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## AIMS AND SCOPE

Slovak Journal of Animal Science (ISSN 1337-9984) is an international peer-reviewed scientific journal published by the National Agricultural and Food Centre – Research Institute for Animal Production Nitra.

SJAS publishes original scientific papers, reviews, and short communications on the topics of biotechnology, husbandry and nutrition of livestock animals, quality of animal products as well as animal environment and behaviour.

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Slovak Journal of Animal Science enters the second half-century of its existence with a new Editor-in-Chief and significant changes to the Editorial Board.

All further progress of SJAS will be built upon the work of past Chief Editors and members of the Editorial Board and it is with gratitude to them as well as the journal's publisher, NPPC – Research Institute for Animal Production Nitra, that I take this position. After many years of acting as a member of the Editorial Board of the journal, I wish to start my first volume as the Editor-in-Chief with a short reflection on the journal's history and a vision for its future.

Last year, Slovak Journal of Animal Science – formerly Journal of Farm Animal Science – celebrated its 50$^{th}$ anniversary. During its many years of existence as a scientific journal, it has undergone significant transformations. From annual publication in the Slovak language it expanded to quarterly issues, first in both Slovak and English, later exclusively in the English language, and it attracted international audience and contributions. Since 2006, all issues have been published online along with the print publication and the journal has also become a partner to the international scientific conference Animal Biotechnology, with the 4$^{th}$ issue of the year annually dedicated to the contributions from this event. Yet, despite the numerous changes to the journal itself as well as the institution of its publisher, SJAS has maintained its original identity and continuity. For this I wish to thank not only the publisher and the previous leadership, but also the authors and reviewers who contributed their time, work and expertise over the years.

Today, SJAS continues to aspire to publish high quality original papers, reviews and short communications from the sphere of animal science with the focus on biotechnology, husbandry and nutrition of livestock, quality of animal products as well as animal environment and behaviour. It is the journal's mission to disseminate the results of valuable research and to expand scientific knowledge. To do so effectively in the modern era requires new approaches to publishing. The aim of the already-noticeable as well as upcoming changes to SJAS is to modernize the digitalization of the entire publishing process, shorten the time from submission of the manuscript to reviews, and improve the accessibility of the articles through international databases.

In the editorial office, we look towards the future of SJAS with optimism. The journal will continue to adapt in order to best serve animal science and the scientific community, with commitment to the research integrity and the highest publishing ethics. We invite you to submit your work to SJAS as we prepare for the next 50 years of publishing.

prof. Ing. Peter Chrenek, DrSc.
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# FUNCTIONAL CHARACTERISTICS OF BOVINE SPERMATOZOA IN RELATION TO THE BODY CONDITION SCORE OF BULLS

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## ABSTRACT

This study was aimed at examining possible impact of the body condition of breeding bulls on the motility and viability of the sperm following freezing-thawing. The breeding bulls (n = 16) of Holstein and Czech Fleckvieh breeds were classified to the body condition score (BCS) grade according to a five-point scale with an accuracy of 0.25 points. The sperm samples of the bulls estimated as BCS grades 2, 3 or 4 were frozen in a programmable freezing device and stored in a liquid nitrogen for approximately one year. Following thawing the motility parameters (total motility – TM; progressive movement – PM) were analysed using the CASA Sperm Vision$^{TM}$ system at the intervals of 0, 0.5 or 2 h post-thaw. Viability parameters of sperm - plasma membrane integrity (*peanut* agglutinin, PNA-FITC), apoptotic rate (Yo-Pro-1) and dead/necrotic cell (propidium iodide /DAPI) rates were analysed at the same day using fluorescent microscope. Only minor non-significant difference was observed between BCS2 and BCS3 bull sperm in all studied parameters. However, significant differences in the sperm parameters were noted between bulls with BCS2 and BCS4. In particular, the BCS4 bulls showed significantly lower ($p < 0.5$) TM and PM than the BCS2 bulls. Moreover, significantly higher proportion ($p < 0.5$) of sperm with damaged plasma membrane and dead/necrotic sperm was revealed in the BCS4 bulls compared to the bulls of BCS2. These observations indicate that higher score of the body condition may negatively affect the quality of bull ejaculates.

**Key words:** bull; BCS; sperm; motility; viability

## INTRODUCTION

Fertility of the bull is a major factor contributing to overall reproductive performance of cattle. Predicting the fertility of bulls is one of key area of agricultural research. Semen quality varies greatly from bull to bull. Semen from certain bulls may be of acceptable quality at collection but does not survive cryopreservation. The freezing and thawing process can adversely affect the nucleus, plasma membrane, acrosomal and mitochondrial membranes of spermatozoa (Chatterjee *et al.*, 2001; Aires *et al.*, 2003; Amirat *et al.*, 2004). This can adversely affect processes required for successful *in vivo* fertilization of the oocyte (Bailey *et al.*, 2000).

Previous studies showed that many factors affect semen quality and the bull fertility including scrotal circumference (abnormal scrotal thermoregulation; Coulter *et al.*, 1997), sperm

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morphology, motility, physical normality of the bull and his mating ability, libido, age and the body condition (Barth *et al.*, 1995). The evaluation of body condition score (BCS) is an assessment of the proportion of muscle and of body fat, which the male has, and it is determined on a scale of 1 to 5, where "1" – being very thin and "5" – being overweight. BCS, or degree of fatness, seems to be the most reliable indicator of well-being of an animal, and, when coupled with changes in body weight, provide a useful indicator to assess reproductive potential (Dunn and Moss, 1992). If the animal is too thin or overweight, breeding strength and quality of the ejaculate may be affected (Neary and Yager, 2002). The quality of bull semen may be positively affected by quality and level of livestock feed. Production of quality ejaculates requires a diet which ensures adequate nutrition levels and the sufficient amount of essential vitamins and mineral substances. Bulls in poor body condition have lower fertilizing ability (Neary and Yager, 2002).

There are only few reports about effect of BCS on sperm characteristics and fertilizing ability of beef bulls (Addas, 2011; Barth and Waldner, 2002; Dunn and Moss, 1992). However, there are no sufficient data about effect of BCS in bulls on the sperm viability in regards to fluorescent markers of the cell death, membrane status and CASA-measured motility. The aim of this study was to evaluate effect of body condition score of breeding bulls on several functional parameters of spermatozoa.

## MATERIAL AND METHODS

### Semen collecting and processing

The observations were made in a bull housing facility and laboratory at a single AI center, where 8 Holstein (H) and 8 Czech Fleckvieh (F) bulls were selected for monitoring. The selected bulls were from 1 to 6 years old and the frequency of semen collection for all was once weekly. One sample of ejaculate was obtained from each bull using an artificial vagina. The BCS of selected bulls was evaluated by a five point scale with an accuracy of 0.25 points at the time of ejaculate collecting according to methodology of body condition scoring especially for H and F breeds, respectively. The optimum BCS on five point scale differed between evaluated breeds in relation to different requirements in accordance with production type - milk in H and dual purpose in F. The volume of semen samples (VOL) was measured using an electronic scale (Scout Pro, OHAUS®), sperm concentration (DEN) using a spectrophotometer (GENESYS 10vis, Thermo Scientific®), and percentage of motile spermatozoa (ACT) subjectively by phase contrast microscopy (LP 3000, Arsenal®) immediately after collecting. In addition, we evaluated the percentage of live sperm by staining before diluting and freezing in accordance with standard methodology as following: a drop of semen was mixed with eosin on a preheated microscope slide, spread, then examined under a phase contrast microscope at 1000x magnification and with oil immersion. We classified a minimum of 100 spermatozoa as either dead (with red heads) or live (with white heads) and expressed this as a percentage rate.

Only fresh semen with required quality (minimum progressive motility 70 % and sperm concentration $0.7 \times 10^6$ $mm^{-3}$) was used for the subsequent processing of samples for observation according to common standards used for producing AI doses. The samples of semen were diluted with AndroMed® (Minitüb, Tiefenbach, Germany), a commercially produced extender containing soybean lecithin extract. Polyvinyl chloride (PVC) straws (0.25 $cm^3$; IMV) were filled, cooled to 4 °C and equilibrated for 90 min. Subsequently, they were frozen in a programmable freezing device (IMV-Digitcool, L'Aigle, France) then plunged into liquid nitrogen for storage.

### Sperm motility analysis

The straws were thawed in a water bath at 37 °C for 1 min. Sperm total motility and progressive movement were measured using a CASA system (Sperm Vision, Minitub Slovakia Ltd) at 0, 0.5 and 2 h after removal from storage. Between these time points the samples were incubated in a saline with 1 % of foetal calf serum (saline-FCS) at 37 °C in the incubator. Spermatozoa were transferred by a pipette into a Leja counting chamber with the depth of 10 µm. The chamber was placed under a Zeiss Axioscope A.1 phase-contrast microscope (Carl Zeiss NTS GmbH, Oberkochen, Germany) with heating plate (37 °C) at 200x magnification. The camera transferred the image into a computer,

where sperm motility was determined by the Sperm Vision software. In each sample at least 6 view fields were counted. The values of a total motility (M) and progressive movement (PM), measured at several time points (0, 0.5 and 2 h) during the day, were summarized and the average values per each day were presented in the tables.

**Fluorescent assays**

For plasma membrane integrity the sperm samples were stained with fluorescence-labelled lectin - *Arachis hypogaea* peanut agglutinin (PNA-FITC; Molecular Probes, Lucerne, Switzerland) in combination with propidium iodide (for the detection of dead/necrotic cells). The sperm samples were unfixed, allowing PNA-FITC labelling only in spermatozoa with disrupted or otherwise damaged plasma membrane, whilst sperm cells with intact membranes remained unstained. Following washing in saline-FCS, the sperm suspension was incubated in staining solution containing 20 µmol.l$^{-1}$ of PNA-FITC and 5 µg.ml$^{-1}$ PI in saline-FCS for 20 min at room temperature. After the incubation, sperm samples were washed in saline and, following centrifugation, 4 µl of the sperm suspension was placed onto a microscope slide, gently mixed with 4 µl of Vectashield mounting medium with DAPI (H-1200, Vector Laboratories Inc., Burlingame, CA, USA), a blue-fluorescent DNA stain which marks nucleoplasma of all sperm cells in samples. The obtained drops were flattened with a coverslip and immediately observed under a Leica inverted fluorescent microscope with respective bandwidth filters for green, red and blue fluorescence.

For detection of apoptosis the sperm were stained with a Yo-Pro-1 specific green fluorochrome (Molecular Probes, Lucerne, Switzerland) in combination with PI for identification of dead sperm cells. Following washing in saline-FCS the sperm suspension was incubated in a staining solution: saline-FCS with 5 µmol.l$^{-1}$ Yo-Pro-1 and 5 µg.ml$^{-1}$ of PI. After 20 min staining at room temperature, the sperm samples were washed in a saline-FCS solution and 4 µl of sperm suspension were placed onto a microscope slide, mixed with 4 µl of Vectashield with DAPI and the drop was flattened with a coverslip. The preparations were immediately evaluated under a Leica fluorescent microscope (Leica Microsystems, Wetzlar, Germany) with special filters for green, red and blue fluorescence. The green fluorescing sperm cells were regarded as apoptotic cells. The cells colored pink (stained by propidium iodide) were considered as dead or necrotic cells.

**Statistics**

The results of a whole or progressive motility were statistically processed after summarization of the values obtained from several measurements. For sperm motility analysis at least 7 view fields per each group were evaluated (at least 750 sperm cells per one experiment). Average values were calculated from three measurements during the day. The sperm images were made by a camera equipped with the Leica fluorescent microscope from and number of sperm cells was counted from a PC monitor. The experiments were performed in five repeats. In each experiment, about 8 - 10 microscopic view fields per each group were photographed. Totally, more than 1500 spermatozoa per each group were counted. Comparisons of arithmetic means between BCS groups were performed by a repeated measure ANOVA and t-test. The statistical analysis was performed with original data using the Statistix analytical software (Version 8.0; Anonymous, 2001). The data in table are represented as the mean ± standard error of the mean.

## RESULTS

**Sperm motility characteristics**

The tested bull (n = 16) were arranged into three groups according to their body condition score (BCS) as belonging to BCS2 (n = 6), BCS3 (n = 6) and BCS4 (n = 4). All examined bulls showed average sperm motility (M) after thawing 42.5 % and progressive movement (PM) was 39.6 % (Table 1.). The higher average motility and progressive movement ($p < 0.05$) were observed in the group of the bulls with BCS2 when compared with the BCS4 bulls. The lowest sperm total M and PM was determined in the BCS4 bulls. In the group of BCS3 bulls an average total M and PM were about 42 % and 39 %, respectively.

**Table 1. Sperm motility parameters in relation to BCS of bulls (mean ± S.E.M.)**

| BCS of bulls | No. bulls | Motility | Progressive movement |
|---|---|---|---|
| BCS2 | 6 | 56.26 ± 2.21[a] | 52.93 ± 2.37[a] |
| BCS3 | 6 | 42.21 ± 2.06 | 39.31 ± 2.02 |
| BCS4 | 4 | 28.94 ± 1.98[b] | 26.61 ± 1.86[b] |
| Average | 16 | 42.47 ± 2.08 | 39.62 ± 2.10 |

a versus b – significant difference between BCS groups at $p < 0.05$

**Sperm viability characteristics**

Similar trends were observed at the evaluation of sperm viability characteristics according to BCS (Table 2.). The ratio of sperm with disintegrated plasma membrane (PNA-positive) was significantly lower ($p < 0.05$) in the BCS2 bulls than in the BCS4 bulls. Similarly, the occurrence of dead/necrotic spermatozoa was lowest in the BCS2 bulls. The highest ratio of apoptotic sperm (Yo-Pro-1-positive) was revealed in the BCS3 bulls. In this group of bulls the ratio of dead/necrotic, apoptotic and the sperm with disintegrated plasma membrane was higher compared with the BCS2 bulls. On the other hand, the highest proportion of dead/necrotic and plasma membrane-disintegrated sperm was observed in the BCS4 bulls.

**Table 2. Sperm viability parameters in relation to BCS of bulls (mean ± S.E.M.)**

| BCS of bulls | No. bulls | PNA | Yo-Pro-1 | PI |
|---|---|---|---|---|
| BCS2 | 6 | 24.03 ± 2.30[a] | 15.62 ± 1.83 | 12.63 ± 1.49[a] |
| BCS3 | 6 | 27.07 ± 2.08[a] | 18.85 ± 1.66 | 15.22 ± 1.62[a] |
| BCS4 | 4 | 35.06 ± 2.72[b] | 15.37 ± 1.56 | 20.25 ± 1.85[b] |

a versus b – significant difference between BCS groups at $p< 0.05$

**Correlations among sperm characteristics**

Highly significant ($p < 0.0001$) positive correlation was observed between the total motility and progressive movement in a whole population of bulls, irrespective to BCS. Moderate negative correlation was observed between the motility and sperm viability parameters: the PNA-, PI- and Yo-Pro-1-positive spermatozoa (Table 3.). When BCS was taken into consideration, the highly significant ($p < 0.0001$) positive correlation was noted between the total motility and progressive movement in the BCS2 and the BCS3 bulls. The high negative correlation between total motility and the apoptotic sperm ratio was determined in the BCS2 bulls ($r = -0.7458$). In the BCS3 group, the high negative correlation was observed between total motility and dead/necrotic sperm ($r = -0.7019$). In the BCS4 bulls a correlation was not determined due to low number of animals available in this group).

