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NUTRIENT CONTENT AND DEGRADABILITY OF DRY MATTER IN WHOLE PLANTS OF MAIZE

Zuzana MLYNEKOVÁ*, Mária CHRENKOVÁ, Zuzana FORMELOVÁ

NPPC – Research Institute for Animal Production Nitra, Lužianky, Slovak Republic

ABSTRACT

The objective of our study was to determine the nutrients content and dry matter degradability in whole plants of maize hybrids dent and dent x flint. Hybrids dent x flint − Mesnil, Chambord, Queen, and hybrids dent − Aude, Meridien, KX 1393, Omero were used. Concentration of crude protein (CP) was higher in dent hybrids (85.0 $g.kg^{-1}$ DM) compared to dent x flint hybrids (78.3 $g.kg^{-1}$ DM). Content of starch ranged from 205.2 $g.kg^{-1}$ DM (KX 1393 − dent) to 329.4 $g.kg^{-1}$ DM (Mesnil), higher for hybrids dent x flint. ADF and NDF were higher in hybrids dent and content of lignin was similar. *In sacco* experiment was carried out in three rumen-cannulated cows with large rumen cannulas. Hybrids dent x flint had in average higher effective dry matter degradability (DMD) − 56.8 % than dent hybrids 54.7 %.

Key words: maize plants; *in sacco* method; degradability

INTRODUCTION

Maize has specific characteristics compared to other feeds. It exceeds all feed crops in yield and net high biological value of corn starch and can be used as fodder and concentrate feed (Sommer, 2001). There are differences in the content of structural and non-structural carbohydrates among hybrids and these differences affect the overall digestibility of organic matter (Gálik *et al.*, 2004). According to Verbič *et al.* (1995), there are big differences among maize hybrids in the content of NDF and ADF in the individual morphological parts of the plant. In particular, the ADF content of the plant (without the cob) significantly affects the effective dry matter degradability (R^2 = 0.99).

From a nutritional point of view, whole maize plant is a mixture of fodder and grain, comprising two components of different nutritional value. The maize plant is characterized by high production of organic matter, the substances which are important for animal nutrition.

The objective of this work was to determine dry matter degradability of whole plants of different maize hybrids by *in sacco* method and their nutrition contents.

MATERIAL AND METHODS

Maize hybrids of the dent type (Aude, Meridien, KX 1393, Omero) and dent x flint (Mesnil, Chambord, Queen) were used in our experiment. Hybrids were grown in the same climatic location (district Trnava – Slovakia). Dry matter degradability in whole maize plants was determined by *in sacco* method (Čerešňáková *et al.*, 2005).

The samples of maize hybrids were harvested at the time of milk-waxy maturity. In the whole plants, original dry mater (DM) and chemical composition

***Correspondence:** E-mail: mlynekova@gmail.com
Zuzana Mlyneková, NPPC − Research Institute for Animal Production Nitra, Department of Nutrition, Hlohovecká 2, 951 41 Lužianky, Slovak Republic

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were determined. Material designed for the determination of degradability was freeze-dried and ground.

These samples were weighed (approx. 2.50 g dry matter) into bags (9 x 15 cm) made of Uhelon 120T with pore size of 48 µm. Minimum of three separate bags for hybrids, incubation time and animals were used. The bags with samples were incubated for 6, 9, 16, 24, 48, 72 and 96 hours. The 0 h time bags were only washed in washing maschine to determine washing losses.

In sacco experiments were carried out in three non-lactating heifers (Holstein Fresian cattle) with large rumen cannulas (an average of 10 cm). The animals were fed twice a day a diet consisting of 70 % forage and 30 % concentrate on a dry matter basis at maintenance level. The ration consisted of maize silage, alfalfa hay, wheat, barley meal (1:1) and vitamin – mineral premix. Nutrient intake to one cow.day^{-1} in our experiment was followed: 9770 g dry matter; 1170 g crude protein; 5050 g nitrogen free extract; 2660 g fibre; 1980 g starch and 650 g ash. Access to water was *ad libitum*.

The content of nutrients was analysed according to the directive of the Commission Regulation (EC) no. 152/2009 from 27th January 2009, which defines the methods of sample collection and analysis for the purposes of official feed quality control. Content of ADF, NDF and lignin was determined according to Van Soest (Lutonská and Pichl, 1983). The parameters of degradability (a: rapidly soluble fraction; b: potentially degradable fraction; c: rate constant of degradation; Edg: effective degradability) were calculated using the equations by Ørskov and McDonald (1979) with outflow rate of 0.06.h^{-1}.

The obtained data on nutrients, the losses with time of incubation and degradability of dry matter in maize hybrids were evaluated statistically using models in statistical package Statistix 8.0.

RESULT AND DISCUSION

Nutrients content is presented in Table 1. Chemical analyses showed that dent hybrids had in the whole plants a higher content of ADF, NDF and crude protein compared to dent x flint hybrids. The highest crude protein content was determined in the KX 1393 hybrid (dent) and the lowest in the Chambord hybrid (dent x flint). An average crude protein content in whole plants was higher in dent hybrids than in dent x flint hybrids (85.0 g.kg^{-1} vs. 78.3 g.kg^{-1} DM), the quality of maize proteins is poor because they are deficient in the essential amino acids, lysine and tryptophan (Shewry, 2007).

Content of starch in WP (whole plants) was the highest in hybrid Mesnil (329.4 g.kg^{-1} DM) and the lowest in Meridien (192.5 g.kg^{-1} DM). The average starch content was higher in hybrids dent x flint. Jurjanz *et al.* (2005) determined the starch content in WP to be 372.0 g.kg^{-1} DM, in our experiment it was less.

Fat content in the whole plants was higher in dent x flint hybrids compared to dent (Table 1).

Using one-factor analysis of variance we determined significant differences between the nutrients content of the studied hybrids (Table 2). Differences among hybrids in the nutrients content of whole plants as well as the dry matter are caused not only by the actual differences between the morphological parts, but also by the share of

Table 1. Content of nutrients in whole plants of different maize hybrids (g.kg^{-1} DM)

Hybrid	Type of hybrids	Dry matter	Crude protein	Fat	Starch	ADF	NDF	Lignin
Mesnil	dent x flint	374.2	78.3	29.8	329.4	232.3	429.2	22.6
Chambord	dent x flint	372.2	73.5	23.2	245.9	231.2	435.8	28.4
Queen	dent x flint	402.5	83.0	30.2	311.7	251.8	454.8	31.9
Aude	dent	436.2	79.5	14.9	260.8	245.8	480.3	26.9
KX 1393	dent	374.5	89.9	19.5	205.2	257.6	489.8	28.5
Meridien	dent	374.7	83.9	22.5	192.5	287.2	565.7	23.7
Omero	dent	375.8	86.7	24.5	253.3	279.8	548.7	30.1

Table 2. One factor analysis of variance in whole plants of different maize hybrids

Parameters		Whole plant		
		Hybrid, A $f_A = 6$	Error $f_e = 7$	Significant comparisons
ADF	MS	944.996	2.914	1:(3,4,5,6,7)**, 2:(3,4,5,6,7)**,3:(7,6)**, 4:(5,6,7)**, 5:(6,7)** , 6:7*
	F	324		
NDF	MS	5682.99	13.20	1:(3,4,5,6,7)**, 2:(4,5,6,7)**, 3:(4,5,6,7)**, 4:(6,7)**, 5:(6,7)**, 2:3*
	F	431		
Lignin	MS	22.4230	1.8354	3:(1,6)**, 7:1**, 1:(2,5)*
	F	12.2		
Crude protein	MS	59.8303	0.8030	1:(5,6,7)**, 2:(3,4,5,6,7)**, 3:5**, 4:(5,7)**, 5:6**, 1:(2,3)*, 3:7*, 4:6*
	F	74.5		

1. Mesnil, 2. Chambord, 3. Queen, 4. Aude, 5. KX-1393, 6. Meridien, 7. Omero
Means with the same letters in the same row are significantly different at P < 0.01++ p < 0.05+
Error – error of experiment

various morphological parts and their ripeness at harvest (Verbič *et al.*, 1995).

Kohler *et al.* (1990) found differences in the content of ADF, NDF and lignin among the cultivars. It corresponds with our results. Among the morphological parts of maize plants and also among maize hybrids there are differences in the chemical composition and it results in differences of the effective dry matter degradability. The nutritional value of maize plants decreased with increasing maturity (Gross and Pesche, 1980). Content of ADF and NDF was higher in hybrids dent and content of lignin was similar (Table 1).

We determined significant differences for parameters of degradability and effective degradability of dry matter of whole plants, with the exception of parameter "b". Dent hybrids in whole plants were less degraded than dent x flint hybrids (Table 4).

The losses with time of incubation were determined for each incubation. For dry matter, higher average losses were determined after 6, 9, 16, 48, and 72 hour incubation in hybrid Mesnil (dent x flint), after 96 hour incubation for dent hybrids – Meridien and Omero (Table 3). With longer incubation time, the differences between hybrids decrease. Higher differences between hybrids were

Table 3. Dry matter disappearance from whole plants of maize hybrids during rumen incubation

Incubation (h)		0	6	9	16	24	48	72	96
Hybrid	Type of hybrids								
Mesnil	dent x flint	30.3	53.9^{abcde}	56.2^{ab}	68.4^{abcdef}	69.2^{b}	81.0^{cde}	82.3^{d}	79.6
Chambord	dent x flint	34.7	47.3^{chjk}	53.1	59.1^{ci}	65.5	74.6	81.4^{c}	79.1
Queen	dent x flint	33.1	45.3^{bgjl}	52.7	62.2^{bgh}	70.0^{a}	76.4^{b}	82.2^{b}	80.7
Aude	dent	34.6	47.1^{aflm}	52.7	58.7^{aj}	65.7	77.2^{a}	80.8^{a}	78.5
Meridien	dent	36.5	52.5^{fghik}	54.6^{c}	58.0^{ek}	66.8	69.2^{abd}	79.7	82.1
KX 1393	dent	34.1	45.2^{dimn}	50.7^{a}	50.9^{dgijk}	60.6^{abc}	72.2^{c}	75.5^{abcd}	80.6
Omero	dent	32.3	47.1^{en}	48.9^{bc}	53.6^{fh}	67.7^{c}	72.1e	79.8	81.9

Means with the same letters in the same row are significantly different at P < 0.01++ p < 0.05+

Table 4. Parameters of degradability and effective dry matter degradability (Edg) of different maize hybrids (whole plants)

Parameters of degradability		a (%)	b (%)	c (%.h^{-1})	Edg DM (%)
Hybrid	Type of hybrids				
Mesnil	dent x flint	42.1^{f}	40.3	0.056^{c}	57.0^{d}
Chambord	dent x flint	38.6^{c}	42.7	0.042	56.6^{c}
Queen	dent x flint	29.9^{bf}	51.1	0.063^{ab}	56.9^{b}
Aude	dent	36.3^{a}	44.6	0.047	56.3^{a}
Meridien	dent	48.4^{abcde}	41.2	0.020^{bc}	55.3
KX 1393	dent	38.6^{d}	47.3	0.027^{a}	52.9^{abcd}
Omero	dent	36.8	47.9	0.036	54.3

Means with the same letters in the same row are significantly different at P < 0.01++ p < 0.05+

determined after 6 and 16 hour incubation (P < 0.01 resp. P < 0.05). The lowest losses were determined for hybrid KX 13 93 in all incubation, except 0 and 48 hour incubation. This hybrid had the lowest dry matter degradability (52.9 %).

Effective degradability of dry matter WP of maize was higher for dent x flint type hybrids (56.8 %) compared to dent hybrids (54.7 %) as well as rate of degradation of fraction "b", parameter "c" (Table 4.). The effective degradability is affected by the rate of degradation "c". Lower degradation values were determined by Jurjanz *et al.* (2005) in whole plants of maize (52.1 %) and "b" parameter was 41.6 %.

CONCLUSION

The content of nutrients was different in hybrids. We found higher effective DMD in WP in hybrid dent x flint compare to dent hybrids. From our results follows that there are differences between maize hybrids in chemical composition and differences in effective DM degradability of maize. The determined results are important, because based on the effective degradability of dry matter as well as other nutrients, it is possible to predict their passage into the small intestine.

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HEAVY METAL LEVELS IN THE TISSUES OF WILD LIVING ANIMALS FROM TWO DISTINCT INDUSTRIALLY EXPLOITED AREAS IN SLOVAKIA

Jana MAĽOVÁ[1]*, Juraj CIBEREJ[2], Pavel MAĽA[1], František ZIGO[2], Boris SEMJON[1]

[1]University of Veterinary Medicine and Pharmacy in Košice, Department of Food Hygiene and Technology, Košice, Slovak Republic
[2]University of Veterinary Medicine and Pharmacy in Košice, Department of Nutrition, Dietetics and Animal Breeding, Košice, Slovak Republic

ABSTRACT

The aim of the presented study was to assess the heavy metal burden in biotopes of wild living animals of two distinct industrially exploited areas in Slovakia. 411 samples of various tissues (lung, liver, kidney, spleen, heart and muscle) of red deer, roe deer, mouflon, chamois, wild boar, European brown hare, fox, European brown marten, European badger, gray wolf, brown bear, wildcat, red squirrel, European polecat, alpine marmot, and European otter were collected from the localities between 2014 and 2018. Concentrations of mercury, cadmium, lead, arsenic, nickel, copper and zinc were determined using Atomic absorption spectroscopy. Significant correlations ($p < 0.05$, $t = 0.03162$) of metal levels in each locality and differences between the animals species were recorded. We have found important heavy metal burden in a relatively clean area – Tatra National Park that is legislatively protected and restricted in any industrial activity. In the Zemplín region, the examined heavy metal levels confirm permanent pollution by intensive heavy industrialization. Mostly mercury (29 %) was the metal that exceeded the legal limits permitted for human consumption, then cadmium (28 %) and lead (23 %). Concentration of chromium did not exceed the limit in any sample. The most burdened animal species was wild boar.

Key words: heavy metal; wild animal; tissue; environment; contamination; plant

INTRODUCTION

Animals act as very important part of substance and energy circulation and they play an important role in ecological stabilization of ecosystems as well. However the role of animals is often underestimated (Kulhavý *et al.*, 2003). Contaminants in wild living animals in Slovakia have been monitored since 1995. The basic aim of monitoring is to have a review of the levels and penetration of substances in selected game and fish species. As animals live in various types of biotope and belong to the primary consumer group, data from this study could be evaluated as an appropriate bioindicator of the actual state of the environment and the ecological balance. Thus we can also notice more information about food products – venison and fish (Krížová and Šalgovičová, 2002).

Metals, which are able to cumulate in soil, vegetation and other living organisms, belong to important environment contaminants. Generally, metals do not undergo chemical degradation but are cumulated in upper soil layers. Progressive transport of metals from soil to plants causes higher concentration in animal tissues (Gallo, 1995). Increased heavy metal levels in animal organs and tissues are induced by respiration

***Correspondence:** E-mail: jana.malova@uvlf.sk, ORCID 0000-0002-5547-9149
Jana Maľová, University of Veterinary Medecine and Pharmacy in Košice,
Department of Food Hygiene and Technology Komenského 73,
041 81 Košice, Slovak Republic

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from air and contaminated food intake. Other sources of environmental pollution are industrial fertilizers, exhaust gas from traffic, urban waste, etc. (Kováč *et al.*, 2005; EFSA 2010; Küttner *et al.*, 2014). The highest metal contamination risk is in the surroundings of metal industries, electric power stations and cement mills, which pollute soil and air with air pollutants and those subsequently pass into the food chain. In comparison with domestic animals wild living animals are influenced by the environmental conditions over the whole year (Niemi *et al.*, 1993; Tataruch, 1995; Kugonič and Zupan, 1999). Metals are cumulated by food because of their solubility and mobility that can cause serious ecological and health danger (Abu Al-Rub *et al.*, 2004). Heavy metal monitoring is very important not only for game but also for humans. Permanent exposure of organism to mildly increased concentrations of metals in environmental components is an actual problem for the human population, especially that living in industrial agglomerations. Chronic professional exposure is hardly diagnosed, the symptoms are not specific. Mostly it manifests as balance disorders of organism and chronic multi-symptomal stages – civilization diseases. In some patients, primary diseases are exacerbated by an increased metal concentration in the organism. Game constantly living in natural conditions is a very important bioindicator of its real pollution situation. Examination of wild living animals is the best way to know the level of heavy metal contamination in the natural environment (Babička and Sedláček, 2000).

In Slovakia, legal limits of heavy metal levels in animal body tissues that are acceptable for human consumption are defined in Food Codex of Ministry of Agriculture of Slovak Republic (Regulation of the Ministry of Agriculture and Rural Development of the Slovak Republic and the Ministry of Health of the Slovak Republic from 11 September 2006 No. 18558/2006-SL.).

The aim of our study was to determine the content of mercury (Hg), cadmium (Cd), lead (Pb), arsenic (As), nickel (Ni), copper (Cu) and zinc (Zn) in the tissues of various species of wild living animals from two different industrially exploited areas in Slovakia, to compare the metal contamination burden between the two areas and among the game species and to evaluate the actual situation of the environmental pollution in the localities.

MATERIAL AND METHODS

Tissue samples of various kinds of game species were examined to detect concentrations of heavy metals (mercury, cadmium, lead, arsenic, nickel, copper and zinc) cumulated in animal organisms from two parts of Slovakia (Figure 1). The first locality was Tatra National Park (TANAP) situated in the central north of Slovakia bordering on Poland. It is known as a legislatively protected area. The second locality, Zemplin region in eastern Slovakia, is characterised by its rich industrial exploitation.

