



## Profile of plasma biomolecules and minerals in various reproductive status of cattle and buffaloes

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### ABSTRACT

The reasons for declining fertility in dairy cattle and buffaloes are being investigated intensely to improve the productivity. The present study was conducted to assess the extent of reproductive disorders and possible causes in cattle and buffaloes. Data on various managemental aspects and reproductive status were recorded. The blood samples were collected from the animals without reproductive problems and reproductively problematic animals. The plasma was separated and analyzed for minerals, hormones and biochemical status. The study revealed that 21% of the animals had reproductive disorders, of which, repeat breeders were predominantly high (51.5%), followed by post-partum anestrus (22.8%), delayed puberty (15.74%) and silent heat (2.4%). Based on history, rectal examination and the plasma progesterone levels, silent estrus was observed in 18% (3/16) of the postpartum anestrus animals and luteal insufficiency was observed in 54% (6/11) of the repeat breeders. The blood urea nitrogen (BUN) level was higher in repeat breeders and normal animals as compared to silent heat. Overall, 50% (77/155) of the animals showed a negative energy balance irrespective of the reproductive status. The 55.5% of the postpartum anestrus (10/18) and silent heat (5/9) animals were in negative energy status. The HDL cholesterol was lower in animals with delayed puberty than in normal animals. The plasma levels of calcium (Ca), magnesium (Mg) and zinc (Zn) were lower in the silent heat as compared to normal animals. The present study revealed the importance of HDL cholesterol in attaining puberty and essential role of Mg in maintaining optimum reproductive efficiency in dairy animals.

**Key words:** Biomolecules, Cow, Fertility, Minerals, Progesterone

Dairying is an integral part of Indian agriculture under the tropical husbandry system. In dairy animals, the problems associated with reproductive disorders like delayed puberty, silent heat, postpartum anestrus and repeat breeding are attributed to imbalanced feeding, deficiency of macro and micronutrients, uterine infections, hormonal imbalances and also the poor semen quality (Selvaraju *et al.* 2012, Gowda *et al.* 2013). The importance of micronutrients and balancing of nutrients for optimum fertility had been reported by many researchers (Selvaraju *et al.* 2009, Gowda *et al.* 2013, Diskin 2016). The macro-minerals such as calcium (Ca), phosphorus (P) and magnesium (Mg) and micro-minerals such as copper (Cu),

zinc (Zn) and cobalt (Co) are essential for basic physiological function such as cell morphology, integrity, motility and metabolism apart from influencing the hormone secretion essential for reproductive function (Kong *et al.* 2015, Diskin 2016).

The plasma biomolecules such as total protein, cholesterol, blood urea nitrogen, non-esterified fatty acids, hormones, etc. play an essential role in initiation of events associated with puberty, regularization of estrous cycles, establishment and successful maintenance of pregnancy. HDL- and LDL-cholesterol are involved in transport of hormones from the site of synthesis to the site of action (Wehrman *et al.* 1991, Azhar *et al.* 1998). The levels of energy and proteins in the body fluids influence the uterine defence mechanism, gamete function and embryo survival in the reproductive tract. It is essential to study the changes in the mineral and biomolecule levels in relation to reproductive status in order to overcome the reproductive failures in dairy animals. Hence the objective of the present study was to assess the extent of reproductive problems and the role of macro- and micro-nutrients in influencing such reproductive conditions.

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## MATERIALS AND METHODS

The dairy farm animals were surveyed from different regions of Karnataka state, India (11°30'–18°30'N, latitudes and 74°–78°30'E longitude), covering 36 villages under different regions, southeastern, south coastal, north coastal, central and northern. The data were collected about management of animals (1142: crossbred cattle, 1039; buffaloes, 103) including the type of feeding, reproductive history and status, body condition score and milk yield. The rectal examination was carried out in reproductively problematic animals to ascertain the condition of reproductive tract status and the blood samples were collected for biochemical, mineral and hormone estimations to corroborate the history and rectal examination observations.

Blood samples (10 ml; n=155) from the animals of various reproductive status were collected from the jugular vein in sterile centrifuge tubes containing anti-coagulant heparin (20 units). The plasma was separated by centrifugation (310 g for 10 min) and maintained at 4°C during transportation and stored at –80°C for further analysis.