**Table 3. Correlations between the sperm motility and other perm parameters according to BCS of bulls**

| BCS of bulls | M/PM r | M/PNA r | M/Yo-Pro-1 r | M/PI r |
|---|---|---|---|---|
| BCS2 | 0.996* | -0.358 | -0.746 | -0.213 |
| BCS3 | 0.997* | -0.626 | -0.530 | -0.702 |
| BCS4 | N/D | ND | ND | ND |
| Average | 0.998* | -0.634 | 491 | -0.584 |

r - Pearson´s correlation coefficient (t-test); no correlation – 0.111 ± 0.333; moderate correlation – 0.334 ± 0.666; high correlation – 0.667 ± 0.999; * $p < 0.0001$; N/D – not determined; M- motility; PM – progressive movement

## DISCUSSION

In this study we investigated the impact of BCS of breeding bulls on motility and viability characteristics of spermatozoa after freezing-thawing. Sperm motility is the most important indicator of the ejaculate quality. Motility of 30 % or higher is considered to be of acceptable quality (Person *et al.*, 2007). Good post-thawing motility parameters in association with low proportion of dead, apoptotic spermatozoa, or spermatozoa with damaged plasma membrane contribute to an improvement of fertilizing ability and higher percentage of embryo cleavage rate (Watson, 2000).

The results of our study demonstrate possible negative effect of higher body condition score of bulls on sperm total motility and progressive movement. Bulls with BCS4 showed considerably lower motility and progressive movement than the bulls with BCS2 or BCS3. Similarly, Coulter *et al.* (1997) reported that bulls, fed with a high-energy diet (having high BCS), showed decreased proportion of motile sperm and very poor progressive movement than bulls fed a moderate-energy diet.

Besides good motility characteristics, low content of apoptotic and dead/necrotic sperm in the ejaculate is also very important for proper fertilizing ability. Presence of high percentage of apoptotic spermatozoa in the semen dose could be one of the reasons for poor fertility of breeding bulls (Anzar *et al.*, 2002) and, similarly, spermatozoa with damaged or inactive membranes will have limited viability and fertilizing potential (Correa and Zavos, 1994). In our study, high negative correlation was found between the total motility and apoptotic sperm occurrence in BCS2 bulls. Similarly, negative correlations between motility and apoptotic sperm rate was determined by Zhang *et al*. (2008) on human spermatozoa. No large difference in the proportion of apoptotic sperm was recorded among tested groups in our experiments. This apoptosis occurrence was the highest in the BCS3 bulls.

For successful fertilization, high proportion of sperm with the intact membrane is an essential requirement for proper sperm cell function (Makarevich *et al.*, 2011). Significantly higher ratio of sperm with damaged plasma membrane was revealed in BCS4 bulls compared to BCS2 group. Only moderate negative correlations were confirmed between PNA-positive sperm and motility rate in the BCS2 and BCS3 bulls. Alm *et al.* (2001) found low but significant correlation between the proportion of viable cells and fertility, suggesting that the plasma membrane integrity evaluation can serve as a quality control method for frozen-thawed spermatozoa in breeding bulls.

The present results imply that overfeeding could adversely affect ejaculate, because overfed bulls have lower semen quality than underfed bulls. The BCS4 bulls showed higher occurrence of dead/necrotic spermatozoa than bulls from BCS2 or BCS3 groups. Similarly, other studies on beef bulls (Barth and Waldner, 2002) demonstrated that significantly fewer bulls with BCS2 or BCS4 grade or greater had satisfactory semen quality than bulls with a BCS3 grade (where satisfactory semen quality comprises at least 70 % of morphologically

normal sperm with the progressive movement of at minimum 60 %). Bulls fed a high-energy diet (having high BCS) showed greater proportion of sperm with various morphological defects than bulls fed a moderate-energy diet (Swanepoel *et al*., 2008).

Body condition or degree of fatness seems to be the most reliable indicator of well-being of an animal, and, when coupled with changes in body weight, provides a useful key to assess reproductive potential (Dunn and Moss, 1992). Our results support an idea that high body condition of breeding bulls may have a negative effect on reproductive characteristics of the ejaculate.

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# RESPONSE OF BROILER CHICKEN TO *IN OVO* ADMINISTRATION OF INORGANIC SALTS OF ZINC, SELENIUM AND COPPER OR THEIR COMBINATION

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## ABSTRACT

This study determined the hatchability, post-hatch growth performance, immune response and bone morphometry of broiler chicken to *in ovo* administration of inorganic salts of zinc, selenium, copper or their combination. A total of 330 fertile eggs of Cobb broiler strain were procured from commercial breeder hatchery, fumigated, weighed and set in the incubator. The eggs were candled on the 14$^{th}$ day of incubation and distributed into five treatment groups: Control (without *in ovo* administration), Group II, *in ovo* administration of 80 µg.egg$^{-1}$ inorganic zinc; Group III, *in ovo* administration of 0.3 µg.egg$^{-1}$ inorganic selenium; Group IV, *in ovo* administration of 16 µg.egg$^{-1}$ inorganic copper and Group V, *in ovo* administration with the combination of the inorganic salts of zinc, selenium and copper. The *in ovo* administration was carried out on the 18$^{th}$ day. The post-hatch chicks were distributed into 5 treatment groups of 6 replicates containing 7 chicks each. Data obtained were subjected to Analysis of Variance in a completely randomized design. The results showed highest percentage of hatchability in Zn-injected hatching eggs. The final weight of birds from Zn-injected eggs was significantly ($P < 0.05$) highest on day 7. On day 35, the final weight and weight gain were significantly ($P < 0.05$) affected by the *in ovo* administration of Zn, Se, Cu and their combination with the highest values obtained in birds on Cu-injected eggs. Birds from Zn-injected eggs, Cu-injected eggs and eggs on the combination of the inorganic salts had significantly ($P < 0.05$) highest proportion of heart than obtained in the control. The tibiae ash of birds from Zn and Se-injected eggs recorded the highest values of 40.96 and 40.37 %, respectively, while the lowest value (35.47 %) was recorded in the tibiae of birds from eggs injected with the combination of the inorganic salts. It was concluded that Zn impacts mostly on hatchability, but the combination of the mineral sources at the ratio injected impacted negatively the growth performance of the broiler chickens.

**Key words:** hatchability; *in ovo* injection; inorganic salts; broiler chicken; gut morphology; tibiae mineralization

## INTRODUCTION

The period of embryonic development becomes a greater proportion of a bird's life to match up with the decreasing generation interval that takes meat birds to achieve market size. Most poultry research (Peebles *et al*., 2005; Collin *et al*., 2007; Elibol and Brake, 2008) is now designed towards realizing gains in genetic and production potential of poultry from advancements made during the incubation period and embryogenesis.

Under a practical condition, birds have access to feed after hatching only between 3 and 4 days and this brings about reduction in the body weight, while the intestine and muscle development are also retarded. Noy and Uni (2010) then suggested that a continuous feeding process could be established to ensure continuous supply of nutrients *in ovo* to the developing embryo, feed and water to the newly hatched chicks within the hatchery. The *in ovo*

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injection is, thereby, adopted and widely used for many purposes, such as fertilizing an avian egg in the shell (Cantrell and Wooten, 2003), injecting avian eggs with immunological material (Jochemsen and Jeurissen, 2002), a trial for sex reversal in birds (Kagmi and Hanada, 1997), increasing the post-hatching body weights of birds by *in ovo* injection of growth promoters (Ohta *et al*., 1999) and enhancing the growth of avian embryo by injecting eggs with a special liquid as nutritional supplements. Nutrients in *in ovo* injection have a lot of benefits: greater efficiency of feed utilization (Bhanja *et al*., 2004); reduced post-hatch mortality and morbidity, improved immune response (Gore and Qureshi, 1997), enhanced early growth by improving intestinal function and development through an enhanced absorption by the villi (Tako *et al*., 2004; Noy and Uni, 2010), and increased skeletal growth (Hargis *et al*., 1989), breast muscle yield (Hajihosaini and Mottaghitalab, 2004) and marketing body weight (Selim *et al*., 2012).

It is noteworthy that the rapid growth rate and increasing incidence of leg problems in broiler chickens are highly related to an acceleration of bone deposition at the periosteal surface, which increases the porosity of the cortical bone, subsequently causing poorer biomechanical properties of the bone (Williams *et al*., 2004). Micro-minerals that are of major importance to bone formation and strength include Cu, Zn, and Mn, but they are greatly reduced in concentration in the egg by the 17$^{th}$ day of incubation (Yair and Uni, 2011). These minerals also participate through their contribution to enzyme activity along metabolic pathways that are related to the formation of the skeletal system (Bao *et al*., 2007). The metalloenzymes of which zinc is grouped, play an important role in the bird's immune response and in hormone production (O'Dell, 1981). The involution of the thymus and small spleen weights are characterized by zinc deficiency and primarily the absence of white cells (Vruwink *et al*., 1993). The authors also reported that inadequate intracellular concentration of zinc also causes damage to the lymphocyte function that is responsible for the ability of T-and B- cell proliferation. Zinc is noted to be responsible for normal growth and maintenance and includes among other functions bone development, feathering, enzyme structure and function as well as appetite regulation for all poultry (Batal *et al*., 2001). Zinc sulphate is highly water soluble, allowing reactive metal ions to promote free-radical formation, which can facilitate reactions that lead to the breakdown of vitamins and ultimately to the degradation of fats and oils, decreasing the nutrient value of the diet (Batal *et al*., 2001). Although, zinc interferes with iron metabolism in chicks, iron-deficient chicks are more susceptible to the effects of zinc toxicity than are iron-adequate chicks (Blalock and Hill, 1988).

Rotruck *et al*. (1973) reported that selenium was first associated with toxicity in 1950's but its importance in the diet was elucidated when it was deemed essential in the prevention of liver necrosis in rats. This was further strengthened in the 1970's, when it was found to be an essential component of the enzyme glutathione peroxidase. Since selenium (Se) deficiency in the poultry diets has shown to cause several pathological conditions that can impact growth and development, research is thereby focused on how to ensure its adequacy. Se is often added to the animal's diet using inorganic Se ($Na_2SeO_3$). It has been reported that Se is readily transferred from breeder hens to the eggs and thus, to the embryo (Paton *et al*., 2002). The authors further reported that the amount of Se that can be derived from the hen's diet is limited, because the maximum level of dietary Se supplementation is limited to 0.3 ppm by the FDA (2002). Hence, the introduction of Se *in ovo* to the incubating embryo was found to be a suitable alternative.

Copper is essential for the normal maturation of collagen (Rucker and Murray, 1978), hence laying hens fed a copper-deficient diet produced eggs with abnormal shell membrane (Baumgartner *et al*., 1978). The activation of lysil oxidase also appears to involve a role of copper as cofactor, which may also act on the regulation of lysil oxidase synthesis (Harris, 1976). A well-structured outer shell membrane is required for mineralization and strong eggshells. In addition, adequate membrane structure allows the separation between the inner and the outer shell membranes, promoting the proper formation of the air chamber immediately after oviposition. This is an important oxygen reservoir used by the embryo during pipping. Copper in the shell seems to be essential for the chick embryo metabolism, as it was shown that shell-less cultures led to a failure in accumulation

of normal amounts of hepatic copper during the latter half of incubation (Richards *et al*., 1984). Copper was also found to play vital role in haemoglobin synthesis and it is associated with many enzymes (Gaetke and Chow, 2003). In the findings of Goel *et al*. (2013), Cu was found to enhance the immune response of broiler chickens on *in ovo* administration of 8 µg.egg$^{-1}$ of inorganic Cu ($CuSO_4$).

Most studies on trace mineral requirements in poultry production (Nollet *et al*., 2007; Jegede *et al*., 2011) focused on supplementation of the diets and not the embryo with a single trace mineral. However, supplementation with a single trace mineral could be a disadvantage because of negative interactions, so that over-supplementation of one trace mineral will interfere with other trace minerals' availability (Watts, 1990; Scheideler, 1991). The most common antagonism occurs between Zn and Cu, and a ratio greater than 4:1 of Zn/Cu can be considered antagonistic (Scheideler, 1991). The author further stated that high levels of dietary Zn will inhibit Cu absorption, resulting in hepatic accumulation and deposition in the egg. High levels of Cu and Fe can interfere with Zn availability and potentially induce anaemia in poultry. Hence, this study assessed the effects of *in ovo* administration of inorganic Zn, Se, Cu and their combination on the growth performance, development of the gastro-intestinal tract, carcass yield, bone (tibia) morphology and mineralization and *in vivo* immune response of broiler chickens.

## MATERIALS AND METHODS

The entire experiment including the feeding trials of broiler chicken were carried out at the Experimental Livestock Unit, Indian Council of Agricultural Research - National Institute of Animal Nutrition and Physiology (ICAR-NIANP), Bengaluru, Karnataka, India.

### Hatching of eggs

Three hundred and thirty fertile eggs of Cobb strain of broiler chickens procured from the commercial breeder hatchery were fumigated, weighed and set into the incubator. On the 14$^{th}$ day of incubation, the eggs were candled and eggs showing viable embryo were distributed into five groups of control and *in ovo* supplemented groups (Table 1).

### *In ovo* supplementation

On 18$^{th}$ day of embryonic age, the eggs showing viable embryo were injected with nutrients into amnion using a 24-gauge hypodermic needle (25 mm long) under laminar flow system, with handling temperature not lower than 35 °C (Bhanja *et al*., 2004). The *in ovo* injection of each treatment was completed within 30 minutes of taking out from the incubator. Before injection, the site was suitably sterilized and the injection was done at the broad end of the egg. Following *in ovo* feeding, the injection site was sealed with a sterile paraffin and the eggs were transferred to hatching compartment.