Figure 1. Map of Slovakia monitored areas – Tatra National Park (A) and Zemplin region (B)

Table 1. The number of samples from game species in Tatra National Park and Zemplin

Animal species	n (Number of samples)	
	TANAP	Zemplín
Wild boar (*Sus scrofa*)	45	13
Red deer (*Cervus elaphus*)	41	30
Fox (*Vulpes vulpes*)	17	20
Roe deer (*Capreolus capreolus*)	11	19
Gray wolf (*Canis lupus*)	14	13
European badger (*Meles meles*)	-	5
Wildcat (*Felis silvestris*)	-	14
Mouflon (*Ovis musimon*)	-	18
Brown hare (*Lepus europaeus*)	-	57
Chamois (*Rupicapra rupicapra tatrica*)	13	-
European brown marten (*Martes martes*)	12	-
Brown bear (*Ursus arctos*)	26	-
Alpine marmot (*Marmota marmota*)	10	-
European polecat (*Mustela putorius*)	9	-
Red squirrel (*Sciurus vulgaris*)	9	-
European otter (*Lutra lutra*)	15	-
Total	222	189

During the huting seasons 2014/2015 – 2017/2018, tissues from hunted or dead animals – red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), mouflon (*Ovis musimon*), chamois (*Rupicapra rupicapra tatrica*), wild boar (*Sus scrofa*), European brown hare (*Lepus europaeus*), fox (*Vulpes vulpes*), European brown marten (Martes martes), European badger (*Meles meles*), gray wolf (*Canis lupus*), brown bear (*Ursus arctos*), wildcat (*Felis silvestris*), red squirrel (*Sciurus vulgaris*), European polecat (*Mustela putorius*), alpine marmot (*Marmota marmota*), and European otter (*Lutra lutra*) – were collected. Twelve species of wild animals were examined in TANAP and nine species from Zemplin. In total, we gained 411 of various kinds of tissue – lung, liver, kidney, spleen, heart and muscle, 222 samples from TANAP and 189 from Zemplin (Table 1). It was not possible to obtain exactly the same type of tissue and number in all animal species. The samples did not have any pathological lesions and were without toxic lead ammunition (weight from 50 g to 200 g). Each sample was stored in a plastic bag at -18 °C in a freezing box until the laboratory test was performed.

In 2018, due to the observed elevated levels in the wildlife samples, the samples of biotope components (bark of trees, leaf litter, needles, moss, grass) were taken from the TANAP area and the concentrations of the measured elements were then determined for the comparison. The samples were taken at the same sites where the sampling was carried out. Sample preparation consisted of thawing, homogenizing by means of a laboratory mixer, a laboratory mill and a laboratory mortar.

After preparation of macerate and wet mineralization with HNO_3 in MDS 2000 pressure microwave oven, sample filtrates were used to estimate heavy metal concentrations.

After the macerate preparation and mineralization, we used the sample filtrates to estimate heavy metal concentrations. Mercury level was determined using Atomic absorption spectroscopy (AAS) with dedicated AMA mercury analyzer (Advance Mercury Analyzer AMA 254 by ALTEC). Cadmium, lead, arsenic, chromium, nickel and zinc were detected by flameless AAS (Varian SpctrAA Zeeman/240) with graphite cell. AAS with flame (AASF) was used to determine copper levels (Varian SpectrAA/600). The heavy metal detection was carried out in an accredited laboratory of State Veterinary and Food Institute in Dolný Kubín. Regarding time restricted hunting season and various legislative conditions, the sampling was not strictly continual and simple process.

Statistical processing of results was carried out by Microsoft Office Excel 2007. Results were expressed as a concentration range ($c_{min.-max.}$), the least square means (average) and median. Tukey's multiple comparison test was used to compare statistical differences among values and $p < 0.05$ was considered a statistically significant difference.[1]

RESULTS AND DISCUSSION

Heavy metal concentration range, average and median in tissue samples (n = 222) of wild living animals from TANAP are shown in Table 2 and Table 3 presents respective data (n = 189) from the Zemplin region. As Lazarus *et al.* (2005) claims, calculated median

1 min. – max. range of values exceeded the legal limit

values are considered the best heavy metal burden representation among wildlife. The result data from the two monitored localities are presented in Table 4.

- Of the 411 examined samples, in 170 samples the values of the elements were found to be over the permitted limit (41.36 %).
- A significant difference ($p < 0.05$; $t = 0.03162$) in total metal burden between the two localities was found. The more contaminated area due to the presence of contaminants is TANAP. This fact is very interesting because of no direct industrial activities in TANAP area. In TANAP,

Table 2. The heavy metal concentration range, average and median in tissues of wild living animals from Tatra National Park

Animal species		Heavy metal concentration in mg.kg^{-1}					
		Hg	Cd	Pb	As	Cu	Zn
Wild boar	$c_{min.-max}$	0.02-0.871	0.003-2.633	0.009-1.894	0.06-2.94	0.15-53.12	10.90-326.5
	Average	0.147	0.284	0.34	0.586	4.451	59.193
	Median	0.106	0.098	0.286	0.53	1.640	36.50
Red deer	$c_{min.-max}$	N.A.	0.0016-1.80	0.002-1.479	0.006-0.456	N.A.	11.67-101.0
	Average	N.A.	0.289	0.264	0.053	N.A.	29.577
	Median	N.A.	0.127	0.134	0.040	N.A.	27.32
Fox	$c_{min.-max}$	0.013-0.987	0.002-1.24	0.033-13.25	N.A.	1.330-50.0	13.87-142.19
	Average	0.231	0.639	1.137	N.A.	8.210	36.672
	Median	0.15	0.15	4.261	N.A.	2.40	25.24
Roe deer	$c_{min.-max}$	0.005-0.094	0.009-0.508	0.024-1.08	0.001-0.09	0.065-42.80	N.A.
	Average	0.031	0.138	0.159	0.031	13.348	N.A.
	Median	0.02	0.093	0.086	0.021	2.03	N.A.
Wolf	$c_{min.-max}$	0.023-0.68	0.010-0.403	0.090-0.153	0.010-0.368	0.26-22.33	7.31-97.68
	Average	0.042	0.082	0.156	0.081	5.376	30.715
	Median	0.042	0.039	0.154	0.021	4.605	22.46
Chamois	$c_{min.-max}$	0.001-0.019	0.002-1.998	0.006-144.25	0.002-0.010	N.A.	0.002-0.241
	Average	0.004	0.316	11.366	0.005	N.A.	16.982
	Median	0.002	0.016	0.084	0.003	N.A.	16.595
Marten	$c_{min.-max}$	0.025-0.274	0.027-0.557	0.053-0.281	0.02-2.63	2.30-31.25	17.66-67.19
	Average	0.100	0.250	0.195	0.366	7.876	39.426
	Median	0.066	0.191	0.132	0.157	6.343	32.615
Brown bear	$c_{min.-max}$	0.001-0.607	0.002-3.38	0.002-2.26	0.005-0.254	1.06-10.92	5.21-57.15
	Average	0.115	0.510	0.305	0.086	8.506	26.207
	Median	0.075	0.280	0.092	0.038	5.685	22.28
Squirrel	$c_{min.-max}$	0.002-0.161	0.003-0.042	0.008-0.373	0.003-0.794	N.A.	N.A.
	Average	0.082	0.009	0.218	0.573	N.A.	N.A.
	Median	0.085	0.005	0.227	0.571	N.A.	N.A.
Marmot	$c_{min.-max}$	N.A.	0.021-1.36	0.007-0.515	N.A.	0.90-73.43	16.55-164.1
	Average	N.A.	0.247	0.132	N.A.	12.937	43.474
	Median	N.A.	0.124	0.058	N.A.	2.77	34.03
European polecat	$c_{min.-max}$	0.008-0.056	0.005-0.50	0.008-0.442	0.007-1.67	1.18-54.69	23.80-46.87
	Average	0.045	0.17	0.284	0.236	22.915	35.691
	Median	0.055	0.094	0.094	0.062	24,17	37.13
Otter	$c_{min.-max}$	0.02-1.21	0.021-0.358	0.05-0.247	0.03-0.439	7.01-25.68	15.08-66.89
	Average	0.470	0.142	0.161	0.191	11.997	30.991
	Median	0.444	0.100	0.158	0.199	12.08	26.09

N.A. = not analyzed

the concentration range of those, which exceeded the limit, was 0.050 – 1.210 mg.kg^{-1}* for Hg, 0.120 – 3.380 mg.kg^{-1} for Cd, 0.101 – 144.25 mg.kg^{-1} for Pb and 0.217 – 2.94 for As. These four are the most frequent heavy metals concentrated in animal organisms there. Nickel is the metal that did not exceeded the limit in TANAP. In Zemplin, the four metals were noticed in the following value ranges respectively: 0.052 – 0.353 mg.kg^{-1}, 0.120 – 6.174 mg.kg^{-1}, 0.107 – 2.414 mg.kg^{-1}, 1.000 – 1.230 mg.kg^{-1}; while there is no value of Cu exceeded the limit.

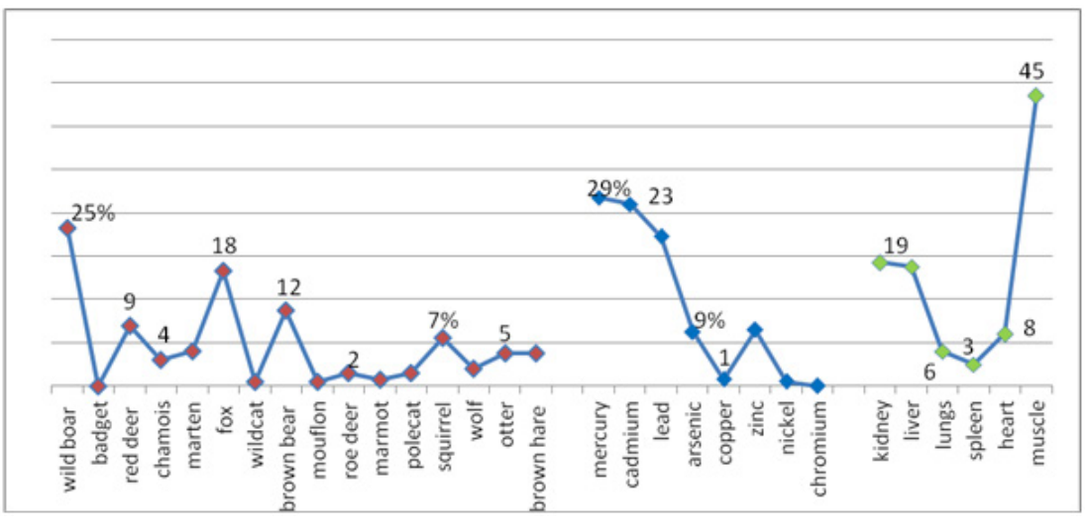
- In TANAP, the highest levels of the four most prevalent metals were detected in following animal species: Hg in otter (median = 0.444 mg.kg^{-1}), Cd in brown bear (median = 0.280 mg.kg^{-1}), Pb in fox (median = 4.261 mg.kg^{-1}) and As in squirrel (median = 0.571 mg.kg^{-1}). In the Zemplin region, it was Hg in wolf, Cd in wild boar, Pb in fox, As in red deer (medians are 0.050; 0.398; 0.279; 0.070 mg.kg^{-1} respectively). We have recorded one special case, when the concentration of Pb reached exceptionally high value (144.25 mg.kg^{-1}) – in chamois (TANAP).
- Generally, the metal most exceeding the legal limits was Hg (0.05 – 1.21 mg.kg^{-1})*, which represents 29 % of all examined samples, then Cd (0.102 – 6.124 mg.kg^{-1}) in 28 %, Pb (0.101 -144.25 mg.kg^{-1}) in 23 %, As (0.217 – 2.94 mg.kg^{-1}) in 9 %, Zn (50.3 – 326.5 mg.kg^{-1}) in 9 %, copper (5.58 – 9.89 mg.kg^{-1}) – 1 %, nickel

Table 3. The heavy metal concentration range, average and median in tissues of wild living animals from Zemplin

Animal species		Heavy metal concentration in mg.kg^{-1}					
		Hg	Cd	Pb	As	Cu	Zn
Wild boar	$c_{min.-max}$	0.001-0.137	0.024-1.53	0.001-0.36	0.001-0.02	0.384-1.294	N.A.
	Average	0.029	0.359	0.188	0.002	0.529	N.A.
	Median	0.002	0.398	0.110	0.001	0.384	N.A.
Red deer	$c_{min.-max}$	N.A.	0.013-0.62	0.002-1.36	0.01-1.23	N.A.	56.17-6.07
	Average	N.A.	0.183	0.304	0.153	N.A.	29.614
	Median	N.A.	0.093	0.095	0.070	N.A.	28.05
Fox	$c_{min.-max}$	0.0015-0.353	0.001-1.11	0.005-1.47	N.A.	N.A.	N.A.
	Average	0.063	0.228	0.296	N.A.	N.A.	N.A.
	Median	0.036	0.031	0.279	N.A.	N.A.	N.A.
Roe deer	$c_{min.-max}$	0.0016-0.09	0.001-0.518	0.003-1.25	0.002-0.823	0.528-42.15	N.A.
	Average	0.024	0.068	0.198	0.164	15.395	N.A.
	Median	0.007	0.009	0.070	0.012	11.380	N.A.
Wolf	$c_{min.-max}$	0.0019-0.82	0.001-0.895	0.005-0.187	N.A.	0.32-5.087	15.64-43.68
	Average	0.140	0.154	0.070	N.A.	4.052	27.848
	Median	0.050	0.028	0.091	N.A.	2.04	21.798
Badger	$c_{min.-max}$	0.0015-0.018	0.001-0.056	0.005-0.091	0.001-0.004	0.17-1.362	N.A.
	Average	0.009	0.024	0.034	0.002	0.839	N.A.
	Median	0,011	0.023	0.026	0.001	1.132	N.A.
Wild cat	$c_{min.-max}$	0.001-0.038	0.002-0.011	0.005-1.47	N.A.	N.A.	N.A.
	Average	0.004	0.005	0.145	N.A.	N.A.	N.A.
	Median	0.001	0.004	0.041	N.A.	N.A.	N.A.
Mouflon	$c_{min.-max}$	0.0015-0.83	0.01-0.518	0.014-1.045	N.A.	N.A.	N.A.
	Average	0.036	0.130	0.258	N.A.	N.A.	N.A.
	Median	0.026	0.074	0.078	N.A.	N.A.	N.A.
Hare	$c_{min.-max}$	0.001-0.075	0.001-6.174	0.004-2.414	0.0001-1.0	0.80-4.806	N.A.
	Average	0.021	0.387	0.221	0.041	2.473	N.A.
	Median	0.002	0.032	0.064	0.030	2.283	N.A.

N.A. = not analyzed

Table 4. The percentage data of the results from the two monitored localities



(0.872 – 1.083 mg.kg^{-1}) in 1 % of the samples. No value exceeded the limit was recorded in chromium.

- In total, the most contaminated animal species (the most data exceeded the legal limit) was wild boar (25 %), then fox (18 %), brown bear (12 %), red deer (9 %), squirrel (7 %), otter (5 %), brown hare (5 %), marten (5 %), chamois (4 %), wolf (3 %), roe deer (2 %), polecat (2 %), marmot (1 %), wildcat (1 %), mouflon (1 %), badger (0 %). The most heavy metal burdened animal species in TANAP was wild boar, then brown bear, fox and squirrel. In Zemplin it was fox, hare, red deer and wild boar. Wild boar was the species with the most significant heavy metal levels in both localities.
- The most attacked organ is the muscle (45 %), liver and kidney (19 %), heart (8 %), lungs (6 %) and the least affected is spleen (3 %).
- From the analysis of the elements in 40 biotope constituents taken from TANAP, the maximum permissible values exceed all three determined elements (Hg, Cd, Pb). The highest over-limit for Hg was measured in the sample of moss (0.893 mg.kg^{-1}).The highest supernatant Cd was in the sample of bark (2.120 mg.kg^{-1}), the lowest in the grass sample (0.082 mg.kg^{-1}). Pb in leaf litter was exceded by more than 85.20 mg.kg^{-1}. The contamination of the constituents was in the following descending order: Moss – Bark – Grass – Leaf litter – Needles (Figure 2).

Several authors (Mauro F *et al.*, 2017) studied the heavy metal environmental burden by heavy metals. In Slovakia, Kováč *et al.* (2005) detected concentrations of Cd, As, and Pb in body tissues of wild living red deer and wild boar game that fell within the legal limit value range (Food Codex, Slovak Republic). However, the authors show differences between individual game species which is a consequence of their distinct way of life, food intake and composition. According to our results, variability in animal species contamination because of different life conditions and feeding manners was also confirmed. Wild boar was the species with significant heavy metal levels in both localities. We suspect wild boar game as typical omnivores to be more exposed to environmental burden. The higher potential risk of food contamination by metals and subsequent metal accumulation in body tissues impend. Between the year 2001 and 2003 concentration values of Pb that exceeded the legal limit (according the Food Codex) in the family Cervidae were recorded in 2.6 % of the examined samples (Šalgovičová and Krížová, 2004). Nowadays, lead has the third position in risk ladder of heavy metal contamination (after Hg and Cd). In our study, Pb exceeded limit in 23 % of the samples what is significantly higher value. Especially two individual cases were significant – in chamois (144.25 kg.mg^{-1}) and wild boar (2.414 mg.kg^{-1}) from TANAP, in fox from the Zemplin region. Bilandžić

et al. (2010) also confirm significant heavy metal (Cd, Pb,Hg) contamination in wild boar in Croatia.

In this study, mercury and cadmium were recorded as the most prevalent in the monitored localities. Other authors also present mercury and cadmium as exceeding the limit levels in various kinds of wild living animals (Kramárová *et al.,* 2005; Piskorová *et al.* 2003; Pompe-Gotal *et al.*, 2009). Contrary to our results, Piskorová *et al.*, (2003) detected chromium as over-limited in 6.6 % of samples. Our research has not revealed dangerous level of chromium. In eastern Croatia, red deer was examined for heavy metal levels in tissues (Lazarus *et al.* 2005). The median concentration of toxic cadmium, mercury, and lead in the kidney were 0.099 $mg.kg^{-1}$, 0.362 $mg.kg^{-1}$, and 0.578 $mg.kg^{-1}$, respectively. In the jawbone, the Pb mass fraction was 0.281 $mg.kg^{-1}$. In comparison, our study provides median values of Cd and Pb concentration in red deer respectively: 0.127 $mg.kg^{-1}$ and 0.134 $mg.kg^{-1}$ in TANAP; 0.093 $mg.kg^{-1}$ and 0.095 $mg.kg^{-1}$ in Zemplin. Mercury was not detected in red deer.

Many authors describe the metal concentration in various types of body tissues (e.g. Andreotti *et al.*, 2016; Bellinger *et al.*, 2013; Bernhoft *et al.*, 2014; Hunt *et al.*, 2009; Juric *et al.*, 2018; Knott *et al.*, 2010). The muscle, liver, kidney and fat samples of 20 roe deer of both sexes originating from a hunting area in central Hungary were investigated by Lehel *et al.* (2017) for the presence of heavy metals such as As, Cd, Hg and Pb, and their contents were evaluated for possible health risk to consumers. Based on the data obtained from the present study, the consumption of organs and tissues of the investigated roe deer could be objectionable from food-toxicological point of view and may pose risk to the high consumers of wild game due to their cadmium and lead contents.