Biochemical profiling of total protein (Total protein test kit, liquid gold, Autospan), blood urea nitrogen (Autospan) and total and HDL cholesterol (cholesterol test kit) were carried out as per the protocol provided in the above-mentioned commercial kits. NEFA estimation was performed using the Cu soap method (Falholt *et al.* 1973) with slight modification. In brief, 1ml of phosphate buffer (pH 6.4, 33 mmol/l) and 6 ml of extraction solvent (chloroform, heptane and methanol in 5:5:1 ratio) were added to 50 µl of plasma sample. As a standard, 50 µl of palmitic acid (0.5 mmol in extraction solvent) was used. These tubes were shaken vigorously exactly for 90 sec, left undisturbed for 15 min followed by centrifugation at 310g. The buffer was carefully removed and 4 ml of extraction solvent, settled at the bottom of the tube was transferred to another tube to which 2 ml of Cu reagent (Cu solution stock, 500 mmol/l), 10 ml; triethanolamine solution (1 mol/l), 10 ml; sodium hydroxide solution (1 mol/l), 6 ml; to the above mixture, 33 g of sodium chloride was added, solution was made up to 100 ml and pH was adjusted to 8.1. Thereafter it was shaken vigorously for 5 min followed by centrifugation at 174g for 5 min and 3 ml of supernatant was transferred to a tube containing 0.5 ml of 1, 5 Diphenyl carbazide solution (4 g/l in ethanol). After mixing carefully, the tubes were incubated for 15 min, and the optical density was read at 550 nm in UV spectrophotometer (Libra S32, Biochrom, UK).

The minerals such as Ca, Mg, Zn, Fe and Cu were estimated in plasma samples by mild acid digestion protocol (Seo *et al.* 2014). In brief, the plasma samples (1 ml) and 2% nitric acid (1 ml) were mixed well and final volume was made up to 10 ml with deionized water and concentrations of all the elements of the sample studied by ICP-OES (Inductively Coupled Plasma-Optical Emission

Spectrometry, Optima 8000, Perkin-Elmer, USA). Plasma P was estimated calorimetrically (Fiske and Subbarow 1925). Briefly, 0.2 ml of plasma was precipitated with 10 ml of 10% TCA and the 5 ml filtrate was used for estimating phosphorus. The plasma inorganic phosphorus was converted to phosphomolybdate on addition of 0.5 ml of 1% ammonium molybdate and subsequently reduced to molybdenum blue on addition of 0.5 ml of 30% stannous chloride. The absorbance was recorded at 660 nm (Libra S32, Biochrom, UK).

The progesterone assay was carried out in blood plasma samples using commercial RIA kit (Immunotech, France). The minimum sensitivity of the kit was 0.03 ng/ml. The intra-assay and the inter-assay variations were 8.15% and 8.66%, respectively.

The analysis was performed using SPSS software (v 23.0, SPSS South Asia Pvt. Ltd). One-way ANOVA with Least Significant Difference was used to assess the difference between groups using Duncan post-hoc test. The significance was set at  $P < 0.05$ . The graphical representation was performed using GraphP Prism software (v 5.0). The values are presented as mean  $\pm$  SEM.

## RESULTS AND DISCUSSION

The study revealed that 21% of the animals had reproductive disorders. Of which, repeat breeders (51.5%) were predominantly high, followed by post-partum anestrus (22.8%), delayed puberty (15.74%), silent heat (2.4%) and other reproductive disorders (7.56%) like cystic ovarian degeneration, under developed genitalia and endometritis. The plasma progesterone analysis coupled with rectal examination indicated that among the postpartum anestrus animals, 18% had silent heat (animals with palpable CL and serum progesterone level more than 1 ng/ml). It was observed that 54% of the repeat breeding animals had luteal insufficiency (these animals were in estrus 10–12 days before rectal examination and had palpable corpus luteum during rectal examination, but the progesterone levels were less than 1 ng/ml). Overall the animals surveyed, the repeat breeding condition (%) in Central (14.3) was the highest, followed by southeastern (13.3), north coastal (13.1), south coastal (11.8) and northern (2.57) regions.

The BUN levels were higher ( $P < 0.05$ ) in normal and repeat breeders as compared to silent heat ( $P = 0.03$ ) and delayed pubertal ( $P = 0.07$ ) animals (Table 1). The lower BUN level in delayed pubertal animals could be due to lowered microbial protein synthesis in the rumen. The protein adequate diet and rumen nitrogen for protein synthesis is an important factor for attaining puberty (Hall *et al.* 1995).