**Table 1. Groups of eggs for *in ovo* injection**

| Treatment group | *In ovo* injection |
|---|---|
| Group I | Control |
| Group II | *In ovo* supplementation with 80 µg.egg$^{-1}$ of inorganic Zinc (Zn sulphate 351.80 µg. 0.5 ml$^{-1}$ deionised water) |
| Group III | *In ovo* supplementation with 0.3 µg.egg$^{-1}$ of inorganic Selenium (Sodium Selenite 0.657 µg. 0.5 ml$^{-1}$ deionised water) |
| Group IV | *In ovo* supplementation with 16 µg.egg$^{-1}$ of inorganic Copper (Copper Sulphate 62.87 µg. 0.5 ml$^{-1}$ deionised water) |
| Group V | *In ovo* supplementation with 80 µg.egg$^{-1}$ of inorganic Zinc, 0.3 µg.egg$^{-1}$ of inorganic Selenium and 16 µg.egg$^{-1}$ of inorganic Copper |

**Post-hatch chick**

A total of 265 eggs were fertile from the 298 egg set (88.93 % fertility) and a total of 234 chicks hatched from 260 fertile eggs (90.00 % hatchability) transited to the hatching compartment. Post-hatch chicks were distributed into 5 treatment groups (Table 1), into 6 replicates of 7 chicks per replicate, and reared in electrically heated battery cages with a provision of wire mesh floor, feeders and waterers under uniform and standard management condition. Standard broiler pre-starter (0-7 d), starter (7-21 d) and finisher (21-35 d) diets were prepared with maize and soybean meal, as the major ingredients (Table 2). Feed and drinking water were provided *ad libitum*. The experiment lasted for 35 days.

**Assessment of the following parameters**

i) Hatching: Egg weight, percentage of hatchability, chick weight and chick-to-egg ratio.

The percentage of hatchability was calculated as follows:

% Hatchability = (№ of hatched chicks/№ of fertile eggs) *100

ii) Growth: Weekly body weight and feed intake were recorded. The feed conversion ratio (FCR) was calculated using the formula:

FCR = (Feed intake/weight gain)

iii) Gut morphometry: On 7$^{th}$ day post-hatch, one chick from each replicate and on 35$^{th}$ day post-hatch, two birds from each replicate were slaughtered by cervical dislocation for gut development studies. Gut morphometry was done by recording the weights of gizzard, proventriculus, liver as well as the weight and length of the duodenum, jejunum, ileum and caecum.

**Table 2. Ingredient and nutrient composition (%) of experimental diets**

| Ingredient | Pre-starter (0-7 days) | Starter (1-3 wk) | Finisher (3-5 wk) |
|---|---|---|---|
| Maize | 57.00 | 58.60 | 62.50 |
| Soybean meal | 37.00 | 36.10 | 31.50 |
| Fat / oil (soybean oil) | 1.87 | 1.65 | 2.20 |
| Limestone | 1.00 | 1.00 | 1.10 |
| Dicalcium Phosphate | 1.75 | 1.75 | 1.75 |
| Salt (NaCl) | 0.35 | 0.35 | 0.35 |
| Lysine | 0.40 | 0.10 | 0.12 |
| Methionine | 0.20 | 0.20 | 0.20 |
| Threonine | 0.18 | 0.00 | 0.00 |
| *Vit & Minerals premix | 0.25 | 0.25 | 0.28 |
| Total | 100.00 | 100.00 | 100.00 |
| ME (kCal.kg$^{-1}$) | 2995.50 | 2991.50 | 3047.75 |
| CP (%) | 22.51 | 21.89 | 20.07 |
| Lysine (%) | 1.52 | 1.26 | 1.15 |
| Methionine (%) | 0.55 | 0.51 | 0.44 |
| Threonine | 0.98 | 0.78 | 0.72 |
| Tryptophan | 0.23 | 0.23 | 0.21 |
| Valine | 0.89 | 0.88 | 0.80 |
| Arginine | 1.37 | 1.35 | 1.21 |
| Ca (%) | 1.00 | 1.00 | 1.00 |
| P, avail. (%) | 0.45 | 0.45 | 0.45 |

*Trace mineral premix 0.1 %, Vit. Premix 0.1 %, B - Complex 0.02 %, Choline 0.05 % and Salt 0.3 %
Trace mineral premix supplied mg.kg$^{-1}$ diet: Mg, 300; Mn, 55; I, 0.4; Fe, 56; Zn, 30; Cu, 4
The vitamin premix supplied per kg diet: Vit. A, 8250 IU; Vit. D3,1200 ICU; Vit. K, 1 mg; Vit. E, 40 IU; Vit. B1, 2 mg; Vit. B2 4 mg; Vit. B12, 10 mcg; niacin, 60 mg; pantothenic acid, 10 mg; choline, 500 mg

iv) Carcass characteristic and weight of immune organs:

Two birds from each replicate were sacrificed through cervical dislocation to evaluate

a) Eviscerated yield, cut-up parts yield (breast, back, drumsticks, and thighs) and giblet yields (gizzard, heart and liver).
b) Weight of immune organs: Weight of lymphoid organs (spleen and bursa only) was expressed as mg.100 $g^{-1}$ live weight at the 35$^{th}$ day.

v) Bone morphology and mineralization

*Tibia-Osteo - morphometry*

(i) Length.
(ii) Proximal width
(iii) Mid shaft width
(iv) Distal width

*Tibia – Osteo-mineralization*

(i) Bone weight
(ii) Calcium
(iii) Phosphorous

**Procedures**

**Tibia bone morphometric measurements**

The left tibial bones were collected and their adhering muscles together with connective tissues were thoroughly removed manually and dipped into a boiling water for 5 minutes to remove any remaining soft tissues. The length, proximal and distal width, as well as the mid shaft width of the tibia bone were measured with Vernier callipers. These were expressed in mm.$kg^{-1}$ live weight.

The tibia weight, tibia length, diaphysis diameter, tibia weight/length index and tibia robusticity index were determined as described by Mutus *et al.* (2006). The bone weight/length index was obtained by dividing the tibia weight by its length (Seedor *et al*., 1991), while robusticity index was determined using the following formula as described by Reisenfeld (1972):

Robusticity index = Bone length/Cube root of bone weight

**Tibia-Osteo-mineralization**

Each tibia was defatted for 16 hours in petroleum ether (boiling point of 60-80 °C), dried and weighed before ashing. The samples were digested with diluted hydrochloric acid (1:2) and the mineral extract was prepared according to AOAC (1995). The extract was used in the estimation of the minerals. The mineral extract from each treatment replicate group was selected and the concentration of Zn, Mn, Cu, Ca and P were determined by Inductively Coupled Plasma Optical Emission Spectrometry (method 6010B).

vi) *In vivo* immune response of the bird

a) Cell-mediated immunity:

Reagents: Phosphate-buffered saline (PBS):
Sodium chloride, 8.0 g; Potassium chloride, 0.20 g; Potassium dihydrogen phosphate, 0.20 g; Disodium hydrogen phosphate, 1.44 g; distilled water, 1 litre; pH 7.2. (The producer of the reagents is Zunche Pharmaceutical Private Ltd., India)

**Procedure**

The cell-mediated immune response to phytohemagglutinin type P (PHA-P) was studied using the method of Corrier and Deloach (1990). At 21 days post-hatch, 0.1 ml (concentration 1 mg.$ml^{-1}$) of PHA-P was injected at 3$^{rd}$ and 4$^{th}$ inter--digital space of the right foot. The left foot served as control and injected with 0.1 ml phosphate--buffered saline (PBS). The foot web index was calculated as a difference between the swelling in the right and left feet before and after 24 hours of injection and expressed in millimetres.

The foot web/pad index was calculated as follows:

Cell-mediated immune response (CMIR): (R2-R1) – (L2-L1)

Where:

R2 = Thickness of right foot web after 24 hours of injection
R1 = Thickness of the right foot web before injection
L2 = Thickness of left foot web after 24 hours of injection
L1 = Thickness of the left foot web before injection

**Humoral immunity**

Reagents: Alsever's solution: Dextrose (Wuhan Yuancheng Gongchuang Tech. Co. Ltd, China), 2.05 g; Trisodium citrate dehydrate (Lianyungang Longyi Industry C. Ltd, China), 0.80 g; Sodium chloride (Zunche Pharmaceutical Private

Ltd., India), 0.42 g; Citric acid (A.+E, Fisher Chemie), 0.055 g; Distilled water, 100 ml; pH 6.5.

**Procedure**

The antibody response to the Sheep Red Blood Cell (SRBC) was studied at 29th day post-hatch, wherein 1 ml of 1 % SRBC was injected i/v to the birds. The SRBC was washed thrice and centrifuged at 704 g for 10 minutes after each washing. After 5 days of SRBC immunization, 2 ml of blood was collected from the wing vein and the antibody titre was recorded by hemagglutination (HA) titre (Siegel and Gross, 1980; Vander Zipp, 1983). The blood was kept in a slightly slanting position for 1 hour to clot. The clot was then allowed to retract after detaching it from the side of the tube. Centrifugation was carried out at 313 g for 5 to 10 minutes for rapid collection of serum.

For the HA test, 50 µl of PBS were poured into each well of the micro titre plate and 50 µl of serum from the chicken were added into the first well. Thereafter, two-fold serial dilution was made up to row 11, while the 12th row was left as a control and then 50 µl of 1 % SRBC were added into each well. The plates were covered and shaken on automatic shaking machine for proper mixing. The micro titre plates were then kept at 37 °C for 1 hour in an incubator. The plates were read under light and the titre expressed as log2 of the highest dilution in which there were complete haemagglutination.

**Statistical analysis**

The data collected during the experiment were subjected to one-way analysis of variance for completely randomized design. Significantly ($P < 0.05$) different means among variables were separated using Tukey test as contained in the Minitab® version 17.1.0 (Minitab, 2013).

## RESULTS AND DISCUSSION

The percentage of hatchability was highest in the Zn-injected hatching eggs resulting from an increase in hatched chicks (Figure 1). This finding corroborates earlier reports (Luscombe *et al*., 2000; Batal *et al*., 2001; Bartsevich *et al.*, 2003) that zinc is essential for embryonic development of all species including poultry. Zn was reported to control the differentiation of many cell types including T-lymphocytes (Staal *et al*., 2001)

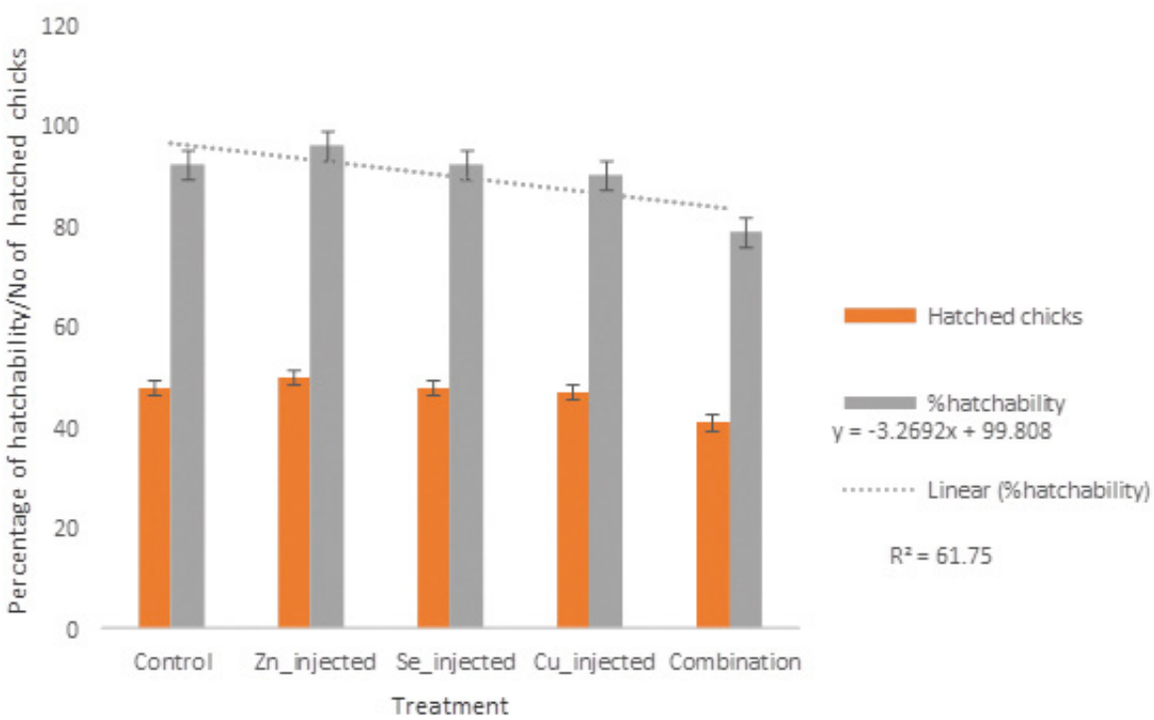


**Figure 1. Effects of *in ovo* injection on percentage of hatchability**

and myeloid precursor cells (Shivdasani, 2001). However, there had been varying hatchability results with *in ovo* administration in broiler chickens; decreased hatchability (McGruder *et al*., 2011); increased hatchability (Bottje *et al*., 2010) and no effect (Zhai *et al*., 2011).

The effect of *in ovo* injection of inorganic salts of Zn, Se, Cu and their combination on growth performance of broiler chickens at days 7 and 35 is shown in Table 3. The final weight of birds from Zn-injected eggs was significantly ($P < 0.05$) highest at day 7 than the values recorded in birds in other treatment groups except in birds from Se-injected and Cu-injected eggs. At day 35, the final weight and weight gain were significantly ($P < 0.05$) affected by the *in ovo* administration of Zn, Se, Cu and their combination. The highest final weight and weight gain were obtained in birds on *in ovo* administration of Cu, though not significantly different from the values recorded in birds from Se-injected eggs and those on the combination of inorganic salts. This is contrary to the report by Richards (1997), that during incubation the mineral use is not constant but shows peak for Zn at 1-2 days post-hatch, particularly in turkey poults. However, the observed development of the birds from Zn-injected eggs at day 7 attest to the role of Zn in post-hatch development by the regulation of the cell turnover (Cui *et al.*, 2003; Joshua *et al*., 2016). The combination of the mineral sources depressed growth possibly from the antagonism occurring between Zn and Cu (Scheideler, 1991). In addition, earlier works (Yair *et al*., 2013; Oliveira *et al*., 2015) indicated that *in ovo* injection of trace minerals singly or combined did not influence increased growth performance of post-hatch chicks. However, Bakyaraj *et al.* (2012) reported that the trace mineral supplemented group (Zn, 80 µg; Se, 0.3 µg; Fe, 160 µg and I, 0.7 µg) gave significantly higher body weight than the control.