In this study, we did not deal with the aspect of metal distribution into individual tissues because of not-equable sample amount. Most of our tissue samples come from hares. Cadmium and lead were the metals, the concentrations of which represented the highest levels in this game. Levels of cadmium, lead and mercury in hare tissues were also examined in south-western Slovakia (Slamečka *et al.*, 1994). Levels of Hg and Cd are significantly increased in body tissues (liver, kidneys), depending on the increasing age of the hares.

During the period from 2002 to 2004 fifteen individuals of brown bear from Carpathians were examined to detect heavy metal levels and their distribution into the body tissues (Čelechovská *et al.,* 2006). The highest concentrations of Cd, Pb, Hg were recorded in kidneys (17.4 ± 5.2 $mg.kg^{-1}$, 1.16 ± 0.39 $mg.kg^{-1}$, 0.39 ± 0.25 $mg.kg^{-1}$). During our study we managed to gain 26 samples of brown bear from the TANAP locality. The metals exceeding

Figure 2. Samples of biotope components (leaf litter, needles, moss, grass)

the legal limit the most were Cd (13 samples), Hg (9 samples), Pb (6 samples), Cu (5 samples), As (3 samples), Zn (1 sample).

In order to detect environmental contamination, vegetation could be used as proper material for laboratory testing. Just as in our study, Bykowszczenko *et al.* (2006) discovered environmental burden with heavy metals in a national park in Poland detecting the content in mosses. Słowiński National Park is also a protected area in the central part of the Polish Baltic coast. Contrary to our results, the Polish research suggest a reduction of heavy metal contamination in this national park over the last 27 years and confirmed that the area is one of the cleanest in Poland and may still serve as a reference background for determining pollution in other areas. Słowiński National Park is under relatively small threat from gas and dust pollution compared with the other national parks in Poland. This is due to its location in a lightly inhabited area of the Baltic coast, far from the industrial centres. Despite the industrial emissions and dust from long-distance transport still present in the area, the natural environment of Słowiński National Park is relatively unaffected. Kozanecka *et al.* (2002) also monitored the heavy metal contamination of pollution-free regions. The stated concentration of Zn, Cu, Pb, Ni, Cr and Cd were very little differentiated considering particular plant species of forest floor. And those were appreciated at the natural level, typical for the unpolluted area. Many substances accumulated in animal and human organisms are not needed for physiological activities. On the contrary, these substances can cause various pathological changes and serious health disorders. Accumulating in body tissues, content of the materials usually increase with aging, thus they start acting as toxic. This is related to e.g. arsenic, cadmium, lead, mercury, etc. (Toman *et al.*, 2003a). The main way of receiving metals into living organisms is food intake, so the monitoring in food-stuff of human and animals is essential (Golian *et al.*, 2004). Grazing animals receive heavy metals from contaminated soil that can create 18 % of total ingested dry mass in cattle and 30 % in sheep. The quickest way for metal absorption is in the middle part of small intestine. Absorbed metals bind to blood cells or blood plasma components (Gallo, 1995). Even the type of grazing can affect the heavy metal levels in the environment. Results of Majid Ajorlo *et al.* (2010) suggest that the excreta of grazing cattle can be an important source of heavy metals in intensively managed pastures in the long-term. However, the metal concentrations were maintained within the normal range and were not high enough to be dangerous from the toxicological point of view.

Research of wild living animals is difficult and very important. It brings a lot of knowledge about the ecological stability changes in select forest localities that can be applied in practice (Begon *et al.*, 1997). In Slovakia, the actual state of forests is disordered by emission and other pollutant effect.

CONCLUSIONS

Generally, physical environmental pollution (air, water, soil) in Slovakia has different tendency in various regions due to new conditions of the market economy that results from permanent changes in the assortment processing. Heavy metal cumulation in ecosystems is a serious problem of environment quality. It is known that the danger of environmental pollution is still impending because of the permanent negative effects of the industrialised country (exhalation, soil contamination, surface and phreatic water contamination, etc.) and motorization. Regarding the life cycle of wild living animals we can suppose that wild animals permanently exposed to external environment have higher levels of metal contamination in comparison to domestic animal breeding in internal stables (Niemi *et al.*, 1993; Kugonič and Zupan, 1999; Košutzký *et al.*, 2003; Krížová and Šalgovičová, 2001). Besides trophy quality production, wild living animals are a suitable bioindicator of biotope quality, as well. Thus it is convenient to use this fact to take care of human life quality (Tataruch, 1995; Krynski *et al.*, 2003; Zmetáková and Šalgovičová, 2008). Animal indicators also help in detecting the amount of toxins present in the tissues of animals (Joanna, 2006; Khatri and Tyagi, 2015).

The results of this study confirm heavy metals contamination even in game that comes from an area preserved and restricted in any industrial activity – Tatra National Park. As presented, reduction or total elimination of the industrial use of an area do not strictly mean elimination of environmental burden there. We suppose that the environment contamination and higher pollutant burden in TANAP

is a consequence of Katowice industrial complex in Poland (Degórska, 2013). Allegedly dominant north air flow supports emission transport from heavy industry to this relatively clean and virgin area (Makovníková and Kanianska, 2003). The results can serve as basic data for next ecological and veterinary study in Slovakia. It is necessary to continue in further parameter collection with subsequent detection of pollutants in other material samples, e.g. in vegetation (moss, conifer needles, etc.) which is another proper bioindicator of environmental contamination. In order to eliminate the negative effect of contaminants on human and animal population in the monitored areas as well as on the whole territory of Slovakia, it is essential: to investigate risk factors in the environment (to know the contamination situation), to decrease production of the metallurgical industry emissions, to reduce agricultural area contamination by restricted pollutant entry through soil, to eliminate the metal absorption in animals (antidote administration, adaptation of food composition), to use legislative and economic means for forcing the polluter to take responsibility for environment pollution losses and costs, to educate the human population in environmental management.

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UTILIZATION OF CYTOCHROME B – MITOCHONDRIAL DNA IN BROILER RABBIT SELECTION

Vladimír PARKÁNYI[1], Ľubomír ONDRUŠKA[1], Ján RAFAY[1,2]*, Dušan VAŠÍČEK[1], Jaromír VAŠÍČEK[1,3], Andrej BALÁŽI[1], Alica NAVRÁTILOVÁ[3]

[1]NPPC – Research Institute for Animal Production Nitra, Lužianky, Slovak Republic
[2]University of Ss. Cyril and Methodius in Trnava, Faculty of Natural Sciences, Trnava, Slovak Republic
[3]Slovak University of Agriculture in Nitra, Nitra, Slovak Republic

ABSTRACT

Cytochrome b haplotypes (GenBank OCU07566, NCBI, USA) of mitochondrial DNA (mtDNA) were described in meat lines of rabbits – cyt b 430 and cyt b 306. The haplotype cyt b 430 represents 571A (190Threonine-T)+877G(292Alanine -A). Haplotype cyt b 306 with polymorphism A571G mtDNA-cell line LEU-RAB is registered as BioSample: SAMN03701526; Sample name: cyt b O.C. (Model organism or animal sample from *Oryctolagus cuniculus*), in NCBI, USA. The vitality of offspring of the cyt b 306 haplotype, represented by the average number of weaned youngs at 42 days of age, was significantly higher compared to the haplotype cyt b 430 (ẍ = 7.00 vs. ẍ = 5.69, t (0.01) = 2.91^{++}). These results are confirmed also by the immunogenetic results determined using flow cytometry. Haplotype cyt b 306 is characterized by a significant increase (P = 0.041^{+} to P = 0.049^{+}) of the flow cytometric parameters at the frequency of T lymphocytes, which results in activation of lymphocytes of the type pT2 and CDS4 (pT2 = 22.02 ± 4.45 % and CD4 = 17.08 ± 3.43 %), compared to the cyt b 430 haplotype, which is represented by significantly lower values of the flow cytometric parameters in activation of lymphocytes of the pT2 and CD4 type (pT2 = 15.32 ± 6.11 % and CD4 = 11.8 ± 4.83 %).
Meanwhile, in the reproduction parameters such as the average number of live-born kits, no differences (8.45 vs. 8.00) were determined between the studied haplotypes (cyt b 306 vs. cyt b 430). Similarly, production parameters represented by carcass value (carcass without the head and skin) of both haplotypes (cyt b 306 vs. cyt b 430) were not influenced by the targeted selection and remained on the same level of 54 % (54.41 vs. 54.26).

Key words: polymorphism; cyt b; rabbit; reproduction

INTRODUCTION

Mitochondrial DNA is traditionally used in population genetics as a selection marker for characteristics of phylogenesis (Avise *et al*., 1987; Zink & Barrowclough, 2008). Many researchers highlighted the functional role of mtDNA and the opportunities for direct utilization of mitochondrial data in ecology and evolution (Ballard & Whitlock, 2004; Gemmell *et al*., 2004; Dowling *et al*., 2008). Multicellular organisms are characterised by a closed circular mtDNA molecule. Animals in general have a small mtDNA (15 – 20 kb) genome containing 37 genes. Genome mtDNA codes 13 enzymes of oxidative phosphorylation (OXPHOS complexes). Rapid changes in amino acid composition influence OXPHOS complexes and have a significant impact on the function of an organism.

Repair mechanisms on the mtDNA level usually eliminate most of the damaging mutations fast. However, unrepaired mtDNA sequences influence the quality of mitochondrial enzyme production

***Correspondence:** E-mail: rafay@vuzv.sk
Ján Rafay, NPPC – Research Institute for Animal Production Nitra, Hlohovecká 2, 951 41 Lužianky, Slovak Republic

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and therefore also the effective share on the total energy metabolism of the cell. Based on the matroclinal inheritance of mtDNA, the mutation haplotype will be present in the population along the female line. This effect was named "mother's curse effect" (Gemmell *et al*., 2004).

The central role in the 3 OXPHOS complexes of mitochondria is played by cytochrome b. Cytochrome b is a protein present in the mitochondria of eukaryotic cells. It forms a part of the electron transport chain and is the primary subunit of the transmembrane cytochrome bc1, known also as ubiquinol-cytochrome c reduction. These complexes participate on the transport of electrons, extraction of protons and generation of the proton-motive force (PMF). Then the proton gradient is used for the production of ATP, therefore these processes play a significant role in cells (Brand, 1997). It is most often used as a mtDNA sequence to determine phylogenetic relationships between organisms. However, cytochrome b finds application in clinical practice as well. Mutations on the cytochrome b cause significant changes in the respiratory tract, which leads to its damages and decreased health of the affected organism (Wei, 1998).

This paper presents the results observed in study of the relationship between cytochrome haplotype frequencies and vitality traits of rabbits.

MATERIAL AND METHODS

Animals

The experimental animals (broiler rabbits) were kept at an accredited facility of the Research Institute for Animal Production Nitra (RIAP) within the National Agricultural and Food Centre in Nitra, Slovakia. Cytochrome b mtDNA was analysed from peripheral blood samples (sampled from *a. auricularis centralis* into heparinised vials) from males (18 animals) and females (24 animals) of a parent generation and their F1 generation of the original meat lines (M91 and P91) of rabbits kept at RIAP Nitra. Females of the parent generation were divided into two groups: 1. – experimental group (12 animals) underwent divergent selection with strict selection criteria (the selected females had to have at least three consecutive litters of 7 – 10 live-born kits). 2. – control group (12 animals) with three consecutive litters with significant variability in the number of live-born kits (1 – 15). The animals were housed in single-level cages under constant light regiment of 14 hours of light and 10 hours of darkness per day. The temperature and humidity in the housing area was continuously monitored using a hydrothermograph, which was situated on the same level as the cages (the average humidity and temperature during the year were maintained at values of 60 ± 5 % and 17 ± 3 °C). The experimental rabbits were fed a commercial diet for growing animals (KV, Tekro Nitra, s.r.o) *ad libitum* and water was supplied through drinkers *ad libitum*. The experiment was approved by the State Veterinary and Food Administration of the Slovak Republic, no. SK CH 17016, SK U 18016.

In the following generations of the progeny of the experimental females selected into the program of divergent selection, in the 24rd to 83rd litter we evaluated also the reproduction and production parameters of the two studied mitochondrial haplotypes (cyt b 430 and cyt b 306), such as the average number of live-born kits per litter, average number of weaned kits, live weight at the age of 75 days, carcass weight and carcass value.

From the gathered results of the particular genotype groups, basic variation-statistical parameters were calculated. Significance of the differences in the arithmetic averages was estimated using the t-test. In order to test a set, Scheffe's test was used. Statistic differences were evaluated on the level of significance $P \leq 0.05$; $P \leq 0.01$ a $P \leq 0.001$.

Cytochrome b-mtDNA (mitochondrial DNA)

To the heparinized rabbit blood samples (Heparin, 25 000 I.U., 6 µl.ml^{-1}) was added a lysis solution (200 µl 1x PCR buffer (50 mM KCl, 20 mM Tris-HCl pH 8.4), enriched with 50 mM DTT, 1 % Triton X-100 and 400 ng.µl^{-1} proteinase K. The samples were incubated overnight at 56 °C. After the incubation, they were deactivated and denaturised for 5 minutes at 96 °C, after which they were cooled down to laboratory temperature. We used technology of amplification of specific small sequences of isolated mitochondrial (mtDNA) male and female rabbit DNA via polymerase chain reaction (PCR). To isolate mtDNA from the heparinised peripheral blood, Maxwell DNA Purification Kit was used. Concentration of DNA

in samples (2.495-2.994 ng.µl^{-1}) was measured using NaNoPhotometer (Implen) spectrophotometer. As a reference solution, we used an elution solution used in DNA elution during purification.

PCR conditions (PTC-200 DNA Engine; BioRad) were 95 °C for 2 minutes, 94 °C for 30 s, 54 °C for 30 s, 35 cycles with the last step of extension at 72 °C for 10 minutes. PCR reaction volume (25 µl) contained 10 mM Tris-HCl (pH 8.6 at 25 °C, 50 mM KCl, 1.5 mM $MgCl_2$, 25 units.ml^{-1} Taq DNA polymerase, 0.2 mM dNTPs each, 5 % glycerol, 0.08 % IGEPAL® CA-630, 0.05 % Tween-20) (New England Biolabs), 10 pmol.µl^{-1} each average (ORYCTO-cyt b-FOR-21nt a ORYCTO-cyt b-REV-20nt) and from 2.495 to 2.994 ng.µ^{-1} from every mtDNA sample. Amplified mtDNA was electrophoreticaly separated to 2 % agarose gel containing ethidium bromide at 80 mA, 120 V in 10 mM lithium borate buffer, pH 8.0 for 90 minutes. PCR products were visualized under UV light and photographed using documentation system MiniBis Pro (Bio-Imaging Systems) (Figure 1).

The detected fragment was 692 bp long, located in the area of *Oryctolagus cuniculus* – cytochrome b (GenBank OCU07566, NCBI, USA) of the rabbit mitochondrial DNA. To locate and restrict the analysed mtDNA sequence, oligonucleotides designed and synthetized under laboratory conditions were applied:

Oligonucleotides for detection of the partial cytochrome b *Oryctolagus cuniculus*:

	Tm
ORYCTO-cyt b-FOR 5'- CTA TCA GCA ATC CCA TAT ATC -3'	54.0 °C
ORYCTO-cyt b-REV 5'- CTT CAT TTG AGG ATT TTG TT -3'	54.0 °C

After evaluation of the amplified PCR product via Agarose electrophorese, the amplicons were then broken by AluI (5 U/ 20 µl) restriction enzyme (New England Biolabs). AluI recognizes a specific sequence 5'- ...AG'CT ...-3' , resp. 3' - ...TC'GA ...- 5', and breaks down the given PCR product, depending on the presence of a given sequence in the amplified sequence under optimal reaction conditions (37 °C), Figure 1.

Flow Cytometry

Samples of peripheral rabbit blood (PB) were taken from *a. auricularis centralis* into heparinized vials. Mononuclear cells from the peripheral blood (PBMC) were isolated using centrifugation with Ficoll according to the original protocol: Isolation of mononuclear cells from human peripheral blood via centrifugation in dense gradient (Miltenyi Biotec, 2008). In each sample, 10 000 to 50 000 cells were measured using flow cytometer FACS Calibur (Becton Dickinson, Mountain View, CA). 7-AAD dye solution (BD Biosciences, USA) was used to eliminate dead cells from the analysis. Frozen and then thawed cells were divided into prepared vials and dyed with various clones of anti-rabbit monoclonal antigens: anti-IgM (NRBM, Bio-Rad AbD Serotec GmbH, Nemecko), anti-CD4 (RTH1A, WSU, Pullman, WA) anti-CD8 (ISC27A, WSU, Pullman, WA), anti-pan T2 (pT2, RTH21A, WSU, Pullman, WA).

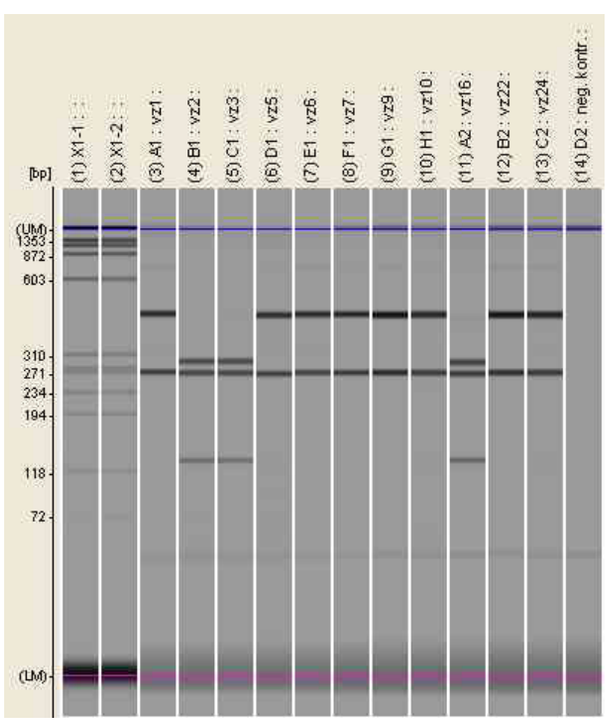

Figure 1. Cytochrom b of rabbit mitochondrial DNA: results of PCR-RFLP (AluI). Microchip electrophoresis MCE® -202 MultiNA.

