



## Phenotypic drift in osmotic fragility of Sahel goat erythrocytes associated with variability of median fragility

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### Abstract

A typical mammalian erythrocyte fragility phenotype (EFP) exhibits a sigmoidal curve of the dependence of fragilities (% haemolysis) on hypotonic saline concentrations, but the goat EFP tends to be hyperbolic. Physiological variation in median erythrocyte fragility (MEF) and the associated EFP of Sahel goats was investigated. Erythrocyte osmotic fragility (EOF) was determined in hypotonic saline using heparinized venous blood from 47 goats (23 males and 24 non-pregnant dry females) aged 1-4 years and weighing  $18.87 \pm 6.32$  (9-30) kg. Packed cell volume (PCV), erythrocyte count and mean corpuscular volume (MCV) were also estimated. Low, medium and high EFP were based on MEF of < 7, 7-8 and > 8 g/L, respectively. MEF of all the goats was  $7.5 \pm 0.6$  (5.9-8.4) g/L. Phenotypic drift from sigmoidal to hyperbolic EOF curve was observed at the lower and upper limits of the phenotypic variation. Frequencies of occurrence of low, medium and high EFP were not different ( $p > 0.05$ ) between males and females. Fragiligrams of low, medium and high EFP separated at erythrocyte fragilities of 10-70% and saline tonicity of 7-8 g/L. The saline concentrations causing fragilities of 10-70% differed ( $p < 0.05$ ) among the phenotypes. There was no correlation ( $r = -0.28, -0.30; p > 0.05$ ) between MEF and MCV or PCV, and between PCV and MCV. In conclusion, phenotypic drift in EOF occurred in Sahel goats without influence by erythrocyte parameters and represented the physiological variability of EOF endpoint estimates that would serve as reference limits in evaluation of erythrocyte membrane defects.

**Keywords:** Erythrocyte size, Median fragility, Osmotic fragility, Phenotypic drift, Sahel goat

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### Introduction

Phenotypic variations in erythrocyte membrane stability have been attributed to differences in erythrocyte size, shape, membrane composition and function, and cytosolic content and metabolism (Harvey, 2010; Olver *et al.*, 2010) and they arise from phylogenetic influences on genetic resources that support adaptations to environmental pressures (Lindenfors *et al.*, 2010). In the arid and semi-arid environments, goats and camels, particularly, have physiological adaptations to cope with low water availability and to adjust to changes in plasma osmolarity during water deprivation and subsequent drinking (Igbokwe, 1997). Goats have the highest erythrocyte osmotic fragility (EOF) among several species of mammals and much higher EOF than camels which have better arid adaptation (Perk *et al.*, 1964; Oyewale *et al.*, 2011). Despite the wide

variation in EOF between goats and camels, both species have similar erythrocyte sizes (Tornquist, 2010). Where interspecies differences in EOF occurred, increased EOF correlated with decreasing erythrocyte size (Schalm *et al.*, 1975; Olusanya & Adepoju, 1979). However, in artiodactylid mammals, erythrocyte size was inversely related to osmotic resistance (Peindo *et al.*, 1992).

EOF is usually depicted graphically by a sigmoidal curve (fragiligram) derived from the dependence of erythrocyte fragilities (% haemolysis) on serial hypotonic buffered saline concentrations. Median erythrocyte (corpuscular or cell) fragility (MEF) is the hypotonic saline concentration that causes 50% haemolysis. The fitting of the sigmoidal curve improves with increasing erythrocyte stability and a shift of MEF to lower saline concentrations.