The Table 4 shows the effects of *in ovo* injection of inorganic salts of Zn, Se, Cu and their combination on organ development and gut morphology of broiler chickens at days 7 and 35. Significant difference ($P < 0.05$) was obtained only in the proportion of heart at day 7. Birds from Zn-injected eggs, Cu-injected eggs and eggs on the combination of the inorganic salts had significantly ($P < 0.05$) highest values: 1.13, 1.13 and 1.11, respectively. The highest heart percentage obtained was relative to the weight of birds in the treatment group but it corroborates the relevance of Zn in embryonic and early post hatch development (Bartsevich *et al*., 2003). The findings at days 7 and 35 are however opposed to the reports by Uni *et al.* (2003), that *in ovo* feeding results in the improvement of the development of gastrointestinal tracts.

**Table 3. Effect of *in ovo* injection of inorganic salts and their combination on growth performance of broiler chicks at days 7 and 35**

| Parameter | Treatment | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control | Zn | Se | Cu | Zn*Se*Cu | SEM | P-Value |
| At day 7 | | | | | | | |
| Initial weight (g.bird$^{-1}$) | 50.08 | 50.78 | 51.12 | 50.12 | 49.20 | 0.25 | 0.13 |
| Final weight (g.bird$^{-1}$) | 182.79$^{b}$ | 191.74$^{a}$ | 186.51$^{ab}$ | 185.95$^{ab}$ | 183.23$^{b}$ | 1.24 | 0.01 |
| Weight gain (g.bird$^{-1}$) | 132.71 | 140.96 | 135.39 | 135.84 | 134.03 | 1.19 | 0.24 |
| Feed intake (g.bird$^{-1}$) | 144.26 | 154.09 | 151.30 | 148.44 | 163.70 | 3.05 | 0.32 |
| FCR | 1.08 | 1.10 | 1.12 | 1.09 | 1.23 | 0.03 | 0.39 |
| At day 35 | | | | | | | |
| Final weight (g.bird$^{-1}$) | 1358.83$^{b}$ | 1367.17$^{b}$ | 1411.00$^{ab}$ | 1481.43$^{a}$ | 1445.40$^{ab}$ | 16.71 | 0.05 |
| Weight gain (g.bird$^{-1}$) | 1308.76$^{b}$ | 1316.39$^{b}$ | 1359.89$^{ab}$ | 1431.38$^{a}$ | 1396.29$^{ab}$ | 16.73 | 0.05 |
| Feed intake (g.bird$^{-1}$) | 2052.69 | 2170.38 | 2127.65 | 2074.70 | 2195.97 | 27.91 | 0.27 |
| FCR | 1.57 | 1.66 | 1.57 | 1.45 | 1.56 | 0.03 | 0.06 |

$^{a,b}$ Means in the same row with different superscripts differ significantly ($P < 0.05$); Zn = Zinc-injected; Se = Selenium-injected; Cu = Copper-injected; Zn*Se*Cu = Combination of Zn, Se and Cu; SEM = Standard error of means

**Table 4. Effect of *in ovo* injection of inorganic salts and their combination on organ development and gut morphology of broiler chicks at days 7 and 35**

| Parameter | Treatment | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control | Zn | Se | Cu | Zn*Se*Cu | SEM | P-Value |
| At day 7 | | | | | | | |
| Live weight, g | 188.97 | 184.85 | 183.47 | 177.66 | 194.01 | 2.65 | 0.386 |
| Proventriculus, % | 1.30 | 1.19 | 1.23 | 1.35 | 1.21 | 0.04 | 0.590 |
| Gizzard, % | 7.47 | 7.67 | 8.45 | 8.36 | 8.01 | 0.18 | 0.371 |
| Liver, % | 3.94 | 3.99 | 3.94 | 4.08 | 4.08 | 0.08 | 0.968 |
| Heart, % | 0.90[b] | 1.13[a] | 0.92[b] | 1.13[a] | 1.11[a] | 0.03 | 0.032 |
| Duodenum length, cm.100 $g^{-1}$ | 9.22 | 9.81 | 10.19 | 10.41 | 9.31 | 0.23 | 0.379 |
| Duodenum, % | 3.03 | 3.02 | 3.24 | 2.99 | 2.82 | 0.06 | 0.394 |
| Jejunum length, cm.100 $g^{-1}$ | 24.53 | 26.56 | 25.94 | 26.99 | 25.08 | 0.46 | 0.430 |
| Jejunum, % | 5.07 | 5.19 | 5.25 | 5.56 | 5.55 | 0.13 | 0.730 |
| Ileum length, cm.100 $g^{-1}$ | 20.10 | 21.49 | 22.86 | 22.41 | 20.64 | 0.51 | 0.395 |
| Ileum, % | 3.21 | 3.71 | 3.89 | 3.27 | 3.67 | 0.14 | 0.467 |
| Caecum length, cm.100 $g^{-1}$ | 3.60 | 3.60 | 3.29 | 4.11 | 3.69 | 0.13 | 0.371 |
| Caecum, % | 1.31 | 1.01 | 1.21 | 1.76 | 1.92 | 0.12 | 0.093 |
| At day 35 | | | | | | | |
| Live weight, g | 1346.67 | 1382.08 | 1367.00 | 1327.67 | 1406.83 | 24.67 | 0.880 |
| Proventriculus, % | 0.81 | 0.63 | 0.79 | 0.76 | 0.81 | 0.04 | 0.628 |
| Gizzard, % | 2.69 | 2.58 | 2.61 | 3.14 | 2.69 | 0.09 | 0.254 |
| Liver, % | 2.44 | 2.39 | 2.49 | 2.66 | 2.53 | 0.05 | 0.625 |
| Heart, % | 0.75 | 0.74 | 0.84 | 0.81 | 0.82 | 0.03 | 0.816 |
| Duodenum length, cm.100 $g^{-1}$ | 2.76 | 2.62 | 2.45 | 2.72 | 2.65 | 0.06 | 0.474 |
| Duodenum, % | 1.31 | 1.29 | 1.33 | 1.28 | 1.33 | 0.04 | 0.993 |
| Jejunum length, cm.100 $g^{-1}$ | 5.80 | 6.02 | 5.63 | 6.38 | 6.32 | 0.12 | 0.235 |
| Jejunum, % | 2.28 | 2.49 | 2.37 | 2.48 | 2.57 | 0.07 | 0.731 |
| Ileum length, cm.100 $g^{-1}$ | 5.89 | 5.97 | 5.41 | 6.15 | 6.32 | 0.13 | 0.192 |
| Ileum, % | 2.32 | 2.19 | 2.24 | 2.17 | 2.55 | 0.06 | 0.316 |
| Caecum length, cm.100 $g^{-1}$ | 1.19 | 1.15 | 1.16 | 1.21 | 1.20 | 0.02 | 0.907 |
| Caecum, % | 1.01 | 0.88 | 1.01 | 1.10 | 1.10 | 0.05 | 0.560 |
| Spleen, mg.100 $g^{-1}$ | 0.12 | 0.13 | 0.14 | 0.16 | 0.14 | 0.005 | 0.099 |
| Bursa, mg.100 $g^{-1}$ | 0.24 | 0.25 | 0.20 | 0.28 | 0.25 | 0.01 | 0.296 |

[a,b] Means in the same row with different superscripts differ significantly ($P < 0.05$); Zn = Zinc-injected; Se = Selenium-injected; Cu = Copper-injected; Zn*Se*Cu = Combination of Zn, Se and Cu; SEM = Standard error of means

**Table 5. Effect of *in ovo* injection of inorganic salts and their combination on cell-mediated immunity and humoral immunity**

| Treatment | Parameter | |
|---|---|---|
| | CMI | Humoral |
| Control | 0.53 | 4.11 |
| Zn | 0.53 | 4.50 |
| Se | 0.57 | 4.69 |
| Cu | 0.58 | 4.87 |
| Zn*Se*Cu | 0.54 | 4.18 |
| SEM | 0.04 | 0.15 |
| P-value | 0.99 | 0.46 |

The effect of *in ovo* injection of inorganic salts of Zn, Se, Cu and their combination on cell--mediated immunity and humoral immunity is shown in Table 5. The observed results of non--significance in the growth of immune organs and response to PHA-P or SRBC suggested that *in ovo* injection of Zn, Se, Cu and their combination might not be immunomodulatory in broiler chickens of improved genetic lines as in the study. This is at variance with the findings on *in ovo* injection of lysine (Lotan *et al.*, 1980), arginine (Kidd *et al*., 2001) and 8 µg.$egg^{-1}$ of inorganic Cu ($CuSO_4$), which were found to enhance the immune response of

**Table 6. Effect of *in ovo* injection of inorganic salts and their combination on bone morphometry and mineralization of tibia bone**

| Parameter | Treatment | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control | Zn | Se | Cu | Zn*Se*Cu | SEM | P-Value |
| Bone morphometry | | | | | | | |
| Bone weight, g | 4.36 | 4.38 | 4.63 | 4.16 | 4.38 | 0.33 | 0.76 |
| Tibia Length, mm | 85.39 | 86.40 | 86.24 | 85.35 | 86.43 | 0.17 | 0.05 |
| Tibia bone weight/length index, mg.mm$^{-1}$ | 50.97 | 50.52 | 53.36 | 48.64 | 50.59 | 3.00 | 0.70 |
| Tibia Length, mm.kg$^{-1}$ | 63.99 | 64.43 | 62.47 | 70.22 | 62.99 | 1.67 | 0.65 |
| Proximal Length, mm | 18.85 | 19.30 | 19.43 | 19.39 | 19.42 | 0.51 | 0.79 |
| Proximal Length, mm.kg$^{-1}$ | 14.09 | 14.45 | 14.10 | 15.88 | 14.16 | 0.37 | 0.55 |
| Distal width, mm | 15.71 | 15.61 | 15.83 | 15.46 | 15.83 | 0.05 | 0.93 |
| Distal width, mm.kg$^{-1}$ | 11.77 | 11.68 | 11.47 | 12.58 | 11.54 | 0.27 | 0.76 |
| Mid-Shaft width, mm | 7.86 | 7.57 | 8.10 | 7.39 | 7.82 | 0.04 | 0.37 |
| Mid-shaft width, mm.kg$^{-1}$ | 5.90 | 5.65 | 5.86 | 6.02 | 5.69 | 0.14 | 0.93 |
| Robusticity index | 5.95 | 6.02 | 5.77 | 6.24 | 6.02 | 0.37 | 0.84 |
| Relative Tibia Bone Density | 0.33 | 0.32 | 0.33 | 0.34 | 0.32 | 0.03 | 0.96 |
| Mineralization of Tibia bone | | | | | | | |
| Ash, % | 36.79$^{ab}$ | 40.96$^{a}$ | 40.37$^{a}$ | 37.77$^{ab}$ | 35.47$^{b}$ | 29.84 | 0.01 |
| Zinc, ppm | 118.52 | 125.55 | 131.50 | 125.77 | 116.96 | 82.72 | 0.75 |
| Manganese, ppm | 3.49 | 3.76 | 4.10 | 3.78 | 3.55 | 2.27 | 0.73 |
| Copper, ppm | 5.06 | 4.21 | 3.92 | 4.12 | 3.95 | 2.79 | 0.15 |
| Calcium, % | 17.17 | 18.79 | 20.00 | 18.25 | 17.51 | 0.55 | 0.55 |
| Phosphorus, % | 9.97 | 10.55 | 11.31 | 10.21 | 9.80 | 6.96 | 0.57 |

$^{a,b}$ Means in the same row with different superscripts differ significantly (P < 0.05); Zn = Zinc-injected; Se = Selenium-injected; Cu = Copper-injected; Zn*Se*Cu = Combination of Zn, Se and Cu; SEM = Standard error of means

broiler chickens (Goel *et al*., 2013).

The effects of *in ovo* injection of inorganic salts of Zn, Se, Cu and their combination on bone morphometry and mineralization of tibia bone are shown in Table 6. Significant (P < 0.05) difference was obtained only in the percentage of ash of the tibia bone. The tibiae ash of birds from Zn and Se-injected eggs recorded the highest values: 40.96 and 40.37 %, respectively, while the lowest value (35.47 %) was recorded in the tibiae of birds from eggs injected with the combination of the inorganic salts. This was similar to the ash obtained in the tibiae of birds from the control and Cu-injected eggs. Significant difference in the tibiae bone ash did not influence the robusticity and relative tibiae bone density. Yair *et al.* (2013) observed that bone ash of birds on *in ovo* injection of Zn, Cu and Mn was increased on 19$^{th}$ day of incubation. However, Bello *et al*. (2014) did not observe differences in the tibia ash concentrations of hatchlings +on *in ovo* injection of different levels of 25 (OH)$D_3$.

## CONCLUSION

Research on *in ovo* feeding has established a new science of neonatal nutrition, and this has engendered greater understanding of the developmental transition from embryo to chick.

Zn impacts mostly hatchability, but the combination of the mineral sources at the ratio injected in this study affected negatively the growth performance of the broiler chickens.

**Conflict of interest statement**

There is absolutely no conflict of interest with any individual or organisation regarding the materials discussed in the manuscript.

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# BITING RATE OF WHITE FULANI CALVES AS INFLUENCED BY SPATIAL DISTRIBUTION OF PASTURE BIOMASS AND SWARD HEIGHT

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## ABSTRACT

Grazing ruminants are faced with the dilemma of making decisions when searching for and defoliating forage to meet their nutrient requirements, particularly in situations of heterogeneity in forage abundance and sward height. Knowledge of sward-animal interaction at bite level is essential for animal intake, performance and productivity of grazing systems. Here, we investigated the effect of two spatial distributions of forage biomass (dense and sparse) assigned to main plot, and three sward heights (10, 15 and 20 cm) allotted to sub-plot in a split plot design, with three replicates totalling six treatments, aimed at assessing the biting rate of White Fulani (WF) calves. The study was carried out between November and December 2015. Biting rate was recorded with the aid of a Chloride UK 8 channel, H.264 digital video recorder and Chloride UK IR waterproof camera equipped with 3.6 mm lens. WF calves altered their biting rate in an attempt to meet their intake requirement on *Panicum maximum*/*Stylosanthes guianensis* sward, notably with decrease in bite number as access time advanced. The calves, grazing pasture with dense biomass, recorded higher bite number during the occupation time ($p < 0.05$). Measurement of grazing bites of WF calves indicates that the herbage could be defoliated by ruminants on tropical pasture relatively easily.

**Key words:** forage biomass; sward height; bite number; *Panicum maximum*; *Stylosanthes guianensis*; White Fulani calves

## INTRODUCTION

The process of harvesting forage is a particularly time-consuming exercise for ruminants, since a large number of bites are required to meet the nutrient intake requirements for maintenance, growth and reproduction (Wendy and Gordon, 2003). Many studies have been carried out to tease out the independent influence of sward height and sward bulk density on bite rate in the temperate region (Dement *et al*., 1995; Gordon and Lascano 1993; Hodgson *et al*, 1994; Launchbaugh, 1996), with paucity of such information in the tropics. However, given the sufficient available information in this respect, the need to predict intake responses of grazing animals to possible changes in structural vegetation and morphology cannot be overemphasized. Therefore, the report of Illius and Hodgson (1996) on the need for studies, that will mechanically explain sward bite interactions, fits

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well within the context of grazing management.