RESULTS AND DISCUSSION

AluI restriction analysis of the PCR product results in the creation of fragments, which were analysed by microchip electrophoresis MCE® -202 MultiNA, Figures 1 and 2. Based on the restriction fragments, 2 types of AluI RFLP -cyt b mt DNA (GenBank OCU07566, NCBI, USA) were detected: 430-262bp and 306-262-124bp. Based on them, the experimental rabbits were divided into 2 haplotype groups cyt b 430 and cyt b 306.

Haplotype cyt b 430 represents 571A (Threonine-T)+877G (Alanine-A). Haplotype cyt b 306 is represented by substitution of nucleotides A571G, that is 571G (Alanine-A) +877G (Alanine-A).

This substitution of nucleotides is the causal result of conversion and translation alteration in the peptide chain, where the essential amino acid Threonin-T is replaced with amino acid Alanin-A, T190A. Haplotype cyt b 430 is characterized by one restriction position (877G; Figure 3). While cyt b 306 is a new haplotype defined by two restriction positions 571G and 877G.

Sudden changes in the sequence of amino acids in cytochrome b influence the expression of OXPHOS mitochondrial complexes of somatic and generative cells and have a large influence also on the function of tissue and organs and therefore on the bodily functions as well.

Due to the aforementioned reason, the occurrence of new haplotypes in cytochrome b mtDNA of rabbits (cyt b 306 and cyt b 430) is studied also from the aspect of prospective benefit as a candidate biomarkers associated with select physiological, functional, selection, reproductive, and production parameters, such as the number of live-born kits in a litter, the average number of weaned kits, live weight at the age of 75 days, carcass weight and carcass value (Table 1).

In the reproduction parameters such as the average number of live-born kits, no significant differences (8.45 vs 8.00) were determined between the studied haplotypes (cyt b 306 vs cyt b 430).

Similarly, the production parameters represented by carcass value of both haplotypes (cyt b 306 vs cyt b 430) were not negatively influenced by the targeted selection and remained at the same level of 54 % (54.41 vs. 54.26).

Despite this, it can be stated that the cyt b 430 haplotype reached higher live weight (g) at the slaughter age of 75 days compared to cyt b 306 haplotype (3076 vs 2884.44). These parameters

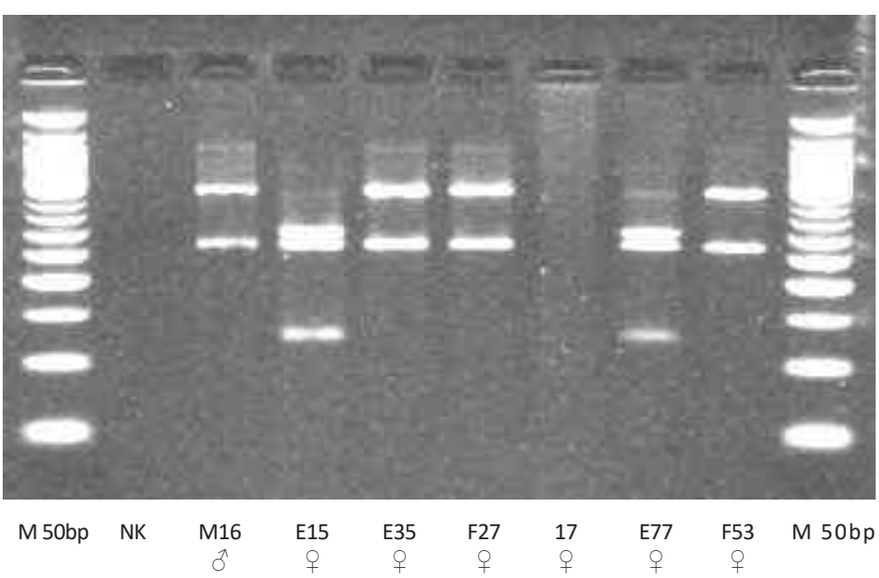

Figure 2. Alu I cleavage of cytochrome b 692 bp PCR product visualised on 2 % Agarose gel by MiniBis Pro (Bio-Imaging Systems). Haplotype cyt b 430 = samples M16, E35, F27, F53; haplotype cyt b 306 = samples E15 and E77; NK = negative control, M = 50 bp DNA Ladder (Jena Bioscience).

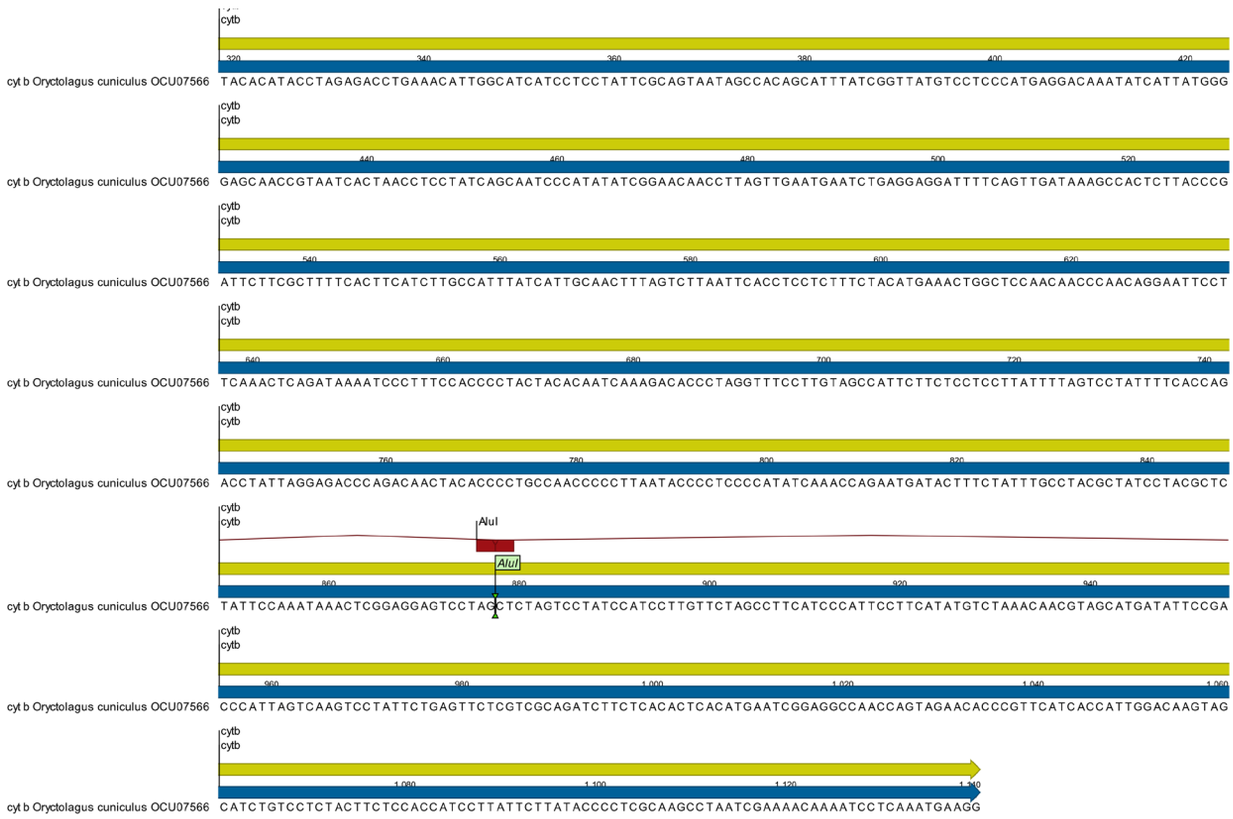

Figure 3. Haplotype cyt b 430: AluI Cytochrome B (GenBank OCU07566 = 430-262b, 571A(190Threonine-T)+ 877G(292Alanine-A)

of cyt b 430 however represent the influence of the higher weight of the head and skin (g) compared to cyt b 306 haplotype (672.00 vs 613.89). The results from evaluation of slaughter weight (g) are significantly favourable to cyt b 430 haplotype (1669.00 vs 1569.44).

However, for an important production and economic parameter – vitality of kits represented by the number of weaned kits at the age of 42 days, haplotype cyt b 306 showed significantly higher values than haplotype b 430 ($\ddot{x}$ = 7.00 vs. $\ddot{x}$ = 5.69, t (0.01) = 2.91^{++}). These results are confirmed also by the immunological data gathered from flow cytometry. The changes in amino acid composition of cytochrome b influence also the OXPHOS complexes of mitochondria and significantly affect the cells of the immune system as well – such as T and B lymphocytes, Table 2. T-lymphocytes are created in thymus in the form of CD4^{+} or CD8^{+} cells. CD4^{+} cells help B lymphocytes during cell-propagated immune response via lyphozine secretion. CD8^{+} cells specialize on cytotoxic killing of other cells, especially virally infected cells or tumour cells under experimental conditions. Their functions can partly overlap, when CD8^{+} cells produce lymphokines and CD4^{+} cells can kill other cells. The most important difference is, however, that CD4^{+} cells recognise antigen peptide in coordination with MHC molecules of Class II while CD8^{+} cells cooperate with Class I MHC molecules (Nossal, 1997). When the CD4^{+} T cells are activated, they start to secrete various types of cytokines (Kelso *et al*., 1991). When the immunity response reaches its peak, however, cases may occur when either (Th)-1 response or (Th)-2 response prevails (Mosmann a Coffman, 1989). Th-1 response causes production of antigens, including production of TgG1 and IgE antigens (Finkelman *et al*., 1990). B lymphocytes are responsible for the production of antigens (Nossal *et al*., 1968). At the beginning, the IgM and B cells can cause an immune response without activation of T cells, but most long-term immunity responses involving IgG, IgA

Table 1. Reproduction and production traits of mtDNA-cytochrome b haplotype rabbits

Traits	mtDNA haplotypes		
	Cytochrome b 306		Cytochrome b 430
1. Number of live born animals per litter: ẍ	8.45		8.00
$s_{\ddot{x}}$	3.50		3.18
df		85.00	
P		0.27	
$t_{(0,05)}$		0.61^{-}	
2. Number of weaning animals at age 42 days: ẍ	7.00		5.68
$s_{\ddot{x}}$	1.46		2.54
df		78.00	
P		0.002	
$t_{(0,01)}$		2.91^{++}	
3. Live weight at 75 days (g): ẍ	2884.44		3076.00
$s_{\ddot{x}}$	159.01		142.14
df		26.00	
P		0.002	
$t_{(0,01)}$		3.16^{++}	
4. Carcass weight (g): ẍ	1569.44		1669.00
$s_{\ddot{x}}$	95.51		83.19
df		26.00	
P		0.005	
$t_{(0,01)}$		2.76^{++}	
5. Carcass utility (%) without head and skin: ẍ	54.41		54.26
$s_{\ddot{x}}$	1.49		0.96
df		26.00	
P		0.39	
$t_{(0,05)}$		0.29^{-}	
6. Weight of head and skin (g): ẍ	613.89		672.00
$s_{\ddot{x}}$	38.22		56.73
df		26.00	
P		0.002	
$t_{(0,01)}$		3.24^{++}	

or IgE antigens requires the help of activated T cells (Miller, 1972). Knowledge of the physiological values is required in order to recognise changes in the distribution of particular types of lymphocytes (Faldyna *et al*., 2001). Haplotype cyt b 306 is characterized by a significant increase ($P = 0.041^{+}$ to $P = 0.049^{+}$) of the flow cytometric parameters in the frequency of the T lymphocytes, which is expressed as activation of pT2 and CD4 (pT2 = 22.02 ± 4.45 % and CD4 = 17.08 ± 3.43 %) type lymphocytes. Compared to the cyt b 430 haplotype, which is represented by significantly lower values of flow cytometric parameters in activation of pT2 and CD4 (pT2 = 15.32 ± 6.11 % and CD4 = 11.48 ± 4.83 %) (Table 2).

From these results it is apparent that genetic polymorphism in cyt b gene is a useful genetic marker in selection and breeding of broiler rabbits.

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Table 2. Influence of haplotype mtDNA=cyt b 306 and cyt b 430 on Flow cytometric parameters for F1 generation at age 62 days (youngs from 56 to 63 days are characterized by maturation of gastrointestinal tract – microbiotical and chemical digestibility

Sample	Flow cytometric parameters/ age of F1 generation	pT2 (%)	CD4 (%)	CD8 (%)	$CD4^+CD8^+$ (%)	IgM (%)	pT2/IgM (ratio)	CD4/CD8 (ratio)	CRP (%)
E15-1 cyt b 306	62 days	17.55	13.27	4.74	0.26	15.13	1.16	2.80	0.04
E15-2 cyt b 306	62 days	28.02	18.32	7.77	0.22	8.83	3.17	2.36	0.02
E77-16 cyt b 306	62 days	20.22	21.19	5.23	0.18	18.06	1.12	4.05	0.07
E77-18 cyt b 306	62 days	22.30	15.52	5.38	0.90	8.46	2.64	2.89	0.17
E17-11 cyt b 430	62 days	6.09	4.63	1.45	0.27	9.53	0.64	3.19	0.02
E17-12 cyt b 430	62 days	10.40	8.16	2.38	0.11	10.21	1.02	3.43	0.03
E17-13 cyt b 430	62 days	17.34	13.92	3.81	0.20	7.44	2.33	3.65	0.03
E5-8 cyt b 430	62 days	16.10	11.11	4.73	0.22	15.87	1.01	2.35	0.07
E35-5 cyt b 430	62 days	23.02	18.67	11.30	0.29	25.97	0.89	1.65	0.08
Statistics	cyt b 306	22.02 ± 4.4	17.08 ± 3.4	5.78 ± 1.3	0.39 ± 0.3	12.62 ± 4.7	2.02 ± 1.04	3.03 ± 0.72	0.08 ± 0.0
	cyt b 430	15.32 ± 6.1	11.48 ± 4.8	4.84 ± 3.48	0.26 ± 0.12	14.25 ± 6.7	1.17 ± 0.59	2.76 ± 0.78	0.04 ± 0.0
		P = 0.049	P = 0.041	P = 0.314^-	P = 0.203^-	P = 0.345^-	P = 0.067^-	P = 0.302^-	P = 0.155^-
		t = 1.8^+	t = 1.99^+	t = 0.50	t = 0.88	t = 0.41	t = 1.66	t = 0.54	t = 1.08

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EFFECTS OF HEAT STRESS ON PHYSIOLOGICAL PARAMETERS AND SERUM CONCENTRATION OF HSP70 IN INDIGENOUS BREEDS OF SHEEP IN NIGERIA

Mabel Omolara AKINYEMI*, Osamede Henry OSAIYUWU, Andrew Enahoro EBOREIME

Department of Animal Science, University of Ibadan, Ibadan, Nigeria

ABSTRACT

Heat stress is one of the most challenging environmental conditions affecting livestock production especially in the tropical regions of the world. The present study was conducted to examine the physiological response and HSP70 secretion in four extensively managed indigenous sheep breeds with little access to shade. Rectal temperature (RT), Skin temperature (ST), Respiration rate (RR) and Heart rate (HR) were taken from 565 adult rams comprising 139 Uda, 88 Yankassa, 221 Balami and 117 West African Dwarf sheep in early morning and midafternoon at the peak of the dry season in Ibadan, South West Nigeria. Extracellular heat shock protein 70 (HSP70) concentration was determined by ELISA. At Temperature Humidity Index (THI) > 82 significant differences were observed between the early morning and midafternoon readings in ST in all the breeds, in RT for WAD and Yankassa and RR in Uda and WAD. In the pooled readings there was a significant difference ($p < 0.05$) between Yankassa and other breeds studied in RR. The concentration of HSP70 ranged from 69.17 to 210.71 $ng.mL^{-1}$ with the highest value recorded for Uda. The investigated breeds differ in their response to heat stress.

Key words: heat stress; sheep; heat shock protein; skin temperature; rectal temperature

INTRODUCTION

Tropical regions are characterized by high levels of temperature and relative humidity which adversely affect animal production (McManus *et al.*, 2009; Naqvi and Sejian, 2010). Thermal stress is a major constraint on animal productivity in the region as it impairs general well-being, growth, protein metabolism, energy and mineral balances, reproduction and productivity. The effect of heat stress is often aggravated when it is accompanied by high ambient humidity (Abdel-Hafez, 2002; Marai, 2007), resulting in increased tissue catabolism in the fat depots and lean body mass and a decrease in anabolic activity occasioned by a decrease in voluntary intake of essential nutrients (Marai and Habeeb, 1998; Marai *et al.*, 2007)

The magnitude of heat stress, defined as the sum of external forces that disperse body temperature from set point, is caused by combined effects of ambient temperature and relative humidity. Several indices have been used to determine the degree of heat stress affecting farm animals, the most common of which is Temperature Humidity Index (THI) (Dikmen and Hansen, 2009). THI value ≤ 74 is considered normal, 75 to 78 is alered status, 79 to 83 is danger status while values above 84 are an emergency (LCI, 1970).

In response to stress, mammals set physical, biochemical and physiological processes into play to counteract the negative effects of heat stress and maintain thermal equilibrium (Silanikove, 1992). This thermoregulatory mechanism ensures the survival and relative ability of the animals to adapt

***Correspondence:** E-mail: larakinyemi@gmail.com
Mabel Omolara Akinyemi, Department of Animal Science,
University of Ibadan, Ibadan, 200284, Nigeria
Tel.: +1 2152526984, +2348055125806

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to the environment. These thermoregulatory characteristics include respiration rate, rectal temperature, skin temperature, pulse rate and heart rate.

Heat shock proteins (HSPs), a large protein family highly conserved across evolutionary lines, allow cells to adapt to environmental changes and play an important roles in environmental stress tolerance and thermal adaptation (Parsell and Lindquist, 1993; Hoffman *et al.*, 2003). These molecular chaperones, which encompass several families, are classified according to molecular weight and play important physiological roles to help cope with heat stress. Among the members of this family, HSP70 is the most temperature sensitive and is induced by various physiological, pathological and environmental stressors (Beckham *et al.*, 2004). In response to heat challenge, blood flow is redirected to the periphery for enhanced heat dissipation and blood flow to the intestines is reduced, resulting in cell damage occasioned by the reduction in the supply of O_2 and nutrients leading to loss of intestinal barrier integrity (Doklandy *et al.*, 2006, Cronje, 2007) and increased intestinal permeability. This often facilitates the penetration of endotoxins, thereby causing inflammatory response (Shapiro *et al.*, 1986; Lambert, 2009). Extracellular HSP70 has been reported to promote inflammatory immune response (Doklandy *et al.*, 2006), thus changes in HSP70 may be an indication of response to thermal challenge.