Decreased stability (increase in EOF) shifts MEF to higher saline concentration leading to a drift of the fragiligram from sigmoidal to hyperbolic curve (Igbokwe & Igbokwe, 2015). Normal values of MEF in goats are not available apart from those estimated from fragiligrams in reports of some controlled experiments with disparities in data presentations (Oyewale, 1991; Oyewale, 1993; Oyewale *et al.*, 1997; Minka & Ayo, 2010; Habibu *et al.*, 2014; Igbokwe & Igbokwe, 2015). Phenotypic variations in EOF may exist in Sahel goat populations under physiological conditions as was previously reported with respect to erythrocyte glutathione concentrations (Igbokwe *et al.*, 1998). Evaluation of the association of EOF with variations in the values of erythrocyte size and packed cell volume (PCV) in goats is yet to be evaluated to ascertain the correlation of these physiological parameters as a means of understanding their dynamic inter-relationships. In this study, phenotypic drift of EOF curves based on the variability of MEF in Sahel goats, under semi-intensive management, was investigated and the correlative strength of erythrocyte size and the associated PCV as modifiers of EOF phenotype was evaluated in order to establish appropriate baseline reference data for use in understanding erythrocyte membrane defects under clinical and experimental conditions (Igbokwe & Igbokwe, 2015).

## Materials and methods

### Animals

Animals for the study were selected from a herd of long-legged Sahel goats reared semi-intensively in the University of Maiduguri farm in north eastern Nigeria. They were tagged and housed in groups in roofed half-walled pens within a fenced farm area. They were offered water and salt lick, fed with cereal offal, grass and legume hays within the pens, and allowed to graze and browse for up to 6 h daily in the surrounding sahelian bushes outside the fence perimeter. They were in good body condition and received regular veterinary attention with regard to deworming, screening and treatment of health challenges. The goats selected for the study included 23 males and 24 non-pregnant dry females with estimated ages of 1-4 years based on dentition and body weights of  $18.87 \pm 6.32$  (9-30) kg.

### Blood sample collection

During the late dry and hot season in April-May, blood sample was collected at 7-8 am in the morning from each animal before leaving the pen. The sample (5 mL) collected from the external jugular vein using syringe and needle was placed into plastic tubes (Silver Health Diagnostics,

Nigeria) containing lithium heparin as anticoagulant. The samples were transported to the laboratory in ice pack and kept within the ice pack without contact with ice until they were analysed within 1-3 hours.

### Determination of erythrocyte parameters

Packed cell volume (PCV) and erythrocyte count (EC) were determined using microhaematocrit method and haemocytometry, respectively; while mean corpuscular volume (MCV) was calculated with PCV and EC values using a standard formula (Schalm *et al.*, 1975).

### Determination of erythrocyte osmotic fragility (EOF)

EOF was determined in a series of hypotonic buffered saline based on the original method of Parpart *et al.* (1947). A stock solution of buffered saline was prepared as follows: 90.0 g sodium chloride (NaCl), 13.65 g disodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4$ ) (BDH, England), 2.34 g sodium dihydrogen phosphate ( $\text{NaH}_2\text{PO}_4$ ) (BDH, England), all made up to 1 L with deionized distilled water, giving a stock solution of 10% NaCl (Ochei & Kolhatkar, 2007). The working solution of 1% NaCl was prepared by dilution of the stock solution from which other lower concentrations of saline were prepared as earlier described (Igbokwe & Igbokwe, 2015).

Each saline solution at various dilutions (5 mL) or deionised distilled water (5 mL) in a tube had an aliquot of a blood sample (5  $\mu\text{L}$ ) added to it, mixed by inversion and allowed to stand for 30 min under room temperature (35-38 °C). After centrifugation of the tubes at  $3000 \times g$  for 15 min, the supernatant of the Haemolysate in each tube was harvested with suction pipette into a curvette and the haemoglobin colour was estimated as absorbance units with a spectrophotometer (ALL PRO, Shibe, Qingdao, China) set at 540 nm, with the supernatants of the tubes containing isotonic solution and deionized distilled water serving as blank (0%) and complete (100%) haemolysis, respectively. The haemolysis (%) at each saline dilution was calculated as percentage of the absorbance at the saline dilution relative to absorbance at complete haemolysis.