Animals grazing tropical pastures have been reported to harvest small bites of leafy materials and are faced with the challenge of meeting their intake requirements (Stobbs, 1973). Where sward conditions are confounded, animals tend to alter their behaviour to maximize intake of forage (Jimoh *et al*., 2017), and this is highly correlated to the quantity of green leaf mass in the sward (Gibb and Orr, 1997). A modified bite feature is, however, expected where the leaf to stem ratio and sward bulk density differ. Soder *et al*. (2009) reported that sward height may result in greater exploitation of upright plant species, and that grazing ruminants may prefer to search for underlying plant species, thereby altering sward dynamics.

There is necessity to re-evaluate the principles of grazing management on tropical pastures to make new management target propositions owing to the influence of sward structure on herbage intake by herbivores (Da Silva and Carvalho, 2005). Moreover, it has been noted that changes in sward structure results from plant growth, defoliation and senescence (Mezzalira *et al*., 2014). This consequently leads to continuous reduction in short-term intake (Fonseca *et al*., 2013) and bite area (Ungar *et al*., 2001) by ruminants and, therefore, results in decreased herbage intake on daily basis (Barret *et al*., 2001; Baumount *et al*., 2004). Arising from this, tropical pastures might impose various levels of constraint to grazing animals due to disappearance of leafy materials from the sward relative to their physiological characteristics.

Grazing behaviour studies in the temperate and sub-tropical regions have shown that cattle (Barrett *et al*., 2001; Robert, 2017) and sheep (Yong *et al*., 2013) have an intensive period of consistent grazing at dawn on daily basis. Hence, investigating the inherent variation associated with the relative ease with which forages could be harvested from pasture sward through biting behaviour within grazing sessions could provide useful information for better management of animals and pasture. Laca *et al*. (1992) stated that sward height and bulk density are the most important cues that determine bite depth and bite area. The difficulty in teasing out the independent effect of sward height and bulk density on bite rate has been widely reported (Hodgson, 1981; Burlison *et al*., 1999; Mitchell *et al*., 1991; McGilloway *et al*., 1999). Therefore, the limited attention paid to variation in cropping or bite rates in a situation where sward height and pasture spatial distribution are confounding in short-term grazing studies may limit our understanding of how ruminants may adjust intake rate in such conditions, because bite rate may be more flexible than the actual time spent foraging (Ruckstuhl *et al*., 2003). In addition, the knowledge of sward-animal interaction at the bite level would provide information on the degree of sown pasture sward utilization by grazing ruminants. The objective of this study was to determine the effects of sward height and spatial distribution of pasture biomass at both main and interaction levels on the bite rate of White Fulani (WF) yearling calves on pasture over a period of 2:00 h.

## MATERIALS AND METHODS

### Experimental site

The experiment was carried out at the Cattle Production Venture unit, Federal University of Agriculture, Abeokuta, Ogun State, Nigeria. The site is located on latitude 7° 10’ N, longitude 3° 20’ E, with altitude of 76 mm within the derived savannah of Nigeria. The area has a mean annual rainfall of 1037 mm and temperature of about 34.7 °C, with relative humidity ranging between 63 - 96 % in the rainy season (April-October) and 55 - 82 % in the dry season (November-March) with an annual average of 82 % (Google Earth, 2015).

### Pasture vegetation

The measurements were made on a *Panicum maximum*/*Stylosanthes guianensis* sward established in 2013. The sward also has *Centrosema pubescens* and *Calopogonium mucunoides* as volunteer species. The volunteer species were present in negligible quantity; hence, the botanical composition of the sward was categorized as grass, legume and weed. The total land area of the pasture is 5 ha, and a demarcated section of the land was used for the present study. The mean proportion for each forage type in the plot with dense biomass are (138, 83, and 63 kg DM) and (88, 146, and 90 kg DM) for those with sparse pasture biomass. Following establishment, the pastureland is used for grazing high producing animals (beef cows) from

the herd. Prior to the current experiment, the field was used for supplemental grazing of lactating dairy cows. The adopted grazing regimes led to the evolution of dense and sparse biomass areas within the pasture field owing to grazing effects such as trampling and treading. The sparse pasture areas cover relatively low land size; hence, no over sowing was carried out. The grass component of the mixture used in our study is widely relished by ruminants in Nigeria (Olanite *et al*., 2006), while the legume component possess the ability to support ruminants during dry season.

**Animals and length of grazing time**

Twelve yearling WF calves (mixed-sex, with average body weight of 82 kg) were selected randomly from the herd and tagged for identification. The animals were turned out for daily grazing routine with other animals in the herd from 10:00 h to 17:00 h. Prior to the commencement of the experiment, the selected animals were separated from the larger herd for two weeks, and had similar experience background (Provenza *et al*., 1995) characterized by transhumant grazing system. Prior to weaning of the calves, the animals do co-graze with their dams on the pasture as a way of introducing them to grazing. The animals were not fasted before the grazing sessions, but were turned out for the trials early in the morning (7:00 – 9:00 h).

**Experimental design**

Two spatial distributions of pasture biomass (dense and sparse) were assigned to the main plot and three sward heights (10, 15, and 20 cm) as the sub-plot. A 15 m × 5 m land area constituted a block and was designated as either dense or sparse pasture. Each block was further partitioned (Boland *et al*., 2011) into a series of 5 m × 5 m plots to have three square shaped plots, with the blocks defined by 3 m space. To ease movement around the blocks, the inter-block spaces were cut down to the ground level. The pastures were cut back six weeks before the onset of the grazing trials to allow for substantial regrowth. The dense sward is characterized with (thick) thoroughly mixed herbage materials, while forages in the sparse pastures were scattered in the plots. We expect that the designated cut back heights will introduce the animals to herbage with varying quality characteristics. Pastures cut to 10 cm height above the ground were perceived to be more succulent but slightly tasking for animals. In contrast, the pasture cut to 20 cm height had higher stem proportion with uneven distribution of leaves. Mechanical mower and sward sticks were used to affirm both pasture heights and uniformity. Simulated grazing samples (*Panicum maximum* and *Stylosanthes guianensis*) were hand plucked from each plot (Mayne *et al*., 1997) and the samples were analyzed for ADF, NDF, Lignin and CP (Boland *et al*., 2011). The field layout of the experiment is shown in Figure 1. The treatments are as listed below:

Treatment 1: Dense pasture biomass cut to 10 cm height
Treatment 2: Dense pasture biomass cut to 15 cm height
Treatment 3: Dense pasture biomass cut to 20cm height
Treatment 4: Sparse pasture biomass cut to 10 cm height
Treatment 5: Sparse pasture biomass cut to 15 cm height
Treatment 6: Sparse pasture biomass cut to 20 cm height

The treatments were assigned randomly to the plots within the dense and sparse pasture biomass blocks, with three replications to give eighteen grazing sub-plots. On departure from the plots, the animals were returned to join the main herd for daily routine grazing.

**Biting frequency and monitoring procedure**

The animals were trained to become accustomed to the experimental procedure (Dummont and Boissy, 2000; Mezallira *et al*., 2014) before the grazing trials. The 120 minutes pasture access time amounts to about 30 % of the animals' daily grazing time. The animals were allowed to join the main herd for daily grazing on days without measurement. On each trial day (grazing session), two calves were put in their respective plots and allowed to graze uninterruptedly, so that 2 blocks (6 grazing sub-plots) were grazed per day, and 6 blocks (18 grazing sub-plots) were grazed per week, and this was done twice (November and December, 2015) at three-week intervals. The same calves grazed the same treatment throughout the trial period. The CCTV gadget was strategically located outside the blocks, and the video surveillance cameras in waterproof enclosures (CSC-3020W) were mounted 4 m above the ground on poles in each plot to monitor the calves. Colour video containing date and time stamps were recorded digitally during grazing sessions onto a computer hard drive (Boland *et al*., 2011) and were later reviewed using a digital media player (Playback

Video Player). The CCTV gadget and the surveillance cameras used have been described by Jimoh *et al*. (2017).

**Data collection**

The biting rate of the animals was quantified by continuous sampling method from the colour video recording (continuous sampling) on each trial day and three days in a week. The access time was divided into four, each comprising of 30 minutes grazing, and herein after referred to as first, second, third and fourth quarters, respectively. This was aimed at monitoring possible fluctuations in bite rate of the animals relative to the treatments imposed. The access time was monitored by a running time background on the CCTV monitor. The authors state that our study is in compliance with the policy on animal ethics. The study was carried out in accordance with the ethical guidelines of the College of Animal Science and Livestock Production, Federal University of Agriculture, Abeokuta committee on the use of animals for experiment.

**Statistical analysis**

The bite rate of the calves in the first, second, third and fourth quarters of the access time, as influenced by spatial distribution of pasture biomass and sward height, were averaged for each treatment prior to statistical analysis (Mezzalira *et al*., 2014). The main and interaction effects of the factors under consideration were tested at significance level of 0.05. The statistical analysis was conducted using General Linear Model (GLM) in R 3.0.1 (R Core Team, 2014). Means were separated using Tukey Honestly Significant Difference (Tukey HSD).

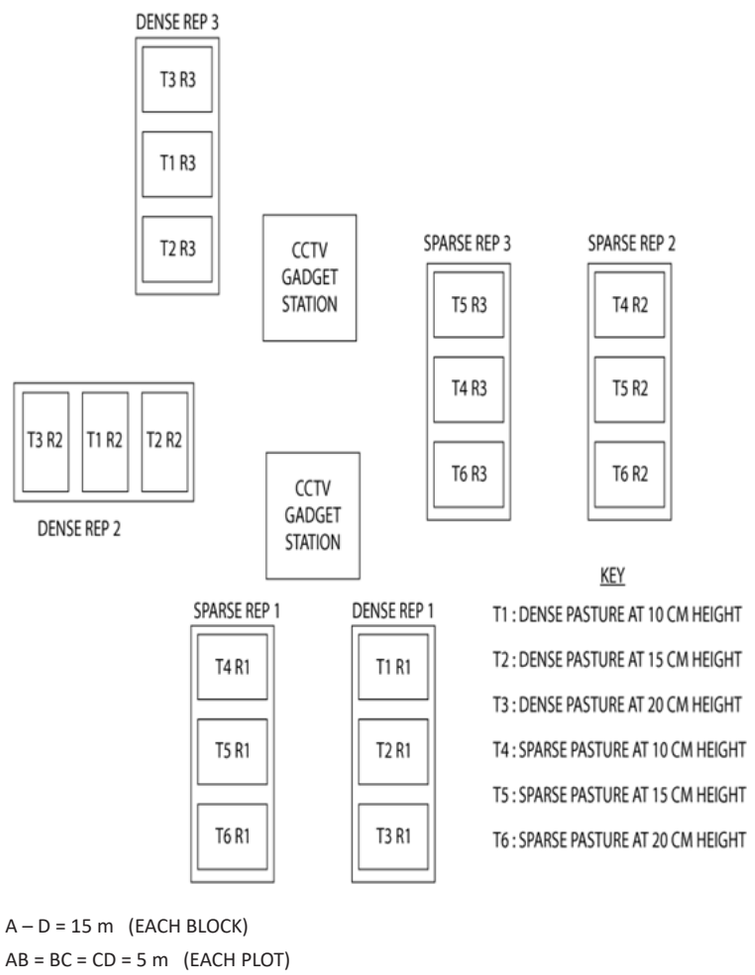


**Figure 1. Field layout of the experimental site (Adapted from Jimoh *et al.*, 2017)**

## RESULTS

The effect of sward height and spatial distribution of forage biomass on the number of bites recorded per minute by WF calves is presented in Table 1. The number of bites recorded in the fourth quarter was significantly affected by sward height, with the plots cut to 10 cm height recording higher bite number (13.71 bites.min$^{-1}$). With exception of the first quarter, the number of bites recorded per minute during the grazing sessions varied under the influenced of spatial distribution of forage biomass. From the second to the fourth quarter, pasture with dense biomass recorded ($p < 0.05$) higher bite rate of 16.47 bites.min$^{-1}$, 15.22 bites.min$^{-1}$ and 13.56 bites.min$^{-1}$, respectively.

The interaction effect of sward height and spatial distribution of pasture biomass on the biting rate of WF calves are presented in Table 2. There were significant differences in the number of bites obtained by the grazing calves across the treatments. In the first quarter, higher biting rate was recorded for plots with dense biomass cut to 10 cm (18.67 bites.min$^{-1}$), while the least was observed

**Table 1. Main effect of sward height and spatial distribution of biomass on number of bites per minute by White Fulani calves**

| | First Quarter | Second Quarter | Third Quarter | Fourth Quarter |
|---|---|---|---|---|
| | bites.min$^{-1}$ | | | |
| Sward height | | | | |
| 10 cm | 17.21 | 15.25 | 13.79 | 13.71$^{a}$ |
| 15 cm | 16.96 | 14.92 | 14.12 | 11.67$^{b}$ |
| 20 cm | 16.38 | 14.88 | 14.04 | 12.00$^{ab}$ |
| SEM | 0.60 | 0.67 | 0.70 | 0.63 |
| Spatial distribution | | | | |
| Dense | 17.44 | 16.47$^{a}$ | 15.22$^{a}$ | 13.69$^{a}$ |
| Sparse | 16.25 | 13.56$^{b}$ | 12.75$^{b}$ | 11.22$^{b}$ |
| SEM | 0.51 | 0.49 | 0.54 | 0.50 |

$^{a,b,c:}$ Means on the same column with different superscripts are significantly different ($p < 0.05$), SEM: Standard Error of Mean, First quarter: First 30minutes of grazing, Second quarter: Second 30 minutes of grazing, Third quarter: Third 30 minutes of grazing, Fourth quarter: Last 30 minutes of grazing

**Table 2. Interaction effect of sward height and spatial distribution of biomass on number of bite.minutes$^{-1}$ by White Fulani calves**

| Spatial distribution | Sward height | First Quarter | Second Quarter | Third Quarter | Fourth Quarter |
|---|---|---|---|---|---|
| | | bites.min$^{-1}$ | | | |
| Dense | 10 cm | 18.67$^{a}$ | 17.17$^{a}$ | 16.33$^{a}$ | 15.75$^{a}$ |
| | 15 cm | 16.67$^{ab}$ | 16.33$^{ab}$ | 14.67$^{ab}$ | 12.5$^{b}$ |
| | 20 cm | 17.00$^{ab}$ | 15.92$^{abc}$ | 14.67$^{ab}$ | 12.83$^{b}$ |
| Sparse | 10 cm | 15.75$^{b}$ | 13.33$^{c}$ | 11.25$^{c}$ | 11.67$^{b}$ |
| | 15 cm | 17.25$^{ab}$ | 13.50$^{c}$ | 13.58$^{abc}$ | 10.83$^{b}$ |
| | 20 cm | 15.75$^{b}$ | 13.83$^{bc}$ | 13.42$^{bc}$ | 11.17$^{b}$ |
| SEM | | 0.81 | 0.85 | 0.88 | 0.81 |

$^{a,b,c:}$ Means on the same column with different superscripts are significantly different ($p < 0.05$), SEM: Standard Error of Mean, SH: Sward height, SD: Spatial distribution of biomass, First quarter: First 30 minutes of grazing, Second quarter: Second 30 minutes of grazing, Third quarter: Third 30 minutes of grazing, Fourth quarter: Last 30 minutes of grazing each

for those with sparse pasture biomass cut to 20 cm height (15.75 bites.min$^{-1}$). Similar bite numbers were recorded for pastures with dense and sparse biomass cut to 15 cm height. Plots with dense biomass maintained at 10 cm height recorded ($p < 0.05$) higher bite number 17.17 bites.min$^{-1}$, while those with sparse biomass cut to 15 cm were observed for the lowest bite rate of 13.5 bites.min$^{-1}$ in the second quarter. Dense pasture biomass cut to 10 cm above ground recorded ($p < 0.05$) higher number of bites (16.33 bites.min$^{-1}$) and the least bite number was observed for plots with sparse pasture biomass cut to 10 cm height (11.25 bites.min$^{-1}$) in the third quarter. The values recorded for plots with dense pasture biomass cut to 15 cm and 20 cm height were statistically similar. Plots with dense pasture biomass cut to 10 cm height were observed for higher bite number (15.75 bites.min$^{-1}$) with the least recorded for plots with sparse pasture biomass cut to 15 cm height (10.83 bites.min$^{-1}$) in the fourth quarter of the access time.

