulphate and/or estrone and calf BWT, estradiol concentration was unaffected by BWT in the present survey. This discrepancy was probably related to differences in calculation of the calf BWT. In this study BWT were calculated for each calf in twins, whereas the previous studies were calculated of the BWT totally. For the LH and FSH concentrations there was no difference at depending on parturition type. In our study population, the incidence of dystocia was within the normal ranges of 8% reported in the literature (1, 2). There is no correlation between the sex of calves and sort of labour. This result indicated that calf sex in cows had no effect on occurrence of dystocia.

As a conclusion, the analyses of estradiol and progesteron could not be used for monitoring in cows with a high risk of dystocia at 7<sup>th</sup> month of pregnancy. However, estradiol concentration appeared to be a predictable indicator of dams bearing a multiple calf. Decreasing in serum Mg concentrations and increasing Ca/Mg ratio are associated with dystocia in cattle. It can be concluded that prepartum supplementation of Mg could be used for reduce the incidence of dystocia. However, magnesium supplementation in pregnant cattle for the prevention of dystocia requires further study.

Nonsignificant decrease in Zn, P and increase in Ca, Cu, Fe in dystocia at the prepartum period as compared to nondystocial ones, which implies evaluation of the these element in 7<sup>th</sup> month of pregnancy does not appear to be useful in the assessment of dystocia. However, these information could be used for formulating appropriate feeding strategies in pregnant cattle to determine the nutrient requirements of the dam for prevent of dystocia.

It should be noted the variances in the timing and stage of the pregnancy are important for determination of the significant differences in mineral and hormone concentrations. For these reasons, more information is needed to determine if prevalence of dystocia could be influenced by maternal serum and hormone concentrations in cattle.

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