### Statistical analysis

A coordinate graphing of the dependence of haemolysis on saline concentrations for each blood sample was plotted to obtain the EOF curve (fragiligram). The mean fragilities (% haemolysis) with standard deviations were calculated for each set of replicates at saline concentrations of 2-8 g/L to obtain the haemolytic effects of the various hypotonic solutions. The saline concentration

(tonicity) at intervals of 10% haemolysis on the graph were read for each blood sample and mean saline concentrations with standard deviations were calculated for the replicates at fragilities of 10-90%. Based on MEF of < 7, 7-8 and > 8 g/L, the EOF of the goats were sorted into low, medium and high erythrocyte fragility phenotypes (EFP). The frequencies of occurrence of EFP in male and female goats were obtained and differences in occurrences compared. All data were summarised as means  $\pm$  standard deviations; means were compared by one-way ANOVA with Tukey post-hoc test; number counts of occurrence were compared by Fisher exact or chi-squared test; and correlation coefficients in linear relationships were obtained using computer software (GraphPad Instat, version 3.05 1992-2000, GraphPad Software Incorporated, USA).

### Results

The PCV, EC, MCV and MEF of the goats are summarized in Fig 1, 2, 3 and 4, respectively. MEF of all the goats (male and female) was  $7.5 \pm 0.6$  (5.9-8.4) g/L. There was no significant ( $p > 0.05$ ) sexual difference in parameters. The fragiligram of

the goats with the minimum and maximum MEF showed a phenotypic drift from sigmoidal to hyperbolic curve at the lower and upper limits of the phenotypic variation (Fig 5). The MEF of the goats had a normal distribution and the frequencies of occurrence of low, medium and high EOF phenotypes were not significantly ( $p > 0.05$ ) different between males and females (Table 1). The medium EFP occurred more frequently ( $p < 0.05$ ) than low and high EFP. The fragiligrams of low, medium and high EFP separated at erythrocyte fragilities of 10-70% and saline tonicity of 6-8 g/L (Fig 6). There were significant ( $p < 0.05$ ) variations in erythrocyte fragilities among the phenotypes with mean fragilities of  $25.9 \pm 27.6\%$ ,  $71.2 \pm 16.6\%$  and  $79.6 \pm 9.5\%$  at saline tonicity of 7 g/L, and  $13.3 \pm 18.1\%$ ,  $22.4 \pm 15.6\%$  and  $64.8 \pm 12.0\%$  at saline tonicity of 8 g/L for goats with low, medium and high EFP, respectively. The saline tonicity causing erythrocyte fragilities of 10-70% were higher in high than medium and low EFP; and higher in medium than low EFP (Table 2). There was no significant ( $p > 0.05$ ) correlation between MEF and MCV or PCV (Fig 7), and between PCV and MCV (Fig 8).

**Table 1:** Occurrence of erythrocyte fragility phenotypes (EFP) among Sahel goats based on median erythrocyte fragilities (MEF)

	Number (%)		
	Low EFP (MEF < 7 g/L)	Medium EFP (MEF 7-8 g/L)	High EFP (MEF > 8 g/L)
Female (n = 24)	4 (16.7) <sup>a</sup>	17 (70.8) <sup>a</sup>	3 (12.5) <sup>a</sup>
Male (n = 23)	2 (8.7) <sup>a</sup>	15 (65.2) <sup>a</sup>	6 (26.1) <sup>a</sup>
Total (n = 47)	6 (12.8)	32 (68.1)*	9 (19.2)

<sup>a</sup> No significant ( $p > 0.05$ ) difference between male and female frequencies

\*Significant ( $p < 0.05$ ) difference from low and high EFP

**Table 2:** Concentrations of saline at various endpoints of haemolysis of erythrocytes from Sahel goats with low, medium and high osmotic fragility phenotypes sorted by median erythrocyte fragility (MEF)