HDL cholesterol was lower ( $P < 0.05$ ) in animals with delayed puberty than normal, repeat breeder and silent heat animals. This signifies the importance of nutrient status for attaining puberty. The HDL cholesterol from the blood is transported to the follicular fluid and could be the source of cholesterol for steroidogenesis of the granulosa cells (Azhar *et al.* 1998). The LDL cholesterol cannot pass

Table 1. Plasma biochemical concentration (Mean±SEM) in dairy animals of various reproductive status in Karnataka

Group	BUN (mg/dl)	Protein (g/dl)	Cholesterol (mg/dl)	HDL cholesterol (mg/dl)	NEFA (mmol/l)
Normal (n=67)	11.9±0.6 <sup>a</sup>	6.7±0.1	164.3±6.3	69.6±3.1 <sup>bc</sup>	0.46±0.01
Repeat breeder (n=44)	11.5±0.7 <sup>a</sup>	6.7±0.1	173.0±7.5	80.5±4.4 <sup>c</sup>	0.46±0.01
Postpartum anoestrus (n=18)	11.2±1.2 <sup>ab</sup>	6.6±0.1	150.4±11.3	62.3±5.9 <sup>ab</sup>	0.48±0.01
Delayed puberty (n=15)	9.5±1.2 <sup>ab</sup>	7.0±0.3	155.1±11.1	50.3±3.7 <sup>a</sup>	0.46±0.01
Silent heat (n=9)	8.1±0.5 <sup>b</sup>	6.9±0.1	156.4±10.5	75.2±7.5 <sup>bc</sup>	0.47±0.02
Normal values range	6.0–18.0	6.0–9.0	70.0–200.0	60.0–100.0	Varies, <0.2–0.3

The superscript (a,b,c) within a column for a particular parameter differ significantly ( $P<0.05$ ). BUN, Blood urea nitrogen; HDL cholesterol, high density lipoprotein-cholesterol; NEFA, non-esterified fatty acids.

through the blood-follicle wall barrier (Savion *et al.* 1982). A positive correlation between blood HDL-cholesterol and follicular fluid HDL cholesterol indicated that the HDL cholesterol is the major source of biomolecules for steroid hormone synthesis (Wehrman *et al.* 1991). The HDL cholesterol as compared to LDL cholesterol is also higher in follicular fluid of an estrogen active follicle (Wehrman *et al.* 1991). The low level of HDL cholesterol in delayed pubertal animals indicated the importance of HDL cholesterol in follicular steroidogenesis and onset of puberty and also for the expression of estrous symptoms. The lowered HDL cholesterol coupled with low BUN in the plasma affected folliculogenesis and steroidogenesis in the delayed pubertal animals. Presence of HDL cholesterol may initiate better follicular growth resulting in onset of estrus in delayed pubertal animals and enhance estrous expression in silent estrus animals.

Negative energy balance was observed in 50% (77/155) of the animals irrespective of the reproductive status. In repeat breeding (22/44) (50%), postpartum anestrus (10/18) (56%), and silent heat (5/9) (56%) of the animals had a negative energy balance. This was in agreement with the earlier report that the negative energy balance during peak stage of lactation is the main reason for anestrus and supplementation of protected fat as an energy source may ameliorate this problem (Gowda *et al.* 2013). The energy balance and dry matter intake during the first month after calving determines the post-partum fertility in dairy animals, even though the level of concentrate supplementation during breeding had minimal effects on conception rate (Diskin *et al.* 2016). The plasma cholesterol

and HDL cholesterol had positive correlation in these animals.

The plasma levels of Ca ( $P<0.05$ ) and Zn ( $P=0.08$ ) were lower in the silent heat animals compared to normal animals. The plasma levels of Mg and Cu were lower ( $P<0.05$ ) in silent heat animals as compared to postpartum anoestrus animals. The plasma levels of P and Fe did not differ among various reproductive status (Table 2). The silent heat animals had higher mineral deficiency i.e. Ca, 88% (8/9), P, 66.7% (6/9), Mg 66.7% (6/9), Zn, 77.8% (7/9) and Cu, 44.4% (4/9). The low Ca in silent estrus animals may be due to depletion of blood Ca in lactating dairy cows. The deficiency in Ca is responsible for milk fever, reduction in muscle contractility which in turn affects rumen function and lower nutrient intake, thus leading to negative energy balance and poor uterine involution (Kumar 2015). The Ca and P levels in the body fluids are regulated by various hormones including vitamin-D. Parathyroid hormone (PTH) regulates the Ca absorption by stimulating bone re-absorption and by enhancing the intestinal absorption of Ca and P. Low calcitriol (vitamin D<sub>3</sub>) is also responsible for decreased Ca absorption (Blaine *et al.* 2014).