The CP content of the grass varied ($p < 0.05$) from 7.17 % to 9.93 % for the plots cut to 20 cm and 10 cm above ground, respectively (Table 3). Spatial distribution of biomass had ($p < 0.05$) effect on the lignin and CP content of the grass. Higher crude protein was recorded for the dense pasture biomass (9.71 %), while the lowest lignin (11.53 %) was observed in this plot. However, this trend was in opposite direction for plots with sparse biomass.

**Table 3. Main effect of sward height and spatial distribution of pasture biomass on the nutritive value of simulated *P. maximum* (Ntchisi) samples grazed by White Fulani calves**

| | NDF (%) | ADF (%) | Lignin (%) | CP (%) |
|---|---|---|---|---|
| Sward height | | | | |
| 10 cm | 59.33 | 39.42 | 12.50 | 9.93$^{a}$ |
| 15 cm | 57.09 | 29.64 | 14.45 | 8.99$^{a}$ |
| 20 cm | 60.31 | 33.54 | 12.69 | 7.17$^{b}$ |
| SEM | 1.03 | 1.84 | 1.35 | 0.48 |
| Spatial distribution | | | | |
| Dense | 57.42 | 33.89 | 11.53$^{b}$ | 9.71$^{a}$ |
| Sparse | 60.76 | 31.59 | 15.00$^{a}$ | 7.45$^{b}$ |
| SEM | 1.48 | 1.58 | 1.02 | 0.37 |

$^{a,b:}$ Means on the same column with different superscripts are significantly different ($P < 0.05$), SEM: Standard Error of Mean, NDF: Neutral detergent fibre, ADF: Acid detergent fibre, CP: Crude protein

**Table 4. Interaction effect of sward height and spatial distribution of forage biomass on the nutritive value of simulated *P. maximum* (Ntchisi) samples grazed by White Fulani calves**

| | | NDF (%) | ADF (%) | Lignin (%) | CP (%) |
|---|---|---|---|---|---|
| Spatial distribution | Sward height | | | | |
| Dense | 10 cm | 59.57 | 35.83 | 10.83$^{b}$ | 11.55$^{a}$ |
| | 15 cm | 54.50 | 32.00 | 10.83$^{b}$ | 10.18$^{ab}$ |
| | 20 cm | 57.83 | 33.86 | 12.71$^{ab}$ | 7.75$^{c}$ |
| Sparse | 10 cm | 61.17 | 34.00 | 14.17$^{ab}$ | 8.31$^{bc}$ |
| | 15 cm | 60.20 | 26.80 | 18.80$^{a}$ | 7.55$^{c}$ |
| | 20 cm | 60.83 | 33.17 | 12.67$^{ab}$ | 6.50$^{c}$ |
| SEM | | 2.63 | 2.55 | 1.66 | 0.37 |

$^{a,b,c:}$ Means on the same column with different superscripts are significantly different ($P < 0.05$), SEM: Standard Error of Mean, NDF: Neutral detergent fibre, ADF: Acid detergent fibre, CP: Crude protein, SH: Sward height, SD: Spatial distribution of biomass

At interaction level, lignin and CP of the grasses differs (Table 4). Plots with sparse pasture biomass cut to 15 cm height recorded higher lignin than other treatments. The highest CP was observed for plots with dense pasture cut to 10 cm (11.55 %) height and the least recorded for sparse pastures cut to 20 cm (6.50 %) above the ground level.

The effect of sward height on CP of *S. guianensis* was significant (Table 5). The highest CP was recorded for the legumes in the plots cut to 10 cm (20.59 %) and the least observed for those in the plots cut to 20 cm height. There was no interaction effect ($p > 0.05$) of sward height and spatial distribution of biomass on the nutritive value of *S. guianensis* (Table 6).

## DISCUSSION

The ease of forage defoliation from the pasture sward was teased out by the biting rate of the animals during grazing, particularly at the onset of grazing. The results from our study indicate variation in the number of bites recorded per minute by the grazing animals under the influence of spatial distribution of pasture biomass (except in the first quarter). Higher bite number was obtained by the calves grazing on pastures with dense biomass from the second to the fourth quarter implying that frequent herbage defoliation was carried out by animals grazing in these plots compared with those grazing in the plots with sparse pasture biomass. In contrast,

**Table 5. Main effect of sward height and spatial distribution of forage biomass on the nutritive value of simulated *S. guianensis* grazed by White Fulani calves**

| | NDF (%) | ADF (%) | Lignin (%) | CP (%) |
|---|---|---|---|---|
| Sward height | | | | |
| 10 cm | 50.50 | 33.00 | 17.50 | 20.59[a] |
| 15 cm | 51.00 | 23.80 | 11.00 | 18.11[ab] |
| 20 cm | 49.50 | 24.00 | 9.25 | 16.06[b] |
| SEM | 5.03 | 2.94 | 2.80 | 0.86 |
| Spatial distribution | | | | |
| Dense | 56.00 | 21.33 | 9.33 | 18.41 |
| Sparse | 48.25 | 27.12 | 12.38 | 17.89 |
| SEM | 3.71 | 2.50 | 1.78 | 0.91 |

[a,b:] Means on the same column with different superscripts are significantly different ($P < 0.05$), SEM: Standard Error of Mean, NDF: Neutral detergent fibre, ADF: Acid detergent fibre, CP: Crude protein

**Table 6. Interaction effects of sward height and spatial distribution of pasture biomass on the nutritive value of simulated *S. guianensis* grazed by White Fulani calves**

| | | NDF (%) | ADF (%) | Lignin (%) | CP (%) |
|---|---|---|---|---|---|
| Spatial distribution | Sward height | | | | |
| Dense | 10 cm | 55.00 | 20.00 | 9.00 | 20.62 |
| | 15 cm | 56.00 | 21.33 | 9.33 | 18.79 |
| | 20 cm | 57.25 | 22.10 | 9.25 | 15.84 |
| Sparse | 10 cm | 50.50 | 33.00 | 17.50 | 20.57 |
| | 15 cm | 43.50 | 27.50 | 13.50 | 17.43 |
| | 20 cm | 49.50 | 24.00 | 9.25 | 16.23 |
| SEM | | 4.13 | 2.95 | 2.31 | 1.04 |

SEM: Standard Error of Mean, NDF: Neutral detergent fibre, ADF: Acid detergent fibre, CP: Crude protein, SH: Sward height, SD: Spatial distribution of biomass

Ruckstuhl *et al*. (2003) reported higher biting rate in areas with lower forage mass, but the higher biting rate, observed for dense plots in our study, could be attributed to defoliation at smaller bites by the yearling calves, as noted by Hilario *et al*. (2017).

The result from this study further affirms the report by McGilloway *et al*. (1999), that intake.bite$^{-1}$ rate was higher for swards with higher bulk density during grazing, particularly at the commencement of grazing. However, the observed decline in biting rate.min$^{-1}$ in both pasture types could be linked to continuous disappearance of herbage materials as access time advances, which invariably reduces instantaneous intake rate, due to the bite size reduction, particularly in sparse plots where forage is limited. Alternatively, decreasing bite rates could be related to increased selectivity, when forage quality is high, as reported by Ferrari *et al*. (1988) in a temperate sward. Mayne *et al*. (1997) concluded that higher intake rate.bite$^{-1}$ could be obtained in short-term grazing studies with dairy cows offered dense grass/legume swards and our result further corroborates this report. The observed close disparity in the bite numbers from the pasture types, although significant, may be due to group foraging, as noted by Dehn (1990).

A positive correlation between sward height and biting rate of grazing dairy cows was reported by Mayne *et al*. (1997), when sward height ranged from 75 to 150 mm and from 170 to 230 mm at varying bulk densities in separate experiments. In our study, we expected bite rate to vary with sward height, but that was obviously not the case, except for the fourth quarter where variation was observed, and this contradicts the report by Laca *et al*. (1992), who listed sward height among factors that affect bite rate of animals grazing temperate swards. Meanwhile, higher bite rate has been linked with newly growing forage with easily digestible plant parts, structure and height of vegetation (Ruckstuhl *et al*. 2003), which is typical of the pasture used in this study.

The rate of herbage defoliation by the animals, especially in the first quarter gave some insight into animals' ability to satisfy intake requirements on tropical pasture. However, the observed variation in bite rate across the treatments is an indication that bite rate could be a more precise intake measurement than grazing time. This is because animals could lower their muzzle into pasture sward without actually harvesting forage, and this is usually estimated as part of grazing time. Where bite rate is high as observed at the onset of grazing in this study, it could be a strategy by ungulates to compensate for reduced bite size, as bite sizes and bite rates are usually inversely related (Spalinger and Hobbs, 1992). Biting rate could, on the other hand, be viewed as a highly flexible behavior, which may be opportunistically utilized to reimburse for higher energy demands of growth (Ruckstuhl *et al*. 2003).

The number of bites per minute, obtained by the animals investigated in this study irrespective of the factors to which they were subjected, was lower than the range of 34-40 bites per minute reported by Nadin *et al*. (2010) for Holstein-Friesian calves grazing winter oats with different structure. It is also lower than the mean of 66.5 bites per minute reported by Orr *et al*. (2005) for yearling beef cattle grazing four intermediate-heading perennial ryegrass (*Lolium perenne* L.) varieties. The observed difference might be attributed to a number of factors including but not limited to the botanical composition of the pasture, sward height, age of the animals, temperate vs tropical environment and sensitivity of the recording gadgets used in the former and the latter studies. However, the study by Jimoh (unpublished data) showed that calves spent longer time grazing *Panicum maximum* than *Stylosanthes hamata*, when both were offered as a mixture. Gibb (1996) noted that several bites defoliated by an animal at a particular feeding station excluding interruption makes a grazing bout, which cover a few square meters and last between 10 to 100 seconds (Andriamandroso *et al*., 2015). Observation from this study conforms to this submission. Several grazing bouts occurring during each grazing session spans from minutes to hours which allows a significant portion of the sward to be explored (Andriamandroso *et al*., 2016).

Bite rate during grazing provides a valuable clue to the relative ease with which herbage is harvested from tropical pasture swards (Stobbs, 1974). In this study, the general linear decline in number of bites severed per minute is in consonance with the report by Stobbs (1974), who reported a decrease in the biting rate of Jersey cows grazing mature Rhodes and Setaria

swards, respectively. Similarly, Boland *et al*. (2011) reported a decline in the bite rate of steers grazing Alfalfa as a novel forage compared to when the steers had been used to the legume (62 bites.min$^{-1}$ vs 66 bites.min$^{-1}$). This clearly indicates that the bite rate of ruminants on both temperate and tropical pastures tends to decline relative to maturity of forage, novelty and bulk density. Moreover, bite.minute$^{-1}$ strongly varied with regard to spatial distribution of pasture biomass indicating that in order to achieve high intake of grazed sown pasture, management strategies should focus on maximizing biting rate on dense swards.

An interesting finding from this research was that calves grazing in dense plots, irrespective of sward height, commenced grazing at a faster rate and continued to graze at a higher rate of biting for a considerable longer period (40 min) than those grazing in sparse plots (10-15 min). However, the rate of decline in bite number was slower in dense plots at 10 cm height, and this could be associated with availability of sufficient prehensible herbage within the plot. The time available for foraging and the biting rate may limit an animals' daily forage intake and therefore affect its growth and survival (Ruckstuhl *et al*., 2003). In addition to satisfying its daily nutrient needs, ruminants grazing tropical pastures must harvest sufficient bites to ascertain the level of sown pasture utilization.

Harvesting and mastication bites were observed during grazing, but this study was focused on harvesting bite. Calves grazing in sparse plots cut to 20 cm height defoliate small amount of herbage, often one or two bites, and this can be linked to reduced dedicated time to forage selection (Chilibroste *et al*., 2007), occasioned by limited forage within the plot. Meanwhile, this does not necessarily lead to reduced jaw movements. On the contrary, calves grazing dense plot cut to 10 cm height were observed to gather large mouthfuls of forage with every tongue sweeps and continue to chew this herbage several times prior to swallowing. Thus, the process of herbage defoliation and mastication of the harvested forage prior to swallowing became confounded at this level, leading to over or under-estimation of harvesting bites. Since the animals were led to the pastures early in the morning, it is possible that the observed differences in biting rate of the animals could be due, in part, to hunger, as noted by Greenwood and Demment (1988), since the animals did not forage overnight.

The structure of pasture influences the time of search, harvest and chewing of forage, which combine to determine forage intake (Daniel *et al*., 2011). The potential for selection was inherent in sparse plots. However, this potential appears to be greatest in sparse plots cut to 20 cm height than for other treatments. This was evident as the animals put in this plot were observed to harvest small bites, perhaps due to maturity and sparse nature of the plot. If animals in this circumstance are able to extend length of time spent grazing in order to meet their intake requirement, animal production is not likely to be affected. Calves grazing in pasture with dense biomass cut to 10 cm height recorded higher bite number from the first to the fourth quarter of the access time than other treatments, and this contradicts the submission of Mezzalira *et al*. (2014) that bite mass; a derivative of bite rate, increases with increasing sward height.It is, therefore, worthy of note that grazing pastures before maturity offers higher intake of herbage and nutritive forage to the grazing animal, but this also depends on the botanical composition of the pasture.