Haemolysis (%)	Concentration of buffered saline (g/L)		
	Low ( MEF < 7 g/L) N = 6	Medium ( MEF 7-8 g/L) N = 32	High (MEF > 8 g/L) N = 9
90	$4.78 \pm 1.25^a$	$4.63 \pm 1.39^a$	$4.16 \pm 1.96^a$
80	$5.67 \pm 0.43^a$	$6.31 \pm 0.78^a$	$6.22 \pm 1.82^a$
70	$5.98 \pm 0.38^a$	$6.84 \pm 0.48^{a,b}$	$7.10 \pm 1.62^b$
60	$6.22 \pm 0.34^a$	$7.19 \pm 0.36^b$	$7.90 \pm 0.45^c$
50	$6.43 \pm 0.29^a$	$7.45 \pm 0.25^b$	$8.26 \pm 0.14^c$
40	$6.72 \pm 0.38^a$	$7.72 \pm 0.27^b$	$8.43 \pm 0.10^c$
30	$6.95 \pm 0.58^a$	$7.94 \pm 0.29^b$	$8.57 \pm 0.07^c$
20	$7.18 \pm 0.67^a$	$8.16 \pm 0.33^b$	$8.67 \pm 0.07^c$
10	$7.14 \pm 0.68^a$	$8.43 \pm 0.34^b$	$8.86 \pm 0.05^c$

<sup>a,b,c</sup> Unmatched superscripts indicate significant ( $p < 0.05$ ) differences between groups on the rows