The majority (>50%) of the repeat breeders were deficient in Zn and Mg and the silent heat animals were deficient in Ca, P, Mg and Zn. However, the minerals level did not differ between the animals having normal and luteal insufficient corpus luteum (Fig. 1). The majority of the delayed pubertal animals were deficient in Mg and Zn. The study also revealed that the majority of the postpartum anestrus animals were deficient in Mg and Zn (Table 3). The mineral levels did not differ between animals with

Table 2. Plasma mineral concentration (mean±SEM) in dairy animals of various reproductive status in Karnataka

Group	Calcium (mg/dl)	Phosphorus (mg/dl)	Magnesium (mg/dl)	Zinc (mg/l)	Iron (mg/l)	Copper (mg/l)
Normal (n=64)	8.85±0.3 <sup>a</sup>	4.54±0.2	1.78±0.1 <sup>ab</sup>	1.05±0.1 <sup>a</sup>	2.09±0.1	1.34±0.1 <sup>ab</sup>
Repeat breeder (n=44)	8.57±0.3 <sup>a</sup>	4.12±0.3	1.79±0.1 <sup>ab</sup>	0.94±0.1 <sup>ab</sup>	1.98±0.1	1.32±0.2 <sup>ab</sup>
Postpartum anoestrus (n=18)	9.26±0.6 <sup>a</sup>	4.13±0.3	1.92±0.2 <sup>a</sup>	0.89±0.1 <sup>ab</sup>	1.82±0.2	1.51±0.3 <sup>a</sup>
Delayed puberty (n=15)	8.54±0.6 <sup>a</sup>	3.76±0.4	1.77±0.1 <sup>ab</sup>	0.89±0.1 <sup>ab</sup>	1.88±0.2	1.35±0.2 <sup>ab</sup>
Silent heat (n=9)	7.04±0.3 <sup>b</sup>	4.00±0.3	1.49±0.1 <sup>b</sup>	0.65±0.1 <sup>b</sup>	2.27±0.2	0.70±0.1 <sup>b</sup>
Normal values range	8.00–10.0	4.0–6.0	1.9–3.2	1.0–2.0	1.0–2.0	0.75–1.5

Superscript (a,b) within a column for a particular parameter differ significantly ( $P<0.05$ ). Superscript (a,b) within the column for particular parameter- Zn differ significantly ( $P=0.08$ ).

Table 3. Percentage of animals showing mineral deficiency in Karnataka

Group	Calcium	Phosphorus	Magnesium	Zinc	Iron	Copper
Normal (n=64)	37.5 (n=24)	35.9 (n=23)	54.7 (n=35)	45.3 (n=29)	9.4 (n=6)	32.8 (n=21)
Repeat breeder (n=44)	31.8 (n=14)	50.0 (n=22)	61.4 (n=27)	70.5 (n=31)	4.5 (n=2)	31.8 (n=14)
Postpartum anoestrus (n=18)	33.3 (n=6)	38.9 (n=7)	61.1 (n=11)	66.7 (n=12)	-	33.3 (n=6)
Delayed puberty (n=15)	33.3 (n=5)	53.33 (n=8)	60.0 (n=9)	80.0 (n=12)	-	13.3 (n=2)
Silent heat (n=9)	88.0 (n=8)	66.7 (n=6)	66.7 (n=6)	77.8 (n=7)	-	44.4 (n=4)

Values in parenthesis indicate the number of animals.

normal corpus luteum function and luteal insufficiency in repeat breeders (Fig. 1). Interestingly, more than 60% of the reproductive problematic animals had Mg deficiency. Importance of Mg in reproduction has been overlooked and the present study suggests that Mg is an essential mineral