Guerriero and Raynes (1990) reported that HSP70 was the main heat shock protein synthesized when lymphocytes of different livestock species were exposed to heat stress *in vitro*.

Sheep (*Ovis aries*) is one of the oldest domesticated species (Pedrosa *et al.*, 2005) and is widely distributed throughout the world due to its high plasticity and adaptability to withstand poor nutrient diets and tolerance to extreme climatic conditions (Kijas *et al.*, 2009). These animals have developed adaptive mechanism that allows their survival at high ambient temperature and humidity. However, despite their tolerance, the productivity is often reduced due to heat stress (Marai *et al.*, 2007).

In Nigeria the four predominant breeds are Balami, Uda, Yankassa and West African Dwarf (WAD). These animals are raised under the traditional system where they are grazed from one location to the other in search of fodder and tethered under the hot sun in open markets with little or no shade to attract buyers.

With the current trend in climate change and its impact on global warming, the performances of these animals are adversely affected due to heat stress in the tropical environment such as Nigeria. The need to urgently address this global trend has become imperative; one vital tool to address this challenge is through careful identification and selection of well-adapted breeds with appreciable tolerance to heat stress. The aim of this study was to compare the thermotolerant ability of the four sheep breeds as indicated by plasma concentration of HSP70 and to also evaluate the physiological response of indigenous sheep breed to elevated climatic stress.

MATERIALS AND METHODS

This study was undertaken at the Akinyele Local Government Area of Ibadan, Oyo State, South West Nigeria (7°22'39" N, 3°54'21" E, and 181 m above mean sea level) at the peak of the dry season (January to April 2015).

Five hundred and sixty-five (565) adult rams of Uda (139), Yankasa (88), Balami (221) and West African Dwarf (117) were used for this study. The animals were sampled from an open market where they are sold. The animals were 1.5 to 3 years old and were judged to be clinically healthy and free from physical abnormalities.

Data on the meteorological variables (Ambient Temperature and Relative Humidity) were monitored and recorded. The Temperature Humidity Index (THI) was calculated from the ambient temperature (AT) and the relative humidity (RH) by the following formula according to Amundson *et al.*, 2006:

$$THI = 0.8 \times AT\ °C + (RH, \%) \times (AT\ °C\text{-}14.4)/\ 100) + 46.4$$

Rectal temperature, Skin temperature, Respiratory rate, Heart rate and Pulse rate were measured in each of the animals studied. Heart rate was measured by palpitation of the jugular artery using a stethoscope. Respiratory rates were recorded by counting the number of flank movements and reported as breaths per minute. Rectal temperatures were recorded in degrees

Celsius using a standard digital thermometer. Pulse rate was determined for each animal by placing fingertips on the femoral artery of the hind limb and counting the number of beats per minute. Skin temperature was measured using non-contact infrared thermometer (VMR Scientific Horiba) on the shaved portion on the back of the animal. Readings were taken in the early morning hours (8:00) and in the afternoon (14:00).

Handling of the animals, which can be classified as a stressor, was minimized when recordings were taken.

The climatic data – ambient temperature (AT, °C) and relative humidity (RH %) – were recorded using a digital hygrometer throughout the study.

Heat Shock Protein 70 (HSP70) Analysis

Blood samples for HSP70 determination was collected from 50 animals per breed from the jugular vein into 5 mL tubes without anticoagulant and was allowed to clot. The samples were then centrifuged at 1400 x g for 10 minutes. Serum separator tube was used to separate the serum. The serum samples were then frozen and stored for laboratory analysis. Serum Hsp70 concentration was measured according to the manufacturers protocol of a commercially available HSP70 Enzyme-linked immunosorbent assay (ELISA) kit (USCN Life Science Inc, Wuhan, China).

Statistical Analysis: Descriptive statistics for the physiological variables was generated for each breed. The data generated were subjected to analysis of variance (ANOVA) using the SAS package (2008). Means generated were separated using DMRT of the same software. Values obtained for the serum concentration of HSP70 for each breed were subjected to analysis and the differences were considered as significant at $P < 0.05$.

RESULTS

The prevalent physiological parameters recorded in the early morning and midafternoon hours in the peak of the dry season in Ibadan, Oyo State Nigeria at THI > 82 are documented in this study (Figure 1).

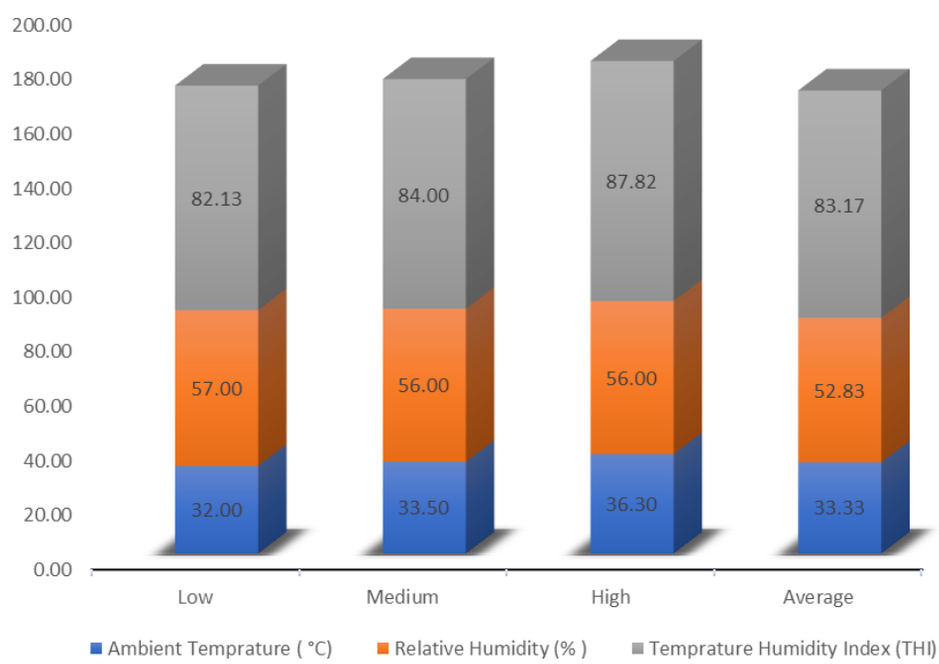

Figure 1. Meteorological Data during the experimental period

The temperature humidity index THI suggests that the animals were exposed to considerable heat load during the period of the study. The observed physiological parameters of extensively reared animals as affected by heat stress in this study are presented in Table 1 and 2. Significant differences were observed in the ST between early morning and midafternoon readings in all the breeds sampled. Differences in RT and RR were also significant in WAD and Yankassa and Uda and WAD respectively. The average daily value of rectal temperature (RT) was highest in WAD and the lowest value was recorded for Yankassa. However, there was no significant difference in the values obtained for Balami and WAD. Pulse rate was significantly higher in WAD (95.41), the lowest value (87.40) recorded for Yankassa was not significantly different from 90.08 observed in Uda. There was no significant difference among the values recorded for heart rate as the values ranged from 98.61 in Uda to 102.24 in WAD.

The result showed no significant difference ($P > 0.05$) in RR between Balami, Uda and WAD but Yankasa was significantly different ($P < 0.05$). The highest value was recorded for Yankasa and the lowest for Uda.

Serum HSP70 differ significantly in the breeds studied ($p < 0.05$). The concentration ranged from 69.17 to 210.71 ng.mL^{-1}, the highest concentration value recorded for Uda while the lowest was for the WAD (Table 3).

Table 1. Effect of Heat Stress on Physiological Parameters of Sheep Breeds at THI ≤ 82

Parameters	Balami	UDA	WAD	Yankasa
RT (°C)	38.34 ± 0.52^{a}	38.16 ± 0.43^{b}	38.43 ± 0.51^{a}	38.14 ± 0.48^{b}
PR (beat.min^{-1})	91.24 ± 12.72^{b}	90.08 ± 11.11^{bc}	95.41 ± 18.78^{a}	87.40 ± 13.12^{c}
HR (beat.min^{-1})	100.82 ± 16.11	98.61 ± 14.75	102.24 ± 18.48	98.72 ± 14.09
RR (breath.min^{-1})	92.38 ± 22.28^{b}	88.84 ± 17.19^{b}	90.65 ± 15.40^{b}	107.39 ± 20.93^{a}
ST (°C)	37.28 ± 0.65^{ab}	37.34 ± 0.50^{a}	37.18 ± 0.32^{b}	37.23 ± 0.76^{ab}

Rectal temperature (RT), Pulse rate, Heart rate (HR), Respiration rate (RR), Skin temperature (ST). Mean ± SD.
Mean with different superscript along the same row are significantly different ($P < 0.05$).

Table 2. Physiological parameters in the studied population as influenced by time of the day

	RT		PR		HR		RR		ST	
Breed	Early	MidDay	Early	MidDay	Early	MidDay	Early	MidDay	Early	MidDay
Balami	38.21	38.31	87.69^{b}	91.06a	95.76^{b}	100.34^{a}	93.26	91.06	36.60^{b}	37.30^{a}
Uda	38.10	38.15	87.97	90.23	95.78	98.76	78.08^{b}	88.86^{a}	37.15^{b}	37.34^{a}
WAD	38.17^{b}	38.42^{a}	91.72^{b}	95.61^{a}	98.07	102.40	80.12^{b}	90.37^{a}	37.02^{b}	37.17^{a}
Yankassa	37.76^{b}	38.13^{a}	86.50	87.39	97.27	98.71	103.23	107.38	36.72^{b}	37.22^{a}

Rectal temperature (RT), Pulse Rate, Heart rate (HR), Respiration rate (RR), Skin temperature (ST).
Mean with different superscript along the same row are significantly different ($P < 0.05$).

Table 3. Effect of Heat Stress on Heat Shock Protein 70 (HSP70) Serum Concentration in Nigerian Indigenous Sheep Breeds

Breed	HSP70 mean (ng.mL^{-1}) ± SE
Uda	210.71 ± 38.75^{a}
Yankasa	173.28 ± 36.24^{a}
Balami	82.13 ± 19.01^{b}
WAD	69.17 ± 19.50^{b}

Mean with different superscript along the same column are significantly different ($P < 0.05$).

DISCUSSION

In this study, physiological parameters at THI 82 indicate that the animals were heat stressed. Readings taken in the early morning hours (8:00) were compared with readings taken in the afternoon (14:00) to evaluate the effect of the change in temperature on physiological parameters. The increase of the body core temperature and rectal temperature (RT) have been considered good indicators of heat stress in animals (Alamer and Al-Hozab, 2004). RT observed in this study ranged from (38.07 to 38.63 °C at 14:00) and is similar to the values reported by Fadare *et al.* (2012) in heat-stressed WAD sheep. However, Buswat *et al.* (2000) reported slightly higher RT values of 39.6, 39.7 and 39.7 °C in Yankassa, Uda and Balami breeds at the peak of the hot season in Bauchi, Northern Nigeria. The disparity in the values may be due to the differences in the climatic region of the study locations. RT values in the early morning (8:00) and midafternoon (14:00) were significantly different in WAD and Yankassa breeds. Rectal temperature is often used as a representative measurement of the core body temperature and has been reported to be perhaps the most reliable indicator of heat stress as it drives other heat stress alleviating mechanisms (Gerbremedhin *et al.*, 2008). This important physiological mechanism offers a valuable window into the stress faced by vulnerable internal organs during periods of extreme hyperthermia and increases when the body is unable to counteract the effect of excessive heat load (Gagnon *et al*., 2010). Alhidary *et al.* (2012) and Lallo *et al.* (2011) also reported high RT values in goats following exposure to high ambient temperature.

Pulse rate increases on exposure to high environmental temperature (Aboul-Naga, 1987) and appears to signal the immediate response of sheep to the environmental stress (Butswat *et al.*, 2000). This increase leads to a rise in blood flow from the core to the surface to allow for more heat to be lost by sensible and insensible means thus as ambient temperature increases pulse rate and blood circulation increases to transfer heat from the core to the surface (Marai *et al.*, 2007). This trait has been reported to be significantly higher in the summer months. In this study pulse rate ranged from 87.40 ± 13.12 and 95.41 ± 18.78 and higher than the values reported for heat stressed Balami, Uda and Yankassa by Butswat *et al.* (2000. However, the least PR value observed in Yankassa in this study was also reported by Butswat *et al.* (2000) The observed accelerated pulse rate (PR) could be due to the redistribution of blood to the peripheral tissues during heat exposure in sheep and goat as reported by Silanikove (2000b). These findings support the previous reports on other sheep breeds (Marai *et al.*, 2009, McManus *et al.*, 2009b).

Heart rate in the studied population ranged from 98.61 ± 14.75 to 102.24 ± 18.48 however no significant difference was observed among the breeds. Significant difference was only observed in the Balami breed between the early morning and midafternoon readings. This trait has been reported to accelerate during the peak hour of the heat load in animals with unrestricted access to water due to cutaneous blood flow (Alexiev *et al.*, 2004.).

Silanikove (2000a) reported that the respiration rate was a practical and reliable measure of heat load and indicated that respiration rate above 80 breaths per minute is an indication of severe heat stress. RR for all the breeds was above the basal respiration rate in sheep (25-30 breaths per min; Hales and Brown, 1974). Significant differences were observed in the early morning and midafternoon readings in Uda and WAD breeds. The observed respiratory rates of Balami, Yankasa, Uda and West African Dwarf (WAD) sheep in this study ranged from (88.84 ± 17.19 to 107.39 ± 20.93 breaths per minute) indicating that the animals were exposed to severe heat stress. This finding is in agreement with Kumar (2005) who also reported that increased respiratory rate (RR) is the first response when animals are exposed to environmental temperature above the thermoneutral zone. Alamer and Al-Hozab (2004) stated that respiration rate can be used as an indicator of heat stress, and to estimate the adverse effects of environmental temperature. The values reported in this study are higher than that reported by Butswat *et al.* (2000) (62.2, 64.9 and 66.0 breaths per minute) in three of the four breeds sampled in this study. This can be attributed to the high relative humidity that characterizes the location of this study. Relative humidity determines the rate at which sweat is evaporated from the skin, increase in RH makes it difficult for the circulating air to absorb sweat from the skin of the animal.

Thus when animals are exposed to high temperature and high relative humidity their ability to lose heat by evaporation is impaired. Thus, heat stressed animals in areas of high humidity which characterizes southern Nigeria may have higher respiration rate (Hales and Brown, 1974).

The mammalian skin provides an excellent pathway for heat dissipation from the body surface to the environment. Thus, skin temperature is the result of the adjustment of skin to blood flow and heat regulation. Skin temperature of sheep differs according to season of the year and time of the day and it becomes higher with an elevation in ambient temperature (Marai *et al.*, 2007). ST readings for 8:00 and 14:00 were significantly different in all the breeds studied. It is corroborated by Marai *et al.* (2007) that ST of sheep differs according to season of the year and time of the day and increases with an elevation in temperature. In this study ST follows the same trend as RT with higher ST recorded in the Uda sheep however the value was not significantly different from that obtained for Yankassa and Balami. The observed range for ST in the studied population is 37.18 to 37.34 °C higher than the values reported by Catanherrra *et al.* (2010) in some Brazilian sheep breeds. but comparable to the range reported by Marai *et al.* (2007) in Ossimi breed. The values obtained for the indigenous sheep under hot conditions could be attributed to prevalent heat stress and more importantly to the shade deprivation experienced by the animals in this study, which has been reported to cause vasodilatation of skin capillary bed and consequently an increase in the blood flow to the skin surface to facilitate heat dissipation (McManus *et al.*, 2009b).

Hsp70 in particular has been shown to respond to both acute, short term stress (Tomanek and Sanford, 2003) and chronic, long term stress (Helmuth and Hofmann, 2001), and its level appears to be the best predictor of heat tolerance (Sorte and Hofmann, 2005). Cellular and extracellular heat shock proteins have been reported to be associated with stress and higher levels of secretion linked to increased resistance (Sorensen *et al.*,2003; Kristensen *et al.*, 2006). In the present study, there were higher levels of Hsp70 expression in the Uda (210.71 ± 38.75) and Yankasa (173.28 ± 36.24) than that of Balami (82.13 ± 19.01) and WAD (69.17 ± 19.50). Uda had the highest HSP70 level and suggests a better physiological coping mechanism in the breed to the prevalent environmental stress. These findings are in agreement with Horowitz (2001) that the changes in HSP transcription are due to intermediate messengers responding to changes in ambient heat but not body temperature, it would appear that once animals receive a significant stress challenge the HSP response is elicited; and according to Xiao *et al.* (2002) reported that HSP70 can confer transient protection against the adverse effects of subsequent heat and chemical or abnormal stresses. The observed difference in the secretion of HSP70 among the breeds in the studied population following heat stress is also similar to the reported increase in the expression of HSP70 when animals are exposed to hot conditions, (Liu *et al.*, 2010) and Cao *et al.* (2009) reported that higher heat stress level led to higher concentration of HSP70 in the testis and epididymis of mice. It has been postulated that differences in HSP concentrations between species may be due to differences in their thermo-tolerance (Agnew and Colditz, 2008). This may also apply within a species. The range in HSP70 concentration in the present study is higher than the range (0.03 to 2.85 ng.mL^{-1}) for Pelibeuy and Suffolk sheep breeds reported by Romero *et al.*, 2013 and for Angus steers (0.54 to 19.75 ng.mL^{-1}) reported by Gaughan *et al.* (2014). The differences in the HSP concentration among individual animals in the current study may reflect within-breed and among-breed variations with respect to thermo-tolerance or stress in general.

The differences in HSP concentration may reflect breed variations, however since a relatively small population was used in the study, further research work will be needed in the indigenous sheep breeds.

CONCLUSION

The physiological parameters recorded and HSP70 secretion during the dry season in this study indicate that the prevailed environmental condition during this season has adverse impact on the indigenous sheep breeds. The effect of heat stress on the extensively managed sheep should be

minimized for their optimum performance through the provision of regular supply of clean water and adequate shade. Further research on the expression of heat shock proteins at the thermoregulatory organs to determine the thermotolerance ability of the breeds is recommended.