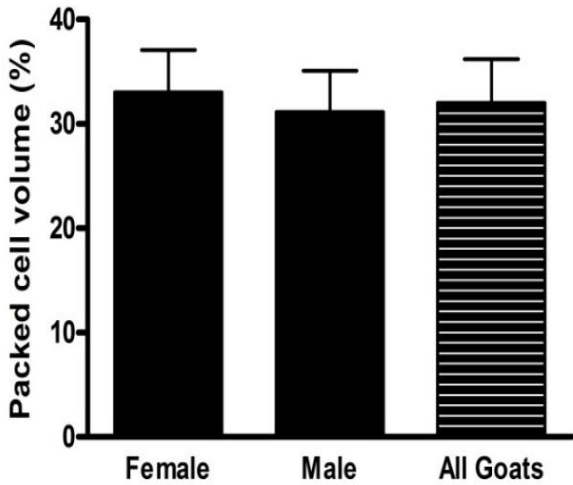


Figure 1: Packed cell volume of Sahel goats

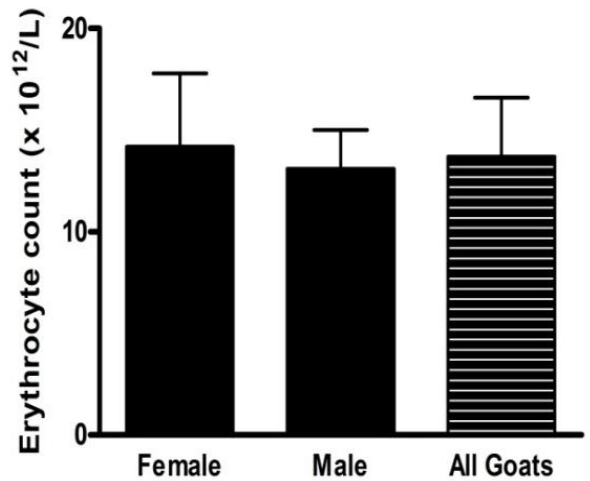


Figure 2: Erythrocyte count of Sahel goats

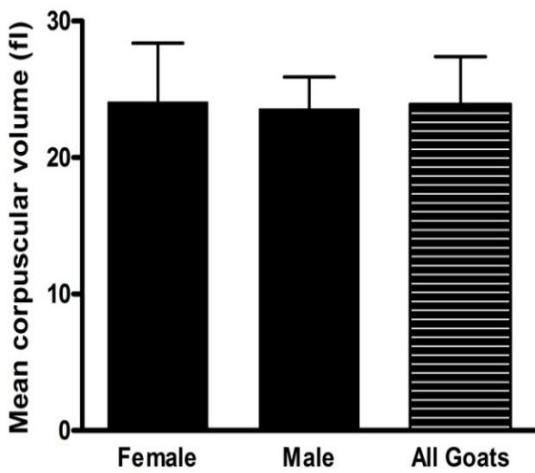


Figure 3: Mean corpuscular volume of Sahel goats

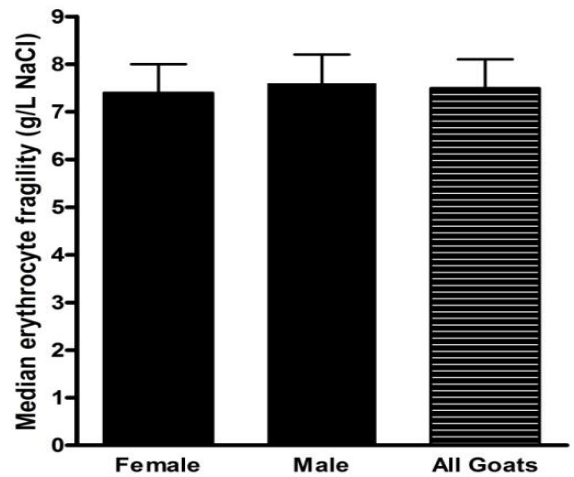


Figure 4: Median erythrocyte fragility of Sahel goats

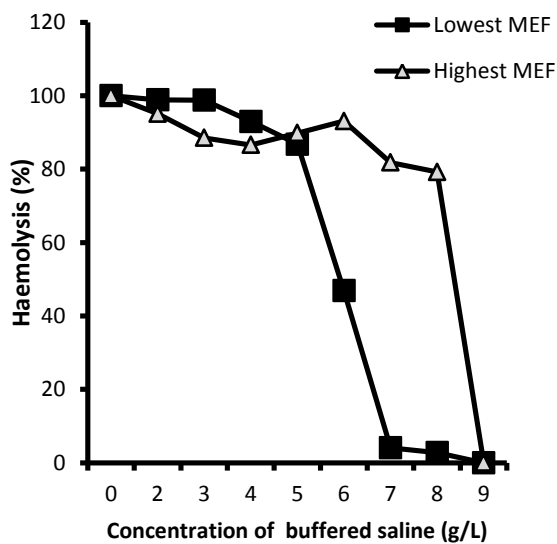


Figure 5: Fragiligrams of Sahel goats showing a phenotypic drift from a typical sigmoidal curve to an atypical hyperbolic curve at the lower and upper limits of phenotypic variation of osmotic fragility based on minimum and maximum limits of median erythrocyte fragilities at saline concentrations of 5.9 and 8.4 g/L

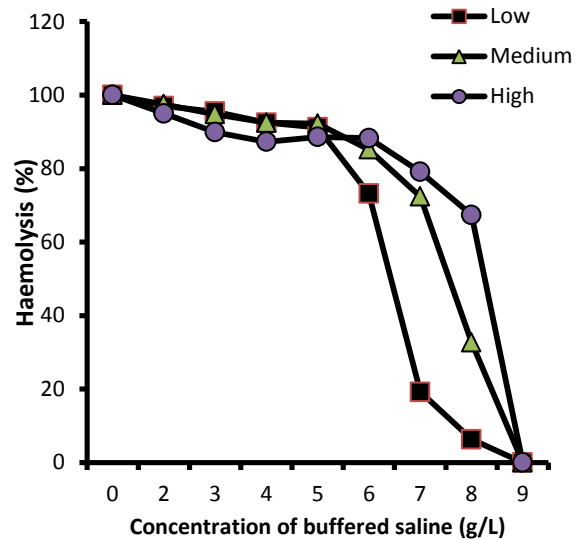
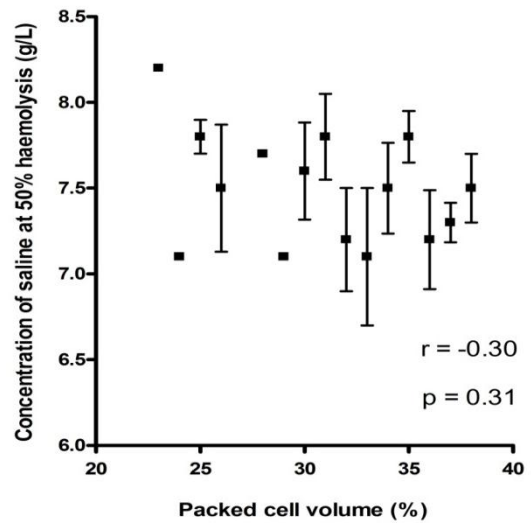
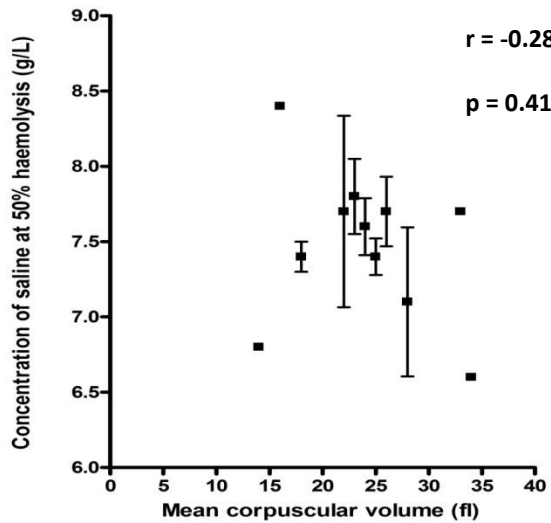