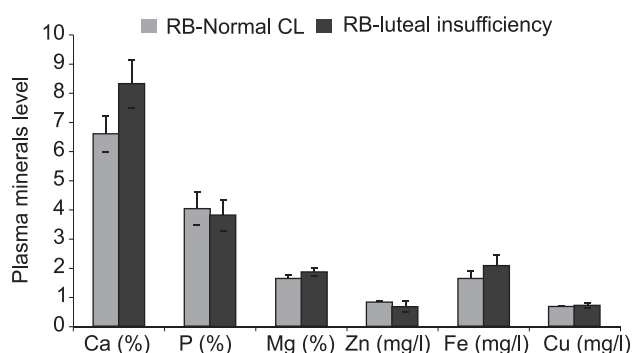


Fig. 1. The mineral levels in repeat breeding animals classified as normal corpus luteum function (n=5) and luteal insufficiency (n=6). Ca, calcium; P, phosphorus; Mg, magnesium; Zn, zinc; Fe, iron; Cu, copper.

for optimum reproductive efficiency. Deficiency of this mineral might lead to spasms in the fallopian tubes (Sas and Kovacs 1981) and may affect gametes and embryo transport in the reproductive tract resulting in either fertilization failure or early embryonic death. Mg deficiency increases cortisol levels in the blood and increases stress to the animals.

As reported in the present study, earlier studies also indicated a significant role of Mg in follicular cell mitosis (Kalmath and Ravindra 2007) and dairy cow fertility, but the specific role of Mg on dairy cow reproduction needs to be established (Patel *et al.* 2015). In superovulatory animals, good responders had high levels of Mg (Verma *et al.* 2008) suggesting the positive influence of Mg to hormonal treatment and follicular function. Increased Mg in uterine fluid than serum on day 6 and 8 of estrus (Hugentobler *et al.* 2007) indicate the critical role of Mg during a certain window of estrous cycles. The relationship between Ca and Mg may be determined based on tissue requirement.

Repeat breeding in Zn deficient animals may be attributed to early embryonic death caused by the unfavorable uterine microenvironment. Zinc deficiency in 45% normal animals, 70% of repeat breeding animals and >70% in delayed puberty and silent heat animals. Similar to the present study, Zn deficiency was reported in the animals of coastal Karnataka region (Gowda *et al.* 2001).

Zinc is an important mineral required for sexual maturity and plays a role in repair and maintenance of the uterine lining. Zinc is essential for proper functioning of the uterus (Alavi-Shoushtari *et al.* 2012) and deficiency of Zn leads to embryonic loss (Graham *et al.* 1995). It is associated

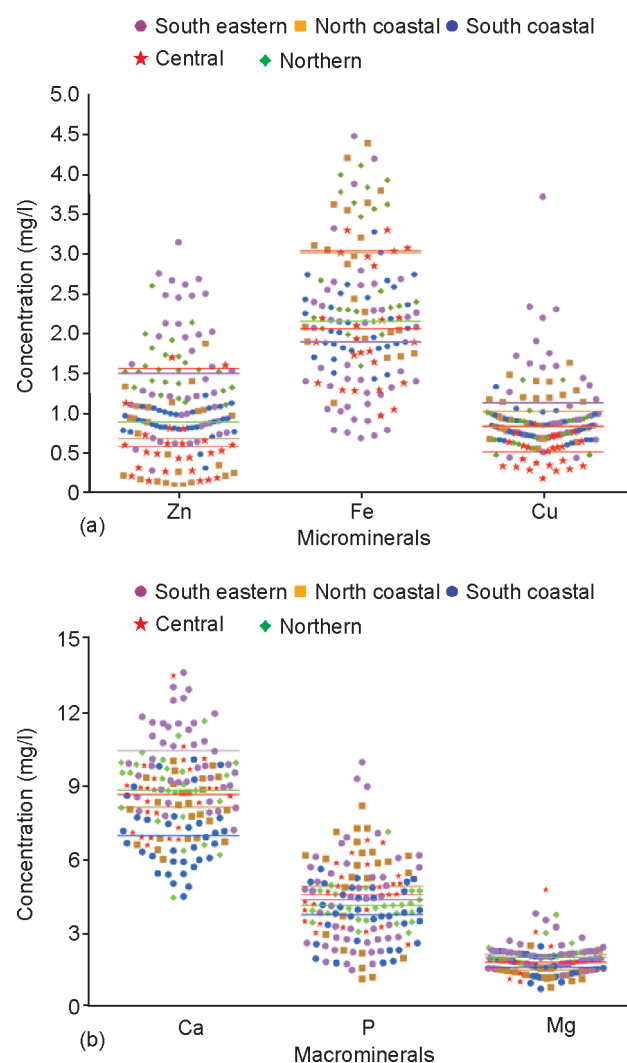


Fig. 2. (a). Microminerals (Zn, zinc; Fe, iron; Cu, copper) distributions among different study regions. The level of Zn deficiency in blood plasma was high in north coastal region and Cu deficiency was high in central region. (b). Macrominerals (Ca, calcium; P, phosphorus; Mg, magnesium) distributions among different study regions. The percentage calcium levels in blood plasma was high in south eastern region and low in south coastal region.