Practically, it is impossible to prevent the accumulation of stem and dead materials in pasture swards, despite variation between species (Stobbs, 1974). Given this situation, the removal of top parts of pasture plants through cut back, especially where senescent materials become dominant; can significantly increase accessibility to young shoots by grazing animals, thereby increasing animal production. Such a practice is however not generally advocated for sown grass/legume tropical pastures because of some deleterious effect on some legumes (Stobbs, 1974). Pasture swards with large quantities of available succulent leafy herbage and accessible for grazing offer higher animal production potential. Whilst further studies are in progress to evaluate the effect of grazing on botanical composition of sown grass/legume sward and evaluation of regrowth potential of such sward, increasing biting rates, rather than increasing time spent foraging, might be the optimal strategy to ensure high intake rate on sown pastures.

## CONCLUSION

There was a progressive decline in the biting rate of WF calves from both pasture types at varying sward height, with the highest recorded for dense pasture biomass cut to 10 cm height. It is concluded that biting rate of WF calves is strongly correlated to sward height and spatial distribution of pasture biomass. There is evidence that offering calves' dense pasture cut to 10 cm height in short-term grazing may lead to increased intake, as a result of reduced bite mass on the shorter sward. The data obtained in this study are valid as an indication of the investigated animals' potential. Measurement of biting rate during early morning grazing session provides insight on the relative ease of pasture defoliation by grazing animals.

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**Conflict of interest**

The authors hereby declare that there is no financial/personal interest or belief that could affect our objectivity on this research. In addition, no potential conflict from individual or group exists regarding this study. We also certify that this research has not being published elsewhere or submitted to other journals for publication.

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# POSSIBLE PHYSIOLOGICAL AND ENVIRONMENTAL FACTORS AFFECTING MILK PRODUCTION AND UDDER HEALTH OF DAIRY COWS: A REVIEW

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## ABSTRACT

The milk production efficiency is affected by many factors, where the quantity and quality are the most important. An important role in the overall milk production economy is not only the current purchase price and the associated costs. The costs of rearing heifers and pregnant animals in relation to future milk production and udder health are very important too. During the rearing of the calf heifers and during the drying off period of the pregnant animals, many factors directly influence the effectiveness of milk production and udder health. Among them, the breeding environment (temperature, ventilation, nutrition etc.), the physiological state (stage and order of lactation, growth intensity of heifers early after birth, duration of drying, the age at first conception or season of calving etc.) and the udder health (the level of somatic cell count at time of dry off and calving) are discussed in the paper.

**Key words:** cows; physiological and environmental factors; milk production; udder; health

## INTRODUCTION

Milk production and milk composition, hygienic safety and technological qualities (udder health – mastitis pathogens) are crucial factors affecting the economy of dairy farms. However, from the point of view of farm economy, the most important factor is the quantity of milk production. Milk quality and hygienic safety are considered important economic factors only when these parameters are decreased, and dairy processor is not willing to pay fixed price. In that case, the income from milk production is, thus, directly related to the milk composition and the udder health. Therefore, for effective economy of dairy farms, the complex husbandry factors affecting milk performance have to be considered. Especially at present, there are big data available about animals and housing systems (regular milk recording, electronic identification of different behavioural activities or milk performances, microclimatic conditions, etc.), which should be used to improve management of dairy farms in a term of precision farming. As it is described in a literature review, many factors influence milk performance during the period of cows' life, but these factors were studied mainly separately under experimental conditions. Therefore, on the base of scientific knowledge related to the factors significantly involved in milk performance, it would be very important for dairy practice to evaluate the effects of most important factors and their economic importance in milk performances more complexly, under certain practical conditions in Slovak dairy farms.

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Thus, the aim of this review was to collect data from literature to summarise the physiological and environmental factors affecting the milk production and udder health of dairy cows with the emphasis on relationships between rearing conditions during prenatal and early postnatal period of heifer calves and their adult production.

**The rearing of heifer calves**

Heifer calves represent the future of each dairy farm. Thought less evidence probably future metabolic, milking and reproductive functions of the cow nowadays is not only a result of genetics, but also the consequence of the metabolic environment during foetal development and postnatal nutrition and management (Bach, 2012). Moreover, the season of birth, but not calving, had a significant influence on milk yield, with winter-born heifers producing less than heifers born in any other season (van Eetvelde *et al*., 2017). Last mentioned authors also pointed out that the lower yielding winter-born heifers had higher insulin concentrations at birth, whereas glucose concentrations were similar. They concluded that heifers born during the hotter months are born with higher peripheral insulin sensitivity, finally leading to a higher first-lactation milk yield. Therefore, during early postnatal period good system of heifer rearing gives an important basis for future milk production and health. The information or data available on farm making optimal management decisions to rear dairy heifers properly. However, the costs of heifer rearing are too high to wait until we have found out that something went wrong during her growing phase (Bach and Ahedo, 2008). The high cost of rearing during this period is associated strongly with the age at breeding and age at conception (Boulton *et al*., 2015). They pointed out that during the period from weaning to conception the good rearing and breeding practice should by well managed to ensure that recommended daily live weight gains are maintained consistently and that high conception rates are achieved.

During the whole period from birth to parturition the development of heifers is influenced by many factors that more or less affect their milk production at the first and probably other lactations. Therefore, any data about animal response to housing are useful for proper rearing of heifers to other farmers. One of the most important data indicating optimal growth rate is body weight. As heifer rearing is a costly investment, dairy farmers have been stimulated to maximize early growth of their calves, mainly by enhanced liquid feeding (van Eetvelde *et al*., 2017). The effects of enhanced liquid milk feeding were intensively investigated on milk performance, especially during the first lactation (Moallem *et al*., 2010; Shamay *et al*., 2015). However, as it was pointed out by van Eetvelde and Opsomer (2017), a little is known about the long-term effects of this "accelerated feeding" on fertility, metabolic health and lifespan of dairy cows. In our dairy practice there are limited data (if any), related to body weight during milk nutrition, prepubertal and postpubertal period or body weight at first mating. There are many results from different studies that high growth rate during first 2-3 week of age of heifers significantly increase their milk production during lactation (Drackley, 2005). Even well fed calves (native milk *ad libitum*) were 5 cm taller, reached puberty onset 23 day earlier, calved 30 d earlier, and produced 453 kg more milk at the first lactation than calves fed milk replacer in restricted amounts (Bar-Peled *et al*., 1997; Shamay *et al*., 2005). In another work, the milk yield was even 1100 kg higher in well fed calves before weaning during their first lactation (Pollard *et al*., 2003). Intensive growth of calves before weening was also related to the shorter age of first calving and tendency of higher milk production (Rincker *et al*., 2011). Also, Ettema *et al*. (2004) found out that higher growth rate during the first 6 months of life has been shown to decrease the age at first calving, reducing rearing costs and shortening the non-productive life of the heifer. Hence, optimization of rearing strategies is necessary, as early body weight accretion is most efficient (Bach *et al*., 2008). At present, at the nutrition of young dairy heifers is a widespread problem, which should be addressed by improved monitoring of growth at regular intervals on both beef and dairy units would aid farmers to optimise their heifer management (Wathes *et al*., 2014). Researchers found that 22 % of the variation in first lactation milk yield was explained by pre-weaning growth, and this effect was three to five times greater than that of genetic merit (Van Amburgh *et al*., 2011). On the other hand, opposite effect is observed

during prepubertal period where the negative effect of excessive rate of weight gain impairs the development udder resulting in lower milk production during future lactation (Sejrsen and Purub, 1997). We also demonstrated positive effect of growth rate of calves during period before weaning and negative ones during prepubertal period on milk yield of Holstein cows in our experimental farm (Uhrinčať *et al*., 2007). In addition, rearing systems of calf heifers also affect their behaviour in response to environmental factors in adults. For example, under stress impact the higher residual milk volume after milking indicates that heifers reared under own nursing cows express higher sensitivity to stressors during whole production life (Mačuhová *et al*., 2009).

Growth intensity of calves during milk nutrition period depends mainly on milk powder composition or amount of feed consumed by calves. However, if environmental conditions are very aggressive (temperature, humidity, airflow etc.) the nutrients are needed for thermogenesis, that reduces the amount of nutrients for body weight gain and in dairy practice it is often possible to notice even body weight loss. For example, a 45 kg calf consuming 500 $g.d^{-1}$ of a typical milk replacer powder will lose body weight when the effective environmental temperature is below 5 °C (National Research Council, 2001). Therefore, the effects of environmental conditions and level of nutrition shortly after birth (colostrum intake), during period of first weeks of calf's life and the prepubertal period on milk performance and their health at adult deserve detail study at the farm level because of significant economic impact. Also weaning is considered as a stressful factor (Weary and Jasper, 2008), which may compromise the immune response of calves for at least 2 weeks after weaning (Hulbert *et al*., 2011), and caused health problems may negatively influence growth of calves (Berge *et al*., 2009)

**The heat stress**

Another very important factor in the calf prenatal development is the effect of environmental temperature on pregnant heifers or cows especially in the last trimester of pregnancy. Calves from cooled cows had greater body weight than calves from stressed cows by heat at birth (42.5 vs. 36.5 kg) (Tao *et al*., 2012). Mentioned differences in body weights between cooled and heat stressed animals were observed also in their calves at weaning time (78.5 vs. 65.9 kg). The differences in the body weight between calves calved to cooled or heat stressed cows could be also related to the development of digestive tracts and other regulatory mechanisms of the body. It was significantly proved that calves from cooled cows had higher total protein, total serum immunoglobulins G and apparently efficient absorption than the calves from heat-stressed cows (Tao *et al*., 2012). Moreover, recent research suggests that heat stress during the last 6 wk of gestation negatively affects fertility and milk production up to and through the first lactation of offspring. A possible explanation for this difference could be the direct effect of the body weight on fertility, as heavier heifers seem to become fertile earlier, or by differences in mammary gland development and altered metabolic efficiency (Monteiro *et al*., 2016). During the dry period, heat stress results in impaired mammary growth, leading to reduced milk yield in the subsequent lactation. Nevertheless, the effects of heat stress on milk composition and quality are inconclusive (Tao *et al*., 2018).

Heat stress affects also gestation length, which was at average 4 d shorter for heat-stressed cows compared with cooled cows. Cooled cows had greater milk production (28.9 vs. 33.9 $kg.d^{-1}$), lower milk protein concentration (3.01 vs. 2.87 %), and tended to have lower somatic cell score (3.35 vs. 2.94) through 280 days in milk (Tao *et al*., 2011). Under practical farming the effect of month of calving on gestation length was also confirmed (Tomasek *et al*., 2017). Maternal heat stress also desensitizes a calf's stress response and alters the foetal development by reducing the hormonal profiles (Guo *et al*., 2016). Recently it was also shown that *in utero* heat stress during late gestation had immediate and prolonged effects on passive immunity, growth and activity patterns in dairy calves (Laporta *et al*., 2017). In pregnant cows the heat stress during the dry period decreased mammary cell proliferation rate (1.0 vs. 3.3 %) at −20 d relative to calving compared with cooled cows (Tao *et al*., 2011). Even cooling cows during the dry period alter immune functions and neutrophil response in the udder to mastitis at early lactation (Thompson *et al*., 2014). The importance of microclimatic conditions during the dry-off period was also pointed out in the study of Thompson and Dahl (2012). They found out increased incidence of postpartum disorders

(retained placenta and mastitis) associated with the exposure of cows to high temperatures during above-mentioned critical period. Thus, this heat stress of the dam during the dry period (and possible effect of a season of calving) compromises the foetal growth, immune and endocrine functions of offspring from birth to weaning, as well as the immune system of the udder. In dairy practice, there are different conditions of housing of pregnant animals, therefore, more detail view on this impact could contribute to the optimal development of offspring during the dry-off period. Based on our practical experiences, there is limited effort of farmers to prevent the effect of heat stress on pregnant animals. The response of animals to heat stress could be evaluated in relation to certain breeds. It seems that Jersey cows appeared to be more heat tolerant than Holstein cows (West *et al*., 2003; Smith *et al*., 2013). Therefore, a breed should be taken into account when the effect of heat stress is evaluated.

**The management of dry-off period**

Very important part for next milk performance after previous lactation is the management of dry-off period. It represents ideal conditions for mammary gland recovery from many aspects – physiological, morphological and immunological (Pezeshki *et al*., 2010). Though there many scientific articles were published that recommend the optimal management of dry period for milk performance there is a need to re-evaluate the influence of the management of dry period due to changes or clear increase in milk production of dairy cows throughout last decades (Annen *et al*., 2004; Grummer and Rastani, 2004). As it was mentioned in last reviews or new results of Chen *et al*. (2015) and Van Hoeij *et al*. (2016), the duration of dry period is important not only for milk yield, but also for metabolic status of cows after parturition and udder health following lactation. The lengths of dry period have some effect on udder health (Sawa *et al*., 2015), but crucial role is played also by the manner of drying of dairy cows – with or without antibiotic injection into the udder (Scherpenzeel *et al*., 2014; Golder *et al*., 2016) and abrupt or gradual cessation (reduced frequency of milking) (Gott *et al*., 2016). Some studies demonstrated that a dry period less than 40 d reduces milk yield in the subsequent lactation, and an 8-wk dry period is optimal (Funk *et al*., 1987; Sørensen and Enevoldsen, 1991). Most of the studies that led to the recommendation of a 60 days pospartum were completed before 1990. Others have shown no production losses following a 30 days dry period (Bachman, 2002; Gulay *et al*., 2003). The effect of dry-off lengths still deserves detail study under practical conditions. Furthermore, short-day length during the dry period also increases milk yield post-partum. Season of the year affects both yield and composition of milk. Seasonal variables known to impact milk yield and composition are photoperiod and thermal environmental variables, such as temperature, wind speed, solar infrared load and humidity (Collier *et al*., 2017).

**The impact of mastitis during the dry-off period**

Mastitis is considered to be the most important and challenging dairy cattle disease, with huge financial impacts. The economic consequences of clinical or subclinical mastitis include loss of milk production, loss of milk sales, lower price for high somatic cell count (SCC) in milk, increased culling rates and cost for veterinary treatments (Petrovski *et al*., 2006; Halasa *et al*., 2007; Huijps *et al*., 2008). Despite this, disease--recording systems compiling data from a large number of farms are still not widely implemented in dairy practice; thus, selection for mastitis resistance, which would improve resistance against other diseases and enhance both fertility and longevity, is often based on genetically correlated indicator traits, such as somatic cell count, udder depth and fore udder attachment (Martin *et al*., 2018).