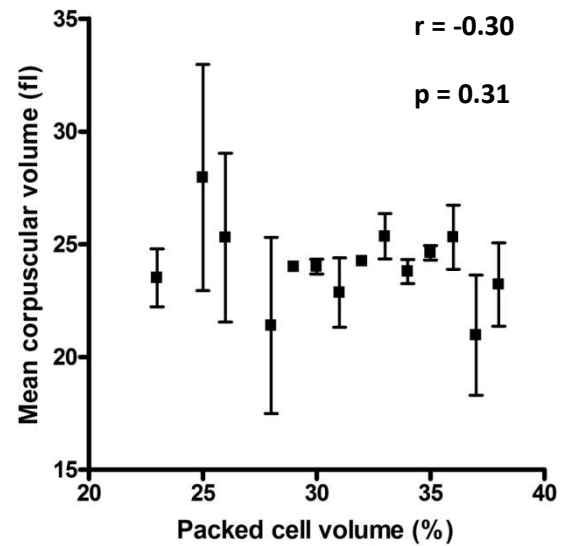
Figure 6: Fragiligrams of the low, medium and high erythrocyte fragility phenotypes in Sahel goats having median erythrocyte fragilities (MEF) in saline concentrations of <7.0 (low), 7.0-8.0 (medium) and >8.0 (high) g/L, respectively



**Figure 7:** Dependence of saline concentrations at median erythrocyte fragilities on mean corpuscular volume and packed cell volume

**Discussion**

The distribution of MEF (5.9-8.4 g/L) in the goat population was normal with most of them (68.1%) having medium MFP. The MEF values may be validated for use as reference interval for interpreting EOF changes in diseases of goats affecting erythrocyte membrane stability. Reports of MEF values seemed to be about 7-8 g/L in West African dwarf (Oyewale, 1991; Oyewale, 1993; Oyewale *et al.*, 1997) and Sahel goats (Igbokwe & Igbokwe, 2015), 8 g/L in Red Sokoto (RS) goats (Habibu *et al.*, 2014), and 6-8 g/L in RS goats during rest and after loading and transportation stress (Minka & Ayo, 2010). Some of these MEF values were read from published fragiligrams where there was no haemolysis in isotonic saline (Oyewale, 1991; Oyewale, 1993; Oyewale *et al.*, 1997; Igbokwe & Igbokwe, 2015) in contrast to where there was isotonic haemolysis of about 20% (Habibu *et al.*, 2014) or 20-40% (Minka & Ayo, 2010). An isotonic haemolysis may be an artefact arising from using blood samples with extracellular haemoglobin due to *in vitro* or *in vivo* haemolysis as shown in the increase of the mean corpuscular haemoglobin concentration (MCHC) to  $63.7 \pm 2.8$  g/dL (Minka & Ayo, 2010) from a lower reference mean (32.3-39.6 g/dL) for goats (Byers & Kramer, 2010). Another source of MEF variation related to methodology for EOF could be the environmental temperature during incubation. Incubation temperatures of 29 and 45 °C produced comparable EOF, but colder temperature of 10 °C increased EOF (Oyewale, 1991) as a result of swelling of erythrocytes in cold temperature (Richieri & Mel, 1985). EOF increased with incubation temperature (Aloni *et al.*, 1977), but remained stable without any variation at incubation temperatures of 37-48 °C (Van der Walt



**Figure 8:** Relationship of the mean corpuscular volume with the packed cell volume of Sahel goats

& Russell, 1978). Therefore, the fluctuation of incubation temperatures within 35-40 °C would not affect EOF endpoint estimates.