with carbohydrate and nucleic acid metabolism and protein synthesis (Smith and Akinbamijo 2000).

Copper is a necessary component of antioxidant enzymes such as Cu-Zn-SOD essential for maintaining tissue integrity and optimal reproductive efficiency. Trace elements like Cu and Zn are components of biomolecules, viz. ceruloplasmin and superoxide dismutase and their deficiency would lead to lowered antioxidant activity and immune response (Pal *et al.* 2014). The Cu-Zn has a role in regulating of reproductive hormones such as progesterone and estradiol (Prasad *et al.* 1989) for augmenting reproductive efficiency. Cu-Zn SOD is involved in the regulation of cell functions such as progesterone production from the luteal cells (Sugino *et al.* 2000) and altered steroidogenesis in Cu and Zn deficient animals might be the reason for poor estrus expression or silent heat. The Zn deficiency leads to retained placenta, embryonic death and irregular estrus (Mudgal *et al.* 2012).

The levels of Ca had positive correlation with Mg ( $r=0.68$ ,  $P<0.001$ ) and Zn ( $r=0.24$ ,  $P<0.05$ ) and Mg had positive correlation with Zn ( $r=0.20$ ,  $P<0.05$ ) also. Similarly, the levels of Cu were positively correlated with Mg ( $r=0.18$ ,  $P<0.05$ ) and Zn ( $r=0.38$ ,  $P<0.05$ ) whereas negatively correlated with P ( $r=-0.17$ ,  $P<0.05$ ). The levels of Fe showed positive correlation with P ( $r=0.21$ ,  $P<0.05$ ). Magnesium activates ROS production (Gulczynska *et al.* 2006) and blocks the entrance of Ca into the cells (Ng *et al.* 1987). In periparturient cows, the lower Ca level is associated with lower Mg level (Goff 2014) and similarly in the present study strong correlation was observed between plasma Ca and Mg irrespective of the reproductive status.

The regional macromineral profile analyses revealed that the southern coastal region was deficient in Ca and northern coastal region was deficient in Mg (Fig. 2b). The percentage Ca in blood plasma was high in southeastern region and low in southern coastal region. The regional micromineral profile indicated that the central region was deficient in Cu (Fig. 2a). The percentage of Zn deficiency in blood plasma was high in southern coastal region and Cu deficiency was high in central region.

The feeding practices under field conditions have a major influence on reproductive status. The occurrence of macro- and micro-nutrients deficiency and relevance of supplementing them in the diet of dairy animals has great impact on dairy animal's productive and reproductive efficiency. Strategic supplementation of minerals through feeds and fodders rich in nutrients along with a mineral mixture specific to a region has been suggested as a practical approach (Gowda *et al.* 2004). The levels of the minerals in the blood are influenced by levels of minerals in the soil, uptake by the plants and feed composition and intake by the animals. If the soil is deficient in these minerals, ultimately the level of these minerals will affect the productive and reproductive efficiency of the animals. In the animal system, the blood composition is majorly influenced by the absorption efficiency. In this regard, levels

of other minerals in the animal diet also affect the absorption and concentration of minerals in the blood.

The limitations in the present study under field conditions were inclusion of animals in different ages, breeds and parity. Animals were reared in different feeding and management status and the clinical and gynaecological observation were based on one time examination. The fixed sampling time with respect to feeding schedule was not maintained and this might also affect especially BUN levels in the animals studied.

The present study revealed that minerals and biomolecules might serve as important indicators for assessing reasons for infertility in dairy animals. The HDL-cholesterol is critical for the timely attainment of puberty and protein is essential for expression of estrous symptoms. Magnesium and Zinc could be the major minerals for successful conception in dairy animals. A balanced feeding strategies focusing on feed intake and energy are needed in order to optimize reproductive efficiency in dairy animals.

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