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INFLUENCE OF EWE ENTRY ORDER INTO MILKING PARLOUR ON MORNING MILK YIELD WITH RESPECT TO EWE AGE AND YEAR OF MEASUREMENT

Jana MARGETÍNOVÁ*, Marta ORAVCOVÁ, Michal UHRINČAŤ

NPPC – Research Institute for Animal Production Nitra, Lužianky, Slovak Republic

ABSTRACT

The objective was to analyse effects of ewe entry order into milking parlour with respect to ewe age and year of measurement on morning milk yield. The measurements (5528) of milk yield mostly in the middle of lactation were taken from 550 ewes of Slovak dairy breed over five years (on average two measurements per ewe and year). Mixed model included fixed factors: milking phase (MP1, MP2 and MP3), ewe age (2, 3 to 10), year (2013, 2014 to 2017) and interaction milking phase x ewe age, and random effect of ewe. Ewes were assigned to milking phases according to their entry order into milking parlour (taking into account batch number and stall number within batch). All fixed factors showed the significant effect on milk yield, except for milking phase: 482 ± 11 ml (MP1), 464 ± 8 ml (MP2) and 444 ± 15 ml (MP3), although ewes with earlier entry order had higher milk yields. Three- to six-year old ewes had higher milk yields than two- and seven- to ten-year old ewes. Significant differences were found predominantly between morning milk yields of three- and four-year old ewes (509 ± 12 ml and 538 ± 10 ml), five- to seven-year old ewes (decreasing from 525 ± 11ml to 471 ± 14 ml) on one side, and milk yields of eight-, nine- and ten-year old ewes (decreasing from 421 ± 17 ml to 311 ± 35 ml) on the other side. According to year of measurement, milk yield increased from 406 ± 12 ml (2013) to 530 ± 10 ml (2015), afterwards decreased and increased again (406 ± 9 ml and 510 ± 26 ml). The significant differences were found only between some years. An interaction milking phase x ewe age showed that milk yields tended to follow patterns found when these factors were analysed individually. Higher milk yields were found in four- and five-year old ewes of MP1 group, lower milk yields were found mostly in nine- and ten-old ewes of MP2 and MP3 groups. Only few levels of this interaction showed significant differences between each other.

Key words: sheep; age; milk production; milking phase

INTRODUCTION

In animal husbandry, ever more issues are emerging in relation to lack of staff, while the demand for hygiene of milk production is constantly increasing. Under these conditions, machine milking appears to be the ideal solution. In machine milking of sheep, it is necessary to ensure good task management, correct parameters of the milking equipment, and not least also the knowledge of and respect to the biological needs of sheep during milking and the manipulation with them. Reaction of the sheep to the milking equipment and their behaviour during the entire process is an important factor, which influences lactation milk yield. Sheep learn relatively quickly to enter and leave a milking parlour. The entire process can be sped up by providing fodder feed during milking. During a period of feed deprivation or hunger, however, this can be a strong incentive for increased aggression and disruption of the existing social structures within the flock. Social hierarchy is a natural and important for characterization of flock animals. It directs their mutual cohabitation, determines the position of the individual

***Correspondence:** E-mail: margetinova@vuzv.sk
Jana Margetínová, NPPC – Research Institute for Animal Production Nitra, Teplická 103, 914 01 Trenčianska Teplá, Slovak Republic

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animals within the hierarchy and their behaviour, and overall allows the flock as a unit to satisfy the needs of all animals.

Behaviour of the livestock, social hierarchy, dominance and order of entry into the milking parlour, and other factors that influence this process have been and remain topics of interest for the experts. Animals on the lower hierarchical levels are more careful, they maintain distance, and during random encounters stop or change directions (Gräser-Hermann and Sambraus, 2001). Their reactions, however, depend also on the situation and are influenced by multiple factors.

While most authors found out that the order of entry into the milking parlour is in the cases of sheep, goats or cattle is not random (Stefanowska *et al*., 2000 in cattle; Keszthelyi and Maros, 1992 and Wasilewski, 1999 in sheep; Margetínová *et al*., 2001 in goats), the results of the studies offer differing and contradictory results.

Age as a factor plays an important role in deciding the social status within a sheep flock (Gräser-Hermann and Sambraus, 2001). Margetínová *et al*. (2003) found out that the older goats enter the milking parlour earlier. However Górecki and Wójtowski (2004) observed (although only for one period of the study) that the younger goats enter the milking parlour earlier. Also, Margetínová *et al*. (2002) reported that the younger ewes entered the milking parlour earlier.

Differing results may be found when an influence of animal entry enter into milking earlier on milk yield is investigated. According to some authors, the animals with higher milk yield enter the milking parlour earlier (Margetínová *et al*., 2003 in goats; Sambraus and Keil, 1997 and Gräser-Herrmann and Sambraus, 2001 in sheep; Polikarpus *et al*., 2014 in dairy cows), whereas Gere *et al*. (2001) reported that animals with lower milk yield enter the milking parlor first (in dairy cows).

The objective of this study was to analyse effects of ewe entry order into milking parlour with respect to ewe age and year of measurement on morning milk yield. Ewes were assigned to three milking phases (MP1, MP2 and MP3) on a base of their entry order into milking parlour (respective batch number and respective stall number within batch).

MATERIALS AND METHODS

The measurements were done with ewes of the Slovak dairy breed kept at the experimental farm of the NPPC – RIAP Nitra located in West Slovakia. The measurements morning milk yield were done under regular production conditions during milking season mostly in the middle of lactation (on average two measurements per ewe and year). A five-year period was included. Ewe entry order into the milking parlour and ewe age were recorded. Morning milk yield measurements were done following ICAR recording guidelines (2014). The first measurement was done within 15 days from the beginning of machine milking. The interval between measurements was 28 days (± 5 days). During milking, ewes were fed with diet supplement of concentrate (100 g per ewe). A total, 550 ewes were included in the analysis. Single-row milking parlour with 24 stands (Farmtec) was used (respective batch number and respective stall number within batch were a base for ewes to be assigned to milking phases: MP1 (48 ewes milked first over individual years), MP2 (ewes neither assigned to ewes milked first nor to ewes milked last) and MP3 (within 48 ewes milked last over individual years). Two measurements of morning milk yields done in the middle of lactation per ewe and year were included in the analysis.

The mixed model methodology using MIXED procedure (SAS, 2009) was applied to study the influence of factors affecting variation of morning milk yield. The model equation was as follows:

$$y_{ijklm} = \mu + MP_i + A_j + MP_i A_j + Y_k + u_l + e_{ijklm}$$

where:

- y_{ijklm} – individual measurements of morning milk yield
- μ – intercept
- MP_i – fixed factor of milking phase (PM1, PM2, PM3); $\sum_i MP = 0$
- A_j – fixed factor of ewe age (2, 3, ..., 10); $\sum_j A = 0$
- MP_iA_j – interaction milking phase x ewe age $\sum_{ij} MPA = 0$
- Y_k – fixed factor of year of measurement (2013, 2014, ..., 2017) ; $\sum_k Y = 0$
- u_l – random factor of ewe (1, 2 to 550); $u_n \sim N(0, I\sigma_n^2)$
- e_{ijklm} – random error; $e_{ijklm} = N(0, I\sigma_e^2)$

Fixed factors included in the model were estimated using the Least Squares Means (LSM) method. Statistical significances of fixed factors were tested by Fischer F-test; statistical significances of individual differences between estimated levels of fixed factors were tested *post hoc* by Scheffe multiple-range tests. Differences were considered statistically significant when $P < 0.05$. Ewe and residual error variances were estimated using the Restricted Maximum Likelihood (REML) method. Estimated variances were used to estimate repeatability of morning milk yield that can be interpreted as the proportion of total 0-variance attributable to among-individual variance:

$$r^2 = \frac{\sigma_I^2}{\sigma_I^2 + \sigma_e^2}$$

RESULTS AND DISCUSSION

Analysis of variance of fixed factors affecting morning milk yield (below referenced as milk yield) is given in Table 1. The fixed factors (ewe age and year of measurement) were statistically significant ($F = 11.19$ and $F = 76.31$; $P < 0.001$). The exception was milking phase with $P = 0.06$ ($F = 2.81$). The fixed factor of interaction milking phase x ewe age was also statistically significant ($F = 2.81$; $P < 0.001$). Differences in milk yield in dependence on individual levels of considered fixed factors are discussed below.

With reference to Table 2, ewes milked first (MP1) had higher milk yield than ewes milked last (MP3) i.e. 482 ± 11 ml vs. 444 ± 15 ml. Remaining ewes (MP2), that were assigned neither to MP1 nor MP3, had milk yield 464 ± 8 ml which fell between values for MP1 and MP3 ewes. No significant differences were found between milk yields of ewes of three groups, although a difference in milk yield between MP1 and MP3 was on the significance limit i.e. $P = 0.07$ and expected pattern with decreasing milk yield in ewes in dependence of their later entry order was revealed. A similar finding about significance limit ($P = 0.05$) was reported by Mačuhová *et al*. (2017) between milk yields of ewes milked first and ewes milked last. These authors performed a wider analysis of milkability traits (including milk yield, although this was evening milk yield) and milk composition traits on ewes of various genotypes: purebred Lacaune and crossbreds of Lacaune (sire breed) with either Improved Valachian (dam breed) or Tsigai (dam breed) ewes. Villagrá *et al*. (2007) also reported non-significant effect of entry order into milking parlour on milk yield of Manchega ewes. In contrast,

Table 1. Analysis of variance (statistical significance of Fisher F-test) for morning milk yield

Trait	Sources of variance – Fixed factors			
	Milking phase	Ewe age	Year of measurement	Milking phase x age
Morning milk yield* (ml)	- (P = 0.06)	+++	+++	+++

$^{+++}P < 0.001$

Table 2. Least squares means and standard errors for morning milk yield by milking phase

Trait	Milking phase		
	MP1 (1) N = 1052	MP2 (2) N = 3420	MP3 (3) N = 1056
Morning milk yield (ml)	482 ± 11	464 ± 8	444 ± 15
Scheffe test		1:3^{-} (P = 0.07)	

Margetínová *et al*. (2003), who analysed three groups of Slovak White goats assigned according to their order entry into milking parlour, found all differences between these three groups significant ($P < 0.05$ or $P < 0.001$).

Analyses of milk yield according to various ewe ages (Table 3) showed that three- to six-year old ewes had higher milk yields than two-year and seven- to ten-year old ewes. The significant differences were found between milk yields of two-year old ewes (444 ± 15 ml), three- and four-year old ewes (509 ± 12 ml and 538 ± 10 ml), five-, six- and seven-year old ewes (decreasing from 525 ± 11 ml to 471 ± 14 ml) on one side, and milk yields of eight-, nine- and ten-year old ewes (decreasing from 421 ± 17 ml to 311 ± 35 ml) on the other side. Also, milk yield of two-year old ewes significantly differed when was compared to milk yields of three oldest ewe groups i.e. eight-, nine- and ten-old year ewes.

Taking into account year of measurement as a fixed factor included in the statistical model (Table 4), milk yield was increasing from 406 ± 12 ml (2013) and 464 ± 10 ml (2014), respectively, to 530 ± 10 ml (2015); afterwards this decreased to 406 ± 9 ml (2016). In 2017, an increase of milk yield by 104 ml (510 ± 10 ml) than in 2016 was observed. The significant differences ($P < 0.01$ or $P < 0.001$) were found when multiple comparisons were done, see Table 4. These justified that year of measurement was of great importance and variability of milk yield over individual years needed to be account for. This meant that environmental conditions (diet, temperature, rain/drought etc.) within investigated flock might vary over years of measurement.

The interaction milking phase x ewe age showed that milk yields tended to follow patterns found when these factors were analysed individually (Figure 1). According to ewe assignment to MP1,

Table 3. Least squares means and standard errors for morning milk yield by ewe age

Trait	Age	N	Estimate	Scheffe test
Morning milk yield* (ml)	2	1180	444 ± 15	$2:8^{+}$, 9, 10^{++}
	3	1120	509 ± 12	$3:7^{+}$, 8, 9, 10^{+++}
	4	937	538 ± 10	4:8, 9, 10^{+++}
	5	711	525 ± 11	5:8,9,10^{+++}
	6	608	503 ± 13	$6:8^{+}$, 9, 10^{++}
	7	415	471 ± 14	7:9, 10^{+}
	8	289	421 ± 17	
	9	171	359 ± 25	
	10	97	311 ± 35	

$^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$

Table 4. Least squares means and standard errors for morning milk yield by year of measurement

Trait	Year of measurement				
	2013 (1) N = 752	2014 (2) N = 1375	2015 (3) N = 998	2016 (4) N = 1420	2017 (5) N = 983
Morning milk yield (ml)	406 ± 12	464 ± 10	530 ± 10	406 ± 9	510 ± 10
Scheffe test	1:2, 3, 5^{+++}; 2:3, 4,5^{+++}; $3:4^{+++}$, $4:5^{+++}$				

$^{+++}P < 0.001$

MP2 or MP3, the youngest i.e. two-year and three-year old ewes appeared to have higher milk yields in MP2 and MP3 groups. When frequency of these ewes over milking phases was investigated (results not shown), they mostly entered the milking parlour later (43 % and 56 % in MP2 and MP3 vs. 28 % in MP1. The oldest i.e. seven-, eight- and nine-year old ewes appeared to have higher milk yield in MP1 group. The exception was the group of ten-year old ewes which appeared to have higher milk yields in MP2 and MP3 groups. When frequency of the oldest ewes over milking phases was investigated, they mostly entered the milking parlour earlier (23 % in MP1 and 17 % in MP2 vs. 14 % in MP3). This might indicate that ewes gain information and experience and form a habit with age. Consequently, older ewes seemed to enter the milking parlour earlier than younger less experienced ewes. In general, higher milk yields were found in four- and five-year old ewes of MP1 group in comparison to milk yields of ewes of these ages of MP2 and MP3 groups. Only few levels of interaction milking phase x ewe age showed significant differences between each other. No difference between milking phases of ewes of the same age were found.

Findings in this study correspond with experiences of farmers who noticed that especially young ewes in their first milking season entered the milking parlour later due to fact they lack of experience. Many authors reported that ewe age plays an important role in behaviour of ewes within flock. Gräser-Herrmann and Sambraus (2001) analysed East Friesian dairy ewes from three different farms and found that ewe age significantly influenced ($P \leq 0.01$) social status of ewes of various ages within flocks. These authors reported the mutual relation between hierarchical status and milk performance of ewes (ewes with higher milk yield entered the milking parlour earlier). Margetínová *et al*. (2003) reported that entry order into the milking parlour is influenced by age and milk performance in the favour of older sheep. Whereas, Margetínová *et al*. (2002) reported that younger ewes entered the milking parlour earlier than older ewes. This was most likely caused by out-of-season mating, due to which older ewes were started to be milked later in respective milking season. Thus, older ewes were introduced into an already existing system with a predominance of younger sheep and this was probably a reason why they entered the milking parlour later. According to Mačuhová *et al*. (2017), ewes entering the milking parlour first had more favourable milk flow parameters (shorter latency time, higher peak flow rate, and higher milk yield in 30 s and 60 s). With cattle, Polikarpus *et al*. (2014) reported that cows with higher milk yield entered the milking parlour first. The positive relationship between entry order

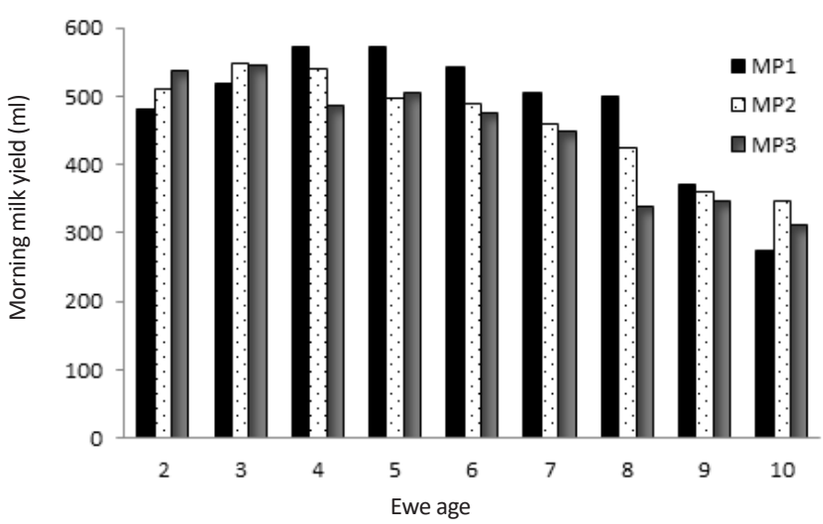

Figure 1. Least squares means for morning milk yield by milking phase x ewe age

into milking parlour and amount of milk was reported by Grasso *et al*. (2007) in cows after first calving (correlation coefficient equalled to 0.22). Littooij and Butterworth (2018) found that older cows were more likely to enter the milking parlour earlier than younger animals.

However, it is necessary to point out another important fact. Social status of an animal within a group is also influenced by feed diet to some extent. Hierarchy and dominance in a group is formed in order to achieve a certain status in a group, which allows respective animal better opportunity to access feed earlier, and often in better quality when comparing to other members of a group. Deprivation, however, causes increased aggression in animals (Syme *et al*., 1974) and in such situations, subdominant animals cease to respect their order in the group. Animals with higher status have stronger predispositions to satisfy their needs. Farmers use feed mixture offered during milking as a stimulus. Thus, animal are strongly motivated and the vision of feed prompts them towards achieving "benefit". This may work as an advantage for older and more experienced animals. The aspect of supplementary feeding is particularly important during dry grazing season (recently occurring in some regions of Slovakia quite often) and causes aggressive competitive behaviour within the group. Under such circumstances, more noticeable aggression is observed in the dominant animals (Erhard, 2004). Because a social hierarchy is not random, weaker animals are forced to be back during milking. Advantageous access to fodder in dominant animals then influences their milk yield. Larger volume of milk creates pressure in milk gland and "forces" animals to earlier milking. Order of entry into the milking parlour may be also influenced by health issues (Polikarpus *et al.*, 2015). Mačuhová *et al*. (2017) suggest that for ewes with inadequate udder anatomy, milking can be painful and therefore they avoid entering the milking parlour early. Moreover, way of animal handling during milking may cause them pain or discomfort. Dimitrov *et al*. (2017) reported an increased fear in primiparous ewes during milking when teat cups are put on i.e. the preparation of younger ewes to and good organization of machine milking are very important. Other authors (Munksgaard *et al*., 2001; Rushen *et al*., 2001; Grasso *et al*., 2007) also recommended that animals need to be handled gently and, if possible, not to be disturbed. Paranhos da Costa and Broom (2001) observed that some animals, if they have the option, favour the same side repeatedly during milking. This phenomenon, however, was not statistically confirmed until now.