The very important part of the review is to point out the research in the biological and economic impact of the prevention and treatment of mastitis, caused by a variety of contagious and environmental pathogens in dairy practice. Mastitis occurred during lactation but more frequently the udder health status is negatively connected shortly after the dry-off time and also new intramammary infections acquired at the end of dry period (shortly before parturition) are affecting udder health after parturition and during following lactation. Therefore, mastitis should be seriously considered in dry-off management (Vangroenweghe *et al*., 2005). Greater last SCC before the conventional drying-off day (no

antibiotics) was associated with a two-time greater risk of at least one case of clinical mastitis in the subsequent lactation (van Hoeij *et al*., 2017). Recently, Bradley *et al*. (2015) found out that dynamics of intramammary infections acquired during the dry period on European dairy farms was not influenced by a cow and quarter factors measured in their study suggest that the herd and management factors may be more influential. A relatively high proportion of dairy cows also have subclinical mastitis (Heringstad *et al*., 2000). Subclinical mastitis might be caused by extended dry period (143 to 250 d), which increases the occurrence of subclinical mastitis during early lactation, has a negative association with reproductive performance (Pinedo *et al*., 2011) and affects milk production and quality, which is characterized by the presence of inflammatory components in the milk (Heringstad *et al*., 2000).

Due to the legislative and consumer pressure on the reduction of antibiotics used in dairy practice, it seems that selective using of antibiotics at drying period could be a useful tool, but only at quarters or udders with low somatic cells without presence of pathogens (Scherpenzeel *et al*., 2014), or alternative methods for infected quarters could be used, such as natural casein hydrolase (Leitner *et al*., 2017). Last mentioned authors pointed out that the decrease in the antibiotic use by drying off quarters without antibiotics significantly increases clinical mastitis (CM) and somatic cell count in milk after parturition, but such increase was not compensated by an increase in antibiotic use for treating CM. Total antibiotic use related to mastitis was thus reduced by 85 % in these quarters. Despite proven efficacy and widespread use of antibiotics before the dry-off status, the use of antibiotics has limitation due to several reasons. Over time the antibiotics reduce the efficacy at the end of dry period (Oliver *et al*., 1990; Pinedo *et al*., 2012). At parturition there are many environmental bacteria (gram-negative) that are not so sensitive against antibiotics. It was found that environmental bacteria are now the most common causes of new mastitis during the dry period (Bradley and Green, 2004). In our previous work with the milk samples from cows suspicious for mastitis, we revealed that many milk samples were microbiologically positive mainly for environmental bacteria (Idriss *et al*., 2013a). On the base of microbiological cultivation, the management can implement effective mastitis control program (Idriss *et al*., 2013b) already used in Nordic countries (Osteras and Solverod, 2009), which is based on treating the cows that are most likely to have intra-mammary infection (Kiesner *et al*., 2016) i.e. the cows with high SCC last day before the dry-off period (Vanhoudt *et al*., 2018). However, in present situation dairy practitioners in Slovakia are not willing to finance the microbiological cultivation, so there is a limited information about mastitis pathogens in dairy practice. One of the reasons of this status is that the most of the samples are bacteriologically negative (Idriss *et al*., 2013b). Therefore, there is necessary to focus on further research to explain the high percentage of microbiologically negative samples of milk from the mastitis cows and to define possible factors involved. Without regular cultivation of milk samples from suspicious cows there is very low chance to reduce mastitis occurrence in the herd.

Another challenge of effective dry-off is high milk yield at the time of drying, which increases a risk of milk accumulation in the udder and possible negative effect on welfare (pressure in the udder) and the udder health (milk leakage) (Ollier *et al*., 2015). The increased yield at drying-off has been associated with an increased risk of new mastitis in the dry period and calving, mainly because of increased risk of leaking milk and intra-mammary pressure (Bradley and Green, 2004; Rajala-Schultz *et al*., 2005). On the other hand, gradually reducing milking frequency of high-producing cows resulted in reduced time spent to anticipating milking and reduced milk leakage after dry-off (Zobel *et al*., 2013). It is possible to conclude that implementation of different management schemes near dry-off for different status of animals may significantly improve milk performance and mammary health within a herd and, thus, the economic efficiency and price competition of dairy farms.

## CONCLUSIONS

As we described above, there are many environmental and animal factors influencing milk performance during the period of cows' life. Therefore, on the base of scientific knowledge related to factors significantly involved in milk

performance, it would be very important for dairy practice to evaluate the effects of most important environmental factors and their economic importance in milk performances more complexly under current practical conditions.

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# DILUTION FACTOR AFFECTS THE ABILITY OF RAM SPERM TO SURVIVE CRYOPRESERVATION: SHORT COMMUNICATION

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## ABSTRACT

In this preliminary study, the fresh and frozen-thawed sperm from the original Valachian sheep breed were analysed for its quality. Semen was collected from two rams by electroejaculation and used for motility analysis using computer-assisted sperm analysis immediately after collection and following 60 and 120 min of incubation at 37 °C, or for rapid freezing. Semen was equilibrated at 15 °C for 20 min and diluted with a commercial diluent OviXcell (IMV Technologies) supplemented with 100 mM trehalose to a ratio of 1:1 (DR 1:1 group) or 1:2 (DR 1:2 group) (v:v). After 90 min of equilibration at 5 °C, the straws were suspended horizontally in liquid nitrogen vapours for 10 min before being plunged into a liquid nitrogen for storage. After one month, the straws were thawed by immersing into a water bath at 38 °C for 60 s. Sperm motility analysis was done immediately after thawing and after 30 and 60 min of incubation at 37 °C, as stated above. Our results showed that the dilution ratio has considerable effect on ram sperm survivability. Higher percentage of total motility and progressive movement ($P \leq 0.05$) was found in the DR 1:2 when compared to the DR 1:1 group. In addition, our results confirmed inter-male variability in the susceptibility to the cryopreservation process. Nevertheless, results of this study are of preliminary character. Experiments using higher number of samples (individuals) as well as other methodology approaches need to be tested in order to find the best procedure for semen cryopreservation of Slovak national sheep breeds.

**Key words:** ram; sperm; cryopreservation; dilution

## INTRODUCTION

Generally, animal gene banks play an important role in agricultural production globally for the present and the future, and in sustaining the most of production systems and community livelihoods. The situation with animal genetic resources in the Slovak Republic is not satisfactory due to the fact that livestock semen doses stored in the gene bank are originated only from several Slovak breeds (Chrenek *et al.*, 2017). Therefore, if there is an opportunity to obtain biological material from valuable breeds, it is desirable to optimize specific cryopreservation process. The original Valachian sheep has the important functions such as maintaining natural landscape and agrotourism, and its historical value has been recognized (Oravcová and Krupa, 2011).

In order to optimize ram sperm cryopreservation in our conditions, we aimed to analyse whether different semen dilution ratios with freezing solution affects the post-thaw quality of Valachian sheep semen.

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## MATERIAL AND METHODS

### Semen collection

Semen was collected by electroejaculation from two original Valachian sheep breed rams (PV1; PV2). The rectum was cleaned of faeces. A three electrode probe 1" for ram and boar with diameter of 2.54 cm and length of approximately 16 cm, connected to a power source that allowed voltage and amperage control, were used (Minitube Electro-ejaculator). The EE regime (automatic mode, type of curve 2 – the power output is linearly increased from 0.5 Volt to 7 Volt) consisted of consecutive series of 2 s pulses of similar voltage, each separated by 2 s break. The initial voltage was 0.5 V, which was increased in each series until maximum of 7 V. Upon reaching a voltage of 7 V, impulses remained at this level until the ejaculation was complete. After collection, the semen was transported to the laboratory in a water bath at 37 °C.

### Sperm quality evaluation and cryopreservation

An aliquot taken from each fresh semen sample was used for motility analysis immediately after collection and following 60 and 120 min of incubation at 37 °C. Semen was diluted in a saline (0.9 % NaCl; Braun, Germany) at the ratio of 1:20, immediately placed into a Leja Standard Count Analysis Chamber (depth of 20 microns; MiniTüb, Tiefenbach, Germany) and evaluated under a Zeiss AxioScope A1 microscope using the CASA system (Sperm VisionTM; MiniTübe, Tiefenbach, Germany). For each sample and repetition, seven microscopic view fields were analyzed for average concentration (CON; $1 \times 10^9$) and percentage of total motility (TM; motility > 5 $\mu m.s^{-1}$) and progressively moving spermatozoa (PM; motility > 20 $\mu m.s^{-1}$). The rest of the semen samples were used for cryopreservation.

Semen was frozen using a rapid freezing method. Individual semen samples were cooled down to 15 °C for 20 min to minimize cold-shock damage. After cooling, an aliquot of semen was diluted in a commercial diluent (OviXcell, IMV Technologies, France) enriched with 100 mM trehalose (Sigma Aldrich, Germany) to a ratio of 1:1 or 1:2 (v:v). Thereafter, the semen was loaded into 0.25 ml plastic straws and equilibrated at 5 °C for 90 min. The straws were suspended horizontally in liquid nitrogen vapours (LNV) 5 cm above the liquid nitrogen ($LN_2$) level for 10 min (-125 to -130 °C) before being plunged into a $LN_2$ (-196 °C) for storage. After one month of storage the straws were thawed by immersing into a water bath at 38 °C for 60 s. Sperm motility analysis was done immediately after thawing and after 30 and 60 min of incubation at 37 °C, as stated above.

### Statistical analysis

Sperm quality between the two dilution ratios and between the two rams was compared by a t-test using a Sigma Plot software (Systat Software Inc., Germany). Values at $P \leq 0.05$ were considered as statistically significant.

## RESULTS AND DISCUSSION

In our study, the fresh and frozen-thawed sperm from two males of original Valachian sheep were analysed for its quality. We aimed to compare the effect of different dilution ratios during the cryopreservation process on individual sperm motility. No difference in CON, TM and PM of fresh semen was found between the two males tested (Table 1).

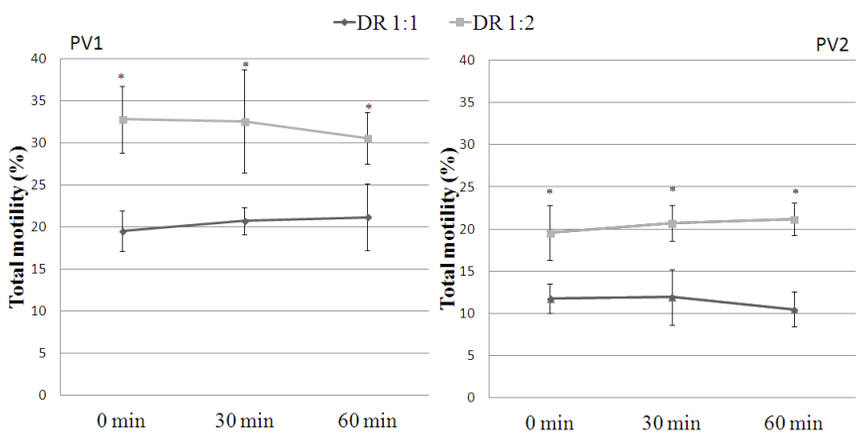
**Table 1. Concentration and motility of fresh sperm from the two Valachian sheep rams**

| Ram | CON (x $10^9$) | TM00 (%) | PM00 (%) | TM60 (%) | PM60 (%) | TM120 (%) | PM120 (%) |
|---|---|---|---|---|---|---|---|
| PV1 | 1.655 ± 0.2 | 78.9 ± 3.1 | 74.5 ± 2.4 | 78.2 ± 6.1 | 74.7 ± 4.6 | 78.6 ± 3.9 | 75.5 ± 4.6 |
| PV2 | 1.725 ± 0.2 | 75.2 ± 2.7 | 69.9 ± 2.4 | 72.4 ± 1.6 | 69.4 ± 2.1 | 77.4 ± 2.8 | 73.5 ± 2.7 |

VOL – volume; CON-concentration; TM – total motility; PM – progressive movement

Using the frozen-thawed semen, the dilution ratio has considerable effect on ram sperm survivability. Total motility was higher (P ≤ 0.05) in semen diluted to a ratio of 1:2 (DR 1:2) when compared to DR 1:1 in both males (PV1; PV2) and at each time point post-thaw (Figure 1). The same trend was noticed in progressive movement (PM; Table 2). It was already shown that sperm concentration at freezing affects post-thaw quality of ram sperm (Alvarez *et al*., 2012).

Moreover, our results confirmed inter-male variability in the susceptibility to the cryopreservation process. Although the fresh semen quality was similar between PV1 and PV2, frozen-thawed semen showed difference (P ≤ 0.05) in TM and PM between the two males (Figure 1; Table 2). Variability in post-thaw quality among males of the same breed has been reported for several species (Waterhouse *et al*., 2006; Lavara *et al*., 2013; Sellem *et al*., 2015; Kulíková *et al*., 2017). Therefore, in order to make a ram semen collection for later use, each individual sample needs to be tested before storage. Nevertheless, results of this study are of preliminary character. Experiments on higher number of samples (individuals) as well as other methodology approaches (different media, equilibration times, addition of other cryoprotectants) need to be tested in order to find the best proceduere for semen cryopreservation of Slovak national sheep breeds.




DR – dilution ratio; PV1, PV2 – two individual males;
* means difference (P ≤ 0.05) between DR 1:1 and DR 1:2

**Figure 1. Differences in total motility between two dilution ratios used**

**Table 2. Differences in sperm post-thaw progressive movement between two dilution ratios and between two individual rams**

| Ram | PV1 | | | PV2 | | |
|---|---|---|---|---|---|---|
| Group | PM00 | PM30 | PM60 | PM00 | PM30 | PM60 |
| DR 1:1 | 7.8 ± 2.2$^{b}$ | 10.2 ± 2.1$^{b}$ | 7.3 ± 2.4$^{b}$ | 6.0 ± 1.4$^{b}$ | 7.6 ± 2.6 | 6.4 ± 0.9$^{b}$ |
| DR 1:2 | 21.2 ± 3.4$^{a}$* | 26.8 ± 4.5$^{a}$* | 22.2 ± 3.7$^{a}$* | 11.7 ± 2.6$^{a}$* | 11.9 ± 1.9* | 12.6 ± 1.6$^{a}$* |

PM-progressive movement at 0, 30 or 60 min of incubation at 37 °C; DR – dilution ratio; $^{a}$ vs $^{b}$ means difference (P ≤ 0.05) between dilution ratios; * means difference (P ≤ 0.05) between rams (PV1; PV2) at specific time of incubation

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