CONCLUSION

Knowledge of the ethological and adaptation abilities of livestock animals, their behaviour and respect to their social and biological needs give the farmer good presuppositions to create optimal breeding conditions. It is necessary to remember that the animals of lower status also need to satisfy their basic needs. To achieve this, also these animals need suitable conditions and stable hierarchy, which has a calming effect on the entire flock. The results of our study can be beneficial with regard to the behaviour of ewes during machine milking.

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EFFECT OF FENCE-LINE WEANING ON EGYPTIAN BUFFALOES' MILK PRODUCTION AND GROWTH PERFORMANCE OF THEIR CALVES

Tamer Mohamed M. HASSAN[1]*, Mayada S. H. MAHMOUD[2], A. S. M. SOLIMAN[1], M. R. EL-MAHDY[1], Hoda Z. HASSAN[2]

[1]Animal Production Department, Faculty of Agriculture, Benha University, Egypt
[2]Buffaloes Breeding Research Department, Animal Production Research Institute, Dokki, Giza, Egypt

ABSTRACT

The objective of the current study was to reduce the stress associated with weaning process for a mother and its weaned calf by increasing the productive performance of the mother and reproductive performance (early return to estrous and conception), increasing the growth rate and some body measurements of calves post-weaning, as well as improving the immunological responses of both mother and calf. Egyptian buffaloes and their calves used in this study (n = 40) were divided into two weaning system groups: 20 buffaloes in fence-line and 20 buffaloes in traditional weaning system. Buffaloes and their calves were placed on opposite sides of strong fence. They had nose-to-nose contact; fence-line visits gradually decreased after the first three days. Milk production and its components were measured. Blood samples were taken for analyzing weekly after calving until 8th week. As a result, regression coefficients of the equation of Wood lactation curve showed that weaning system had different ($P < 0.001$) *a*, *b* and *c* parameters. Buffaloes with fence-line weaning had the highest ($P < 0.001$) milk production and milk component values during 8 months of lactation. Fence-line weaning calves had superior ($P < 0.001$) performance than traditional weaning calves. Immunoglobulin content (immunity) was increased in buffaloes and their calves weaned using the fence-line system. Calves' sex and dams parity effects showed the normal expression. In conclusion, the fence-line system can provide to gain more milk yield, calf growth performance and immunity, and therefore, we recommend this system for buffalo's producers.

Key words: fence-line; milk; calve performance; immune response; Egyptian buffaloes

INTRODUCTION

Weaning time can be stressful for dams and their calves. Under traditional weaning systems, changes in environment, diet composition and pathogen exposure can reduce performance of animals and cause health problems. In response to these challenges, interest in the fence-line weaning has grown in recent years. Fence-line weaning is a management system in which the calves are removed from their dams but are allowed to see, hear and smell their dams. Depending on the fencing used, physical contact may also be possible. It has the potential to reduce stress related to transport, changes in environment and diet adaptation. Fence-line weaning may also reduce labor demands and costs associated with dry lot facilities (Price *et al*., 2003). Preconditioning is implemented around weaning time and is designed to enhance immune system function while minimizing stress (Lalman *et al*., 2002). At two weeks of age, the calf should be introduced to good quality green feed and concentrates formulated to meet the requirements of calves, as a calf starter. This stimulates the rumen

***Correspondence:** E-mail: tamer.mohamed@fagr.bu.edu.eg
T.M.M. Hassan, Animal Production Department, Faculty of Agriculture, Benha University, Egypt

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to grow and function properly. However, the larger period that the calf has an access to the large supply of milk, the less period will be its urge to supplement its diet with other foods; whole milk should be provided to the calf until 15 days of age at a level of 1/10th of the calf's body weight (Salama *et al*., 1989). After feeding the milk, calves are offered a dry calf starter and good quality hay simultaneously from the second week of age.

The objectives of the current study were to reduce the stress associated with weaning process for buffaloes and its weaned calves by increasing milk production of the dams and enhancing the growth performance of calves post-weaning, as well as improving the immunological responses of all experimental animals.

MATERIAL AND METHODS

The present study was carried out at the experimental farm of El Nataf El Kadem in Kafer El-Sheikh Governorate, Buffalo Breeding Research Department, Animal Production Research Institute, Agriculture Research Center, Ministry of Agriculture, Egypt. The field experiment lasted from November 2015 to April 2016. Samples of milk and blood were analyzed at the Buffalo breeding Research Department, Animal Production Research Institute.

Experimental design

Forty native lactating buffalo cows randomly taken after parturition were used in this study. Buffalo dams were balanced in parity and in the quota of nutrition, under the same veterinarian control and were without reproductive diseases.

The experimental animals were divided into two groups: the first groups was fence-line weaning buffalo cows and their newborn and the second was the separated weaning calves which stayed after parturition with their mothers and took colostrum through the first three days after birth; then they were separated from their mothers.

Weaning strategies

Usually, the calves were weighed immediately after birth and every week until weaning at 3.5 months of age. After weaning, the calves were weighed monthly for 3 months (until 5-6 months of age).

Traditional weaning

Buffalo-cows were kept together with its calf in the same place where their calves will stay. Then buffalo-cows were removed to a new location out of the vision and sound of their calves, but the calves were left in familiar surroundings.

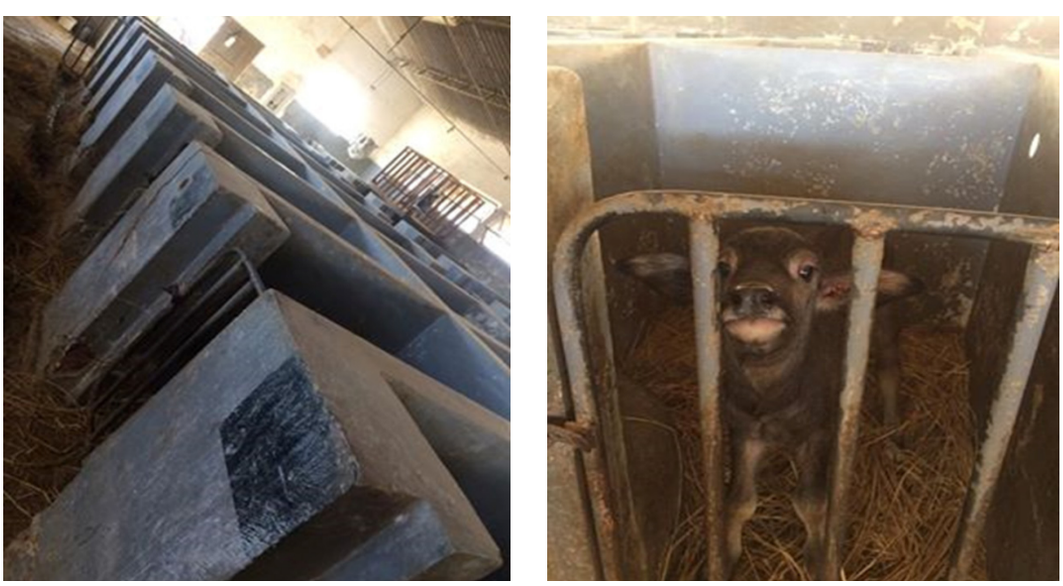

Figure 1. Traditional weaning system

Fence-line weaning system

The current study describes the progressive weaning system (fence-line), as well as the growth rate of calves during weaning and post-weaning phase (6 months) compared to the traditional weaning system. There are many ways to wean calves, but not all methods used are created equally. Traditional calf weaning models can cause high stress in calves and later lead to the reduced appetite, loss weight and suppressed the immunity. Fence-line weaning offers an effective strategy to reduce stress and side effects that come with it during this critical time with fence-line weaning, calves and cows are kept in the same barn. They are separated by a barrier but they can hear and see each other.

Calf feeding system

Calves were artificially fed whole buffalo milk twice a day till weaning age, according to the schedule shown in Table (1). Calves were weaned when their consumption of dry matter from roughage and calf starter per head reached 2 % of birth weight according to Salama and Mohy-El Deen (1996).

At the second week of age clover hay and calf starter were offered *ad libitum*, with clean water and salt lick block until weaning age. Calf starter was composed of 25 % corticated cottonseed meal, 20 % yellow corn, 26 % wheat bran, 15 % linseed meal, 4 % rice bran, 7 % molasses, 2 % limestone and 1 % common salt. Chemical composition of buffalo milk, clover hay and calf starter is shown in Table (2).

The experimental ratios used after weaning were formulated from Concentrate Feed Mixture, CFM (as the concentrate part of the ratios, 75 % of Total Digestible Nutrients, TDN), clover hay (12.5 % of TDN) and rice straw (12.5 % of TDN). The chemical composition of feed stuffs used for formulating the experimental ratios presented after weaning are shown in Table (3). Animals were fed to concentrate feed mixture, clover and rice straw according to NRC (1985) recommendations for the requirements of growing calves.

CFM (concentrate feed mixture) consisted of undecorated cotton seed cake (20 %), linseed cake (15 %), wheat bran (12 %), yellow corn (30 %), rice bran (16 %), limestone (2 %), molasses (3 %), salt (1 %) and vitamins A, D, E (1 %).

Calf diets were weighed and offered to the animals twice daily at 8:00 am and at 4:00 pm with allowance of 15 % refusal in equal quantities for each group. Both of the consumed diets and

Figure 2. Fence-line weaning system

Table 1. Feeding system (amount of buffaloes milk, kg/day/calf)

Item	Calf age (weeks)													
	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Morning	2.3	2.7	2.7	2.7	2.3	2.3	1.8	1.8	2.7	2.3	1.8	1.4	1.3	0.85
Evening	2.3	2.3	2.7	2.3	2.3	1.8	1.8	1.4	_	_	_	_	_	_

Table 2. Chemical composition of buffalo milk, clover hay and calf starter

Item	Buffalo milk	Clover hay	Calf starter
Moisture	84.90	9.71	11.07
Dry matter	15.05	88.94	90.66
Crude protein	25.52	12.30	18.60
Crude fiber	-	28.50	3.25
Ether extract	43.17	2.70	2.93
NFE	25.41	44.00	68.68
Ash	5.90	12.50	6.54

Table 3. Chemical composition of the concentrate feed mixture, clover hay and rice straw (on Dry Matter, DM basis)

Feedstuffs	DM	On DM basis (%)					
		OM	CP	CF	EE	Ash	NFE
CFM	93.94	89.94	14.45	13.34	2.98	10.06	59.17
Clover	91.09	88.45	14.29	28.89	1.62	11.55	43.65
Rice straw	90.56	81.60	2.42	36.76	1.38	17.40	42.04

refusals, if any, were recorded daily. Clean and fresh water with salt lick were also offered *ad libitum*. All calves were weighed individually to the nearest kg in the morning before feeding throughout the experimental period (from birth to six months of age). Birth, weaning age (at 3.5 months of age) and six month body weight were recorded. Average daily weight gain, total feed intake, feed conversion ratio and economical feed efficiency were then calculated by following equation: Feed conversion = total feed intake / body weight gain, kg.

Economical feed efficiency = benefit of body weight gain, $ / cost of feed consumed, $. The date were based on the prices of calves and feed ingredients in the market during the experimental period.

Buffaloes' diet

Dam buffaloes were feeding Egyptian clover (*Trifolium alexandrium*), when available, in addition to rice straw and different amounts of integrated concentrate feed mixtures (48 % decorticated cotton seed cake, 21 % wheat bran, 20 % maize, 5 % rice polish, 3 % molasses, 2 % limestone and 1 % sodium chloride) according to the norms established by the Animal Production Research Institute.

In the summer, Egyptian clover (*Trifolium alexandrium*) was replaced with a silage. Amounts of rations given to the animals were determined according to animal body weight and its level of milk production. The ration was offered twice daily. Buffaloes were feeding individually, according

to Animal Production Research Institute norms (APRL, 1997).

Milk sampling and analysis

Approximately 20 ml of milk samples were pre-warmed at 40 °C in water bath and thoroughly mixed in clean glass beaker. Milk analysis was carried out for total solids, fat, proteins, lactose, and non-fat solids using Milk-Scan kit (Lactostar™, Funk Gerber, Germany) at the Dairy Services Unit, Animal Production Research Institute, Sakha, Kafr El-Sheikh Governorate.

Blood samples and analysis

Blood samples were collected in heparinized test tubes, after collection the blood samples were centrifuged at 4000 rpm for 15 minutes. The clear plasma was then aspirated and stored at -20 C˚, until assay for blood immunoglobulin. The single radial immune diffusion technique was used to quantify total immunoglobulin IgG in blood plasma (Bind ARIDtm Binding site limited, Birmingham, UK) according to the method described by Fahey and McKelvey (1965). The method of IgG quantification involves antigen diffusing radially from cylindrical well through an agarose gel containing an appropriate non-specific antibody. Antigen antibody complexes are formed as a precipitation ring. The ring size increases until an equilibrium. There is a linear relationship between the ring diameter and antigen concentration using (IgG) liquicolor® Kit (traceable to ERM470 (CRM470) from BEN-biochemical Enterprise (Milano, Italy).

Estimating the curve parameters of milk production

In this work, the shape of the curve parameters of milk production of Egyptian buffaloes was studied using the gamma type function (Wood, 1967), which was described as sufficiently good for modeling extended lactations (Abdel-Salam *et al*., 2011). The following gamma-type function was used for describing the lactation curve of all parameters: $Y_n = an^b e^{-cn}$

The constants a, b and c were calculated by using a general linear model (GLM) procedure of SAS software (SAS, 2004); where Y_n – is the test-day milk (kg), in the n^{th} month of lactation, *a* – is the initial yield, *b* – describes the rate of production increase up to the peak during the ascending phase, *c* – describes the rate of yield decrease during the descending phase, and *e* – is a base of natural logarithms. The NLIN procedure of SAS software was used for fitting the gamma type function according to Wood (1967).

Statistical Analysis

Buffalo milk components

Statistical analysis of data was carried out applying the SAS package (2008), according to the following model:

$$Y_{ijkl} = \mu + X_i + M_j + Sex_k + P_L + (XWSP)_{ijkL} + B(X)_{ijkL} + E_{ijkLm}$$

Where:

Y_{ijkl} – is the dependent variable (studied traits; fat, protein, lactose, total solid and solides not fat) of the m^{th} record on the L^{th} parity, k^{th} calf sex, j^{th} test day and i^{th} fence-line and traditional weaning;

μ – the overall mean of studied trait;

X_i – the effect of the i^{th} fence-line and traditional weaning, i = 1 and 2;

M_j – the effect of the j^{th} month of lactation, j = 1, … and 8;

S_k – the effect of the k^{th} calf sex, k = 1 and 2;

P_L – the effect of the L^{th} parity, L = 1, 2 and 4;

$b_{y/x}$ – the regression coefficient of the studied trait on dam birth weight;

$(XWSexP)_{ijkL}$ X_i – the effect of the fence-line and traditional weaning;

TD_j – the effect of the test day;

S_k – the effect of the calf sex;

P_L – the effect of the parity;

E_{ijklm} – random error assumed N.I.D. (0, $\sigma^2 e$).

Differences among means were checked according to Duncan (1955).

Calf performance

Statistical analysis of data was carried out applying the SAS package (2008), according to the following model:

$$Y_{ijkl} = \mu + X_i + W_j + S_k + P_L + (XWSP)_{ijkL} + B(X)_{ijkL} + E_{ijkLm}$$

Where:

Y_{ijkl} – is the dependent variable (studied traits) of the m^{th} record on the lth parity, k^{th} sex, j^{th} sampling week and i^{th} fence-line and traditional weaning;

μ – the overall mean of studied trait;
X_i – the effect of the i^{th} fence-line and traditional weaning, i = 1 and 2;
W_j – the effect of the j^{th} sampling week, j = 1^{st}, … and 8^{th};
S_k – the effect of the k^{th} calf sex, k = 1 and 2;
P_L – the effect of the l^{th} parity, L = 1^{st}, 2^{nd} and 4^{th};
$b_{y/x}$ – the regression coefficient of the studied trait on birth weight;
$(XWSexP)_{ijkL}$ X_i – the effect of the fence-line and traditional weaning;
W_j – the effect of the sampling week;
S_k – the effect of the calf sex;
P_L – the effect of the parity;
E_{ijklm} – random error assumed N.I.D. (0, $\sigma^2 e$).

Differences among means were checked according to Duncan (1955).

RESULTS

Lactation curve

Regression coefficients of the equation of lactation curve (Wood, 1967) showed that weaning system had different (P < 0.001) *a*, *b* and *c* parameters. Buffaloes with fence-line weaning had the highest (P < 0.001) milk production during 8 months of lactation (Table 4). Figure 3 shows the lactation curve of Egyptian buffaloes according to weaning system (fence-line and traditional weaning system).

Milk components

Buffaloes that weaned their calves by fence-line weaning system tended to have the highest milk components (fat, lactose, total solids and solids-not-fat) than that weaned their calves by traditional weaning system. The differences between means of buffaloes milk components due to weaning system effect were highly significant (P < 0.001). Buffaloes milk fat and total solids during the eighth month of lactation were higher and significant (P < 0.001) than other months of lactation. It could be seen that second, seventh and eighth parities of buffaloes had a higher (P < 0.01) milk fat (9.15 %) than buffaloes at first (8.69 %) and second (8.04 %) parities. Buffaloes that born male calves had a higher (P < 0.01) milk protein and solids-not-fat (4.15 and 10.03 %, respectively) than those born female calves (3.82 and 9.81 %, respectively), as shown in Table 5.

Calf growth performance

It could be seen from Table 6 that the differences between means of buffalo calf body weight at weaning and sixth month of age, due to weaning system effect, were highly significant (P < 0.001), but there is no significant effect for calf birth weight. Calves that weaned by fence-line weaning system had a higher body weight at weaning (3.5 months of age) and sixth month of age (109.31 and 155.47 kg, respectively) than that weaned by traditional weaning system (99.23 and 143.56 kg, respectively). Male calves had almost heavier body weight at birth, weaning and sixth month of age (34.73, 104.69 and 150.06 kg, respectively) than female calves (34.59, 104.28 and 149.38 kg, respectively). Calves from fourth or more parities had a heavier (P < 0.01) body weight at birth, weaning and sixth month of age (35.44, 110.04 and 155.44 kg, respectively) than calves from second

Table 4. Regression coefficient of the equation of Wood lactation curve by buffaloes calves weaning system

Item	*a* (kg)	*b*	*c*
Weaning system			
Fence-line weaning	5.2[a]	0.58	-0.25
Traditional weaning	4.7[b]	0.43	-0.21

Means within a column with different letters differ (P < 0.05); *a* – is the associated parameters with the initial milk production; *b* – is the associated parameter with the rise in milk production to peak lactation; *c* – is the associated parameter with the decrease in milk production from the peak to the end of lactation.

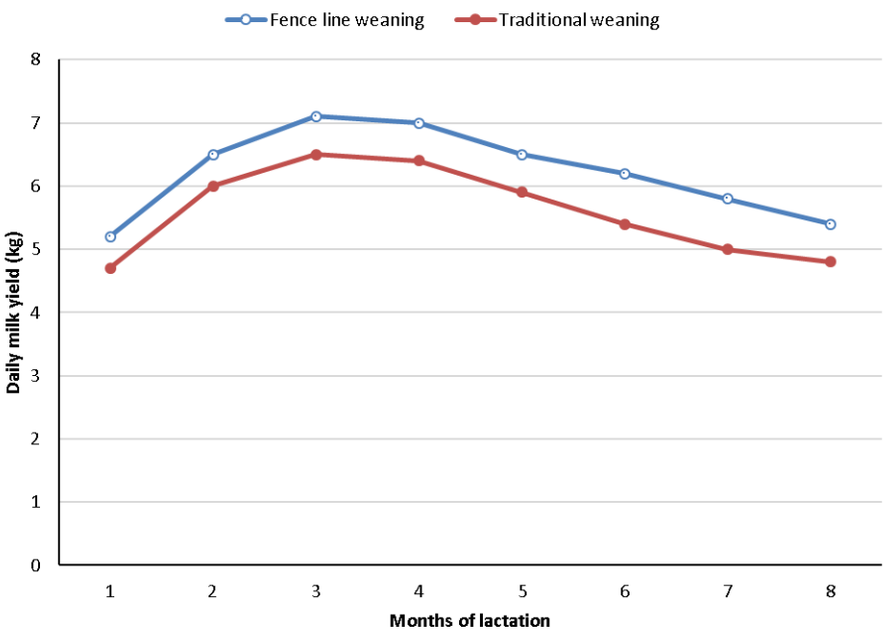

Figure 3. Lactation curve of Egyptian buffaloes according to weaning system

Table 5. Least-squares means and standard errors (LSM ± SE) of buffalo milk components (%)

Classification	N	Fat	Protein	Lactose	Total solids	Solids-not- fat
Weaning system						
Fence-line	122	8.93 ± 0.32	3.712 ± 0.07^{b}	5.762 ± 0.05^{a}	19.15± 0.33^{a}	10.21± 0.09^{a}
Traditional weaning	130	8.65 ± 0.20	4.257 ± 0.04^{a}	4.538 ± 0.03^{b}	18.27 ± 0.2^{b}	9.61 ± 0.06^{b}
Month of lactation						
First	40	8.01 ± 0.29^{b}	4.01 ± 0.06	5.09 ± 0.05	17.92 ± 0.30^{c}	9.89 ± 0.09
Second	40	9.51 ± 0.29^{a}	3.97 ± 0.06	5.18 ± 0.05	19.47 ± 0.30^{a}	9.95 ± 0.08
Third	40	8.15 ± 0.29^{b}	4.05 ± 0.06	5.08 ± 0.05	18.07 ± 0.30^{b}	9.91 ± 0.08
Fourth	36	8.51 ± 0.31^{b}	4.00 ± 0.07	5.16 ± 0.05	18.47 ± 0.32^{b}	9.96 ± 0.09
Fifth	36	7.99 ± 0.31^{c}	3.94 ± 0.07	5.03 ± 0.05	17.76 ± 0.32^{c}	9.76 ± 0.09
Sixth	28	8.53 ± 0.49^{b}	4.06 ± 0.11	5.23 ± 0.08	18.57 ± 0.51^{b}	10.04 ± 0.15
Seventh	18	9.17 ± 0.56^{a}	3.93 ± 0.12	5.08 ± 0.09	18.96 ± 0.58^{b}	9.79 ± 0.17
Eighth	14	9.10 ± 0.81^{a}	3.90 ± 0.18	5.34 ± 0.14	19.13 ± 0.83^{a}	10.03 ± 0.25
Parity number						
First	100	8.69 ± 0.22^{b}	4.11 ± 0.05^{a}	5.11 ± 0.04	18.76 ± 0.23	10.07 ± 0.07^{a}
Second	52	8.04 ± 0.39^{a}	4.12 ± 0.09^{a}	5.14 ± 0.07	18.04 ± 0.41	10.01 ± 0.12^{a}
Fourth or more	100	9.15 ± 0.29^{a}	3.73 ± 0.06^{b}	5.19 ± 0.05	18.83 ± 0.31	9.68 ± 0.09^{b}
Sex of calf						
Male	152	8.59 ± 0.22	4.15 ± 0.05^{a}	5.08 ± 0.04^{b}	18.63 ± 0.23	10.03 ± 0.07^{a}
Female	100	8.65 ± 0.27	3.82 ± 0.05^{b}	5.22 ± 0.05^{a}	18.46 ± 0.27	9.81 ± 0.08^{b}

$^{a, b, c}$ Means within any classification, followed by different letters are significantly different ($P < 0.05$)
N – number of milk samples

parity (35.08, 102.09 and 147.37 kg, respectively) and calves from first parity (33.44, 101.34 and 146.36 kg, respectively).

Calves that weaned by a fence-line system had a higher average daily gain from birth to 6 months of age than by using traditional weaning system (670.71 vs 607.50 grams). The differences between means of calf's average daily gain from birth to 6 months of age due to weaning system effect were highly significant (P < 0.001). Male calves exceeded female calves in average daily gain from weaning to 6 months of age (650.00 vs. 600.00 grams). Calves from fourth or more parities had a higher (P < 0.001) average daily gain than calves from second and first parity (670 and 620 grams, respectively), as presented in Table 7.

Calves that weaned by the fence-line system had a higher, but not significant, total feed intake from birth to 6 months of age than by the traditional weaning system (539.78 vs. 520.57 kg), except for total feed intake from birth to weaning (P < 0.001). Total feed intake was higher for male calves than for females

Table 6. Least-squares means and standard errors (LSM ± SE) of buffalo calf body weight (kg)

Classification	N	Birth weight	Weaning weight	Six months weight
Weaning system				
Fence-line	20	34.76 ± 0.53	109.31 ±1.39^{a}	155.47 ± 1.46^{a}
Traditional	20	34.56 ± 0.39	99.23 ± 1.04^{b}	143.56 ± 1.09^{b}
Calf sex				
Male	24	34.73 ± 0.36	104.69 ± 0.96	150.06 ± 1.01
Female	16	34.59 ± 0.53	104.28 ± 1.42	149.38 ± 1.49
Parity number				
First	14	33.48 ± 0.45^{b}	101.34 ± 1.17^{b}	146.36 ± 1.23^{b}
Second	10	35.08 ± 0.53^{a}	102.09 ± 1.39^{b}	147.37 ± 1.46^{b}
Fourth and more	16	35.44 ± 0.55^{a}	110.04 ± 1.45^{a}	155.44 ± 1.53^{a}

$^{a, b}$ Means within any classification, followed by different letters are significantly different (P < 0.05)
N – number of calves

Table 7. Least-squares means and standard errors (LSM ± SE) of buffalo calf average daily gain (grams)

Classification	N	Birth to weaning	Weaning to six months	Birth to six months
Weaning system				
Fence-line	20	710.00 ± 12.60^{a}	615.00 ± 1.00^{a}	670.61 ± 7.00^{a}
Traditional	20	620.00 ± 9.00^{b}	591.00 ± 1.00^{b}	607.50 ± 5.00^{b}
Calf sex				
Male	24	670.00 ± 8.00	650.00 ± 0.87^{a}	640.00 ± 4.00
Female	16	660.00 ± 12.00	600.00 ± 1.00^{b}	640.00 ± 5.00
Parity number				
First	14	650.00 ± 7.00^{b}	600.00 ± 1.00^{b}	620.00 ± 5.00^{b}
Second	10	640.00 ± 8.00^{b}	610.00 ± 1.00^{a}	620.00 ± 5.00^{b}
Fourth and more	16	710.00 ± 9.00^{a}	610.00 ± 1.00^{a}	670.00 ± 6.00^{a}

$^{a, b}$ Means within any classification, followed by different letters are significantly different (P < 0.05)
N – number of calves

(547.55 vs 536.45). Calves from four and more parities had a higher total feed intake than calves from second and first parities (546.21, 540.23 and 531.44 kg, respectively), as shown in Table 8.

Calves that weaned by the fence-line system had an optimum feed conversion ratio from birth to 6 months of age than weaned by the traditional weaning system (4.47 vs. 4.76). The differences between means of buffalo calves feed conversion ratio, due to weaning system effect, were significant (P < 0.05), except for feed conversion ratio from birth to 3.5 months of age. Male and female calves had nearly the same feed conversion ratio from birth to 6 months of age (4.74 vs. 4.67). Calves from different parities had the same feed conversion ratio (4.73, 4.78 and 4.55, for second, third and fourth or more parity, respectively), as presented in Table 9.

Calves that weaned by the fence-line system had almost the same economical feed efficiency of calves that weaned by traditional weaning system (1.35 vs. 1.24). The differences between means of calves' economical feed efficiency due to weaning

Table 8. Least-squares means and standard errors (LSM ± SE) of buffalo calf total dry matter feed intake (kg)

Classification	N	Birth to weaning	Weaning to six months	Birth to six months
Weaning system				
Fence-line	20	268.68 ± 0.15[a]	270.80 ± 1.11	539.78 ± 1.01[a]
Traditional	20	246.82 ± 0.11[b]	273.75 ± 0.82	520.57 ± 0.74[b]
Calf sex				
Male	24	276.37 ± 0.11	271.18 ± 0.77	547.65 ± 0.69
Female	16	266.93 ± 0.16	269.52 ± 1.13	536.55 ± 1.01
Parity number				
First	14	266.21 ± 0.14[c]	265.23 ± 0.99	531.44 ± 0.89[b]
Second	10	269.21 ± 0.16[b]	270.86 ± 1.14	540.23 ± 1.03[ab]
Fourth and more	16	273.15 ± 0.17[a]	273.95 ± 1.23	546.21 ± 1.11[a]

[a, b] Means within any classification, followed by different letters are significantly different (P < 0.05)
N – number of calves

Table 9. Least-squares means and standard errors (LSM ± SE) of buffalo calf feed conversion ratio (feed intake / weight gain)

Classification	N	Birth to weaning	Weaning to six months	Birth to six months
Weaning system				
Fence-line	20	3.61 ± 0.11	5.87 ± 0.01[b]	4.47 ± 0.02[b]
Traditional	20	3.79 ± 0.09	6.18 ± 0.008[a]	4.76 ± 0.02[a]
Calf sex				
Male	24	3.95 ± 0.08	5.98 ± 0.008	4.74 ± 0.01
Female	16	3.83 ± 0.12	5.97 ± 0.012	4.67 ± 0.02
Parity number				
First	14	3.92 ± 0.104	5.90 ± 0.011	4.73 ± 0.025
Second	10	4.02 ± 0.120	5.98 ± 0.012	4.78 ± 0.08
Fourth and more	16	3.65 ± 0.129	6.03 ± 0.013	4.55 ± 0.031

[a, b] Means within any classification, followed by different letters are significantly different (P < 0.05)
N – number of calves

system effect were significant ($P < 0.05$), except from birth to 3.5 months of age. The differences between means of calves economical feed efficiency due to sex and parity effects were not significant, as presented in Table 10.

Immune response of buffaloes and their calves

Buffalo dams that weaned their calves by the fence-line system had a stronger immunity than those weaned their calves by traditional weaning system. The differences between means of buffalo dams' immunoglobulin, due to weaning system

Table 10. Least-squares means and standard errors (LSM ± SE) of economical feed efficiency (benefit of weight gain, $ / cost of feed consumed, $)

Classification	N	Birth to weaning	Weaning to six months	Birth to six months
Weaning system				
Fence-line	20	1.07 ± 1.77	2.55 ± 0.31^{a}	1.35 ± 0.42^{a}
Traditional	20	0.96 ± 1.31	2.42 ± 0.12^{b}	1.24 ± 0.31^{b}

$^{a, b}$ Means within any classification, followed by different letters are significantly different ($P < 0.05$)
N – number of calves

Table 11. Least-squares means and standard errors (LSM ± SE) of dams and calves' immunoglobulin (IgG) concentration

Classification	N	Dams	Calves
Weaning system			
Fence-line	160	588.51 ± 5.76^{a}	522.91 ± 3.59^{a}
Traditional	160	435.28 ± 4.74^{b}	451.32 ± 3.76^{b}
Sampling time			
First week postpartum	40	520.41 ± 8.37^{ab}	473.69 ± 7.74^{b}
Second week postpartum	40	512.38 ± 8.37^{ab}	482.66 ± 7.74^{b}
Third week postpartum	40	531.66 ± 8.37^{a}	475.11 ± 7.74^{b}
Fourth week postpartum	40	515.04 ± 8.37^{ab}	490.14 ± 7.74^{ab}
Fifth week postpartum	40	510.66 ± 8.37^{ab}	496.26 ± 7.74^{ab}
Sixth week postpartum	40	497.04 ± 8.37^{b}	510.21 ± 7.74^{a}
Seventh week postpartum	40	498.26 ± 8.37^{b}	509.79 ± 7.74^{a}
Eighth week postpartum	40	509.71 ± 8.37^{b}	487.75 ± 7.74^{b}
Parity number			
First	112	517.56 ± 4.89	488.69 ± 2.89
Second	80	508.85 ± 7.06	490.54 ± 5.54
Fourth and more	128	509.27 ± 6.49	482.11 ± 4.36
Sex of calf			
Male	192	510.78 ± 4.02	488.10 ± 2.32
Female	128	513.01 ± 5.92	486.13 ± 3.71

$^{a, b}$ Means within any classification, followed by different letters are significantly different ($P < 0.05$)
N – number of calves

effect were highly significant (P < 0.001). Calves that weaned by the fence-line system had stronger (P < 0.001) immunity defense mechanism than those weaned by traditional weaning system. The differences in mean values of immunoglobulin between dams and calves, due to sampling time effect were significant (P < 0.001). Third week postpartum showed higher level of dam's immunoglobulin than other weeks postpartum, while sixth and seventh weeks postpartum showed higher levels of calf's immunoglobulin. The differences between means of buffalo's immunoglobulin, due to parity and sex of calf, were not significant, as shown in Table 11.

DISCUSSION

Lactation curve

Lactation curve shows that fence-line weaning system was better in in Egyptian buffalo milk production than traditional weaning system. Many investigators obtained similar results. In particular, Tonhati *et al*. (2008) have found that milk yield in dairy buffaloes, mainly during the first six months of lactation, could be adopted as a selection criterion to increase total milk yield. Ibrahim (2012) analyzed lactation curve of Egyptian buffaloes and has found that increasing enrollment period increases daily milk yield and total milk yield, according to the regression line. Also, Kisac *et al*. (2011) stated that stay of calves with their dams within 21 days after birth is not recommended for high-yielding dairy cows.

Milk components

Fence-line weaning had a significantly (P < 0.01) positive effect on milk components (fat, protein and lactose), as observed by Bampidis *et al*. (2012) on Greek buffaloes. Similarly, Kisac *et al*. (2011) observed that the weaning of calves from mother at different ages significantly affected (P < 0.05) milk composition (fat, protein, lactose and total solids). Gajbhiye *et al*. (2019) found that stage of lactation and parity number had a significant effect on Gir cow's milk fat (P < 0.05). Third parity cows of Sheko cattle in Ethiopia had the higher (P < 0.01) milk components than the cows of other parities (Bayou *et al*., 2015).

Growth performance of calves

The superiority of fence-line weaned calves in body weight could be due to decreasing of stress factors, which reflected in a better health and increase in the solid feed consumption compared to the traditionally weaned calves. These results agree with those reported by Kisac *et al*. (2011), who concluded that the calves reared with their mothers for a longer time reached higher live weight at weaning and at the age of 90 days. It is so, because native milk suits the animals and calves receive it according to their needs. Brown (2013) stated that fence-line weaning can decrease the stress and increase the performance in growing calves. Baily *et al*. (2016) observed that growing calf performance during the feedlot receiving period was improved by pasture weaning in combination with fence-line contact with dams compared to a dry lot weaning plus dam separation. Kumar *et al*. (2017) observed that average birth weight of Murrah Buffalo did not differ significantly (P > 0.05) for weaned and suckled calf groups. However, average body weight at 90 days of age was significantly (P < 0.05) higher in the suckled group (122.77 kg) compared to those in the weaned group (113.12 kg) calves. Also, Yilmaz *et al*. (2013) found that male calves had heavier body weight at birth than the average of female ones (40.541 vs. 38.375 kg). Parity number of dam had a significant (P < 0.01) effect on calves' body weight at birth, weaning and sixth month of age (Bayou *et al*., 2015). Fence-line weaning enhanced average daily weight gain in calves. The present results agree with the finding of Price *et al*. (2003), who observed an increase in average daily gain of Angus cattle calves, which had been weaned by fence-line system, compared to calves being completely separated from their dams. Kisac *et al*. (2011) reported that average daily gain is affected (P < 0.01) by weaning the calves from mother at different ages (7, 14 and 21 days); the third group had the optimum average daily gain from birth to 90 days of age (550, 660 and 740 grams, respectively). Brown (2013) concluded that fence-line weaning system can cause an increase in calf performance compared to other traditional weaning systems. Bailey *et al*. (2016) have found that calves with fence-line weaning had the highest average daily gain than those with a dry lot weaning.

Immune response of buffaloes and their calves

The superiority of fence-line weaned dams and calves in immune response could be due to decreasing of stress factors which, which was reflected in a better health and increasing performance of its calves compared to the traditionally weaned calves (Brown, 2013). Also, Coleman *et al*. (2015) concluded that immunoglobulin (IgG) can be used as a predictor for early growth and high milk production in dairy cattle.

CONCLUSION

Fence-line weaning reduces the stress associated with weaning process for both buffaloes and its weaned calves, which is resulted in the improving milk production and calf growth performance as well as in the enhancing immune response. Thus, we recommend that buffalo producers should wean their calves by the fence-line system to gain more milk production, calf growth performance and immune response.

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