The phenotypic drift of EOF from sigmoidal to hyperbolic curve seemed to be a physiological phenomenon. Previous reports of fragiligrams of EOF in goats were hyperbolic (Oyewale, 1991; Minka & Ayo, 2010; Habibu *et al.*, 2014; Igbokwe & Igbokwe, 2015). In the present study, there were small populations of goats with increased osmotic resistance (12.8%) having fitted sigmoidal fragiligrams at MEF of < 7 g/L or increased osmotic susceptibility (19.2%) having an increasing fitting of hyperbolic fragiligram at > 8 g/L. MEF variability was not affected by sex, although sex was reported to have the potential to affect EOF (Habibu *et al.*, 2014). Furthermore, MCV and PCV

did not correlate with MEF, showing that the erythrocyte parameters were not relevant as physiological factors affecting MEF. MCV correlated with  $\text{Na}^+\text{K}^+\text{ATPase}$  activity in mammalian erythrocytes (Katyukhin *et al.*, 1998) and erythrocytes from sheep with extra  $\alpha$ -gene were larger (with increased MCV) and had increased EOF (Pieragostini *et al.*, 2003). Erythrocyte volume was altered in mice lacking K-Cl cotransporters and osmotic sensitivity was increased (Rust *et al.*, 2007). Reticulocytes and young erythrocytes have higher MCV than mature erythrocytes and have higher activity of K-Cl cotransporters that mediate volume reduction and affect osmotic resistance (Quarmyne *et al.*, 2011). The present study indicated that changes in MCV did not correlate with PCV implying that no abnormal change in erythrocyte mass occurred to affect erythropoiesis that could have possibly influenced MCV.

The phenotypic variations and drift in osmotic fragility of Sahel goat erythrocytes may be explained by variations in genetic expressions within the population. Genetic factors have been reported to produce clusters of strains within species with differing EOF (Dewey *et al.*, 1982; Schaefer & Dewey, 1989; Armsby *et al.*, 1996; Pieragostini *et al.*, 2003). Decreased EOF in a strain of mice, compared to another strain, was directly controlled by their genotype (Dewey *et al.*, 1982) and the osmotic resistance allele was autosomal and recessive to the susceptible one (Schaefer & Dewey, 1989). Gene effect on EOF seemed to influence erythrocyte membrane ion transport in mice (Norman & Dewey, 1985) and correlated with milk fat traits in dairy cattle (Krogmeier *et al.*, 1993). Although the variation of erythrocyte glutathione concentrations in Sahel goats was considered to be influenced by genetic factors, no further studies had been available, so far, to suggest genetic influence on erythrocyte parameters in Sahel goats.

Adaptation to environmental or nutritional stress might have played some role in the phenotypic drift of EOF, but the extent of its involvement could not be directly ascertained. Stress leads to production of cortisol and related substances by the adrenal gland and the interaction of stress hormone with erythrocytes in circulation may

change their osmotic stability. Osmotic resistance of erythrocytes of rats was decreased after repeated injection of hydrocortisone (Nezhentsev, 1981). Similarly, EOF of goats was increased after transport stress, but supplemental administration of ascorbic acid to the stressed goats reduced the effect on EOF (Minka & Ayo, 2010). Endogenous ascorbic acid synthesis occurs in the liver microsomes of goats (Chatterjee *et al.*, 1960) and adaptation to stress in goats would seem to depend on their capacity to upregulate ascorbic acid biosynthesis to counter stressful situations, thereby mediating the fluctuations in the osmotic stability of erythrocytes. It would seem that stress-adapted goats have decreased EOF.

The present study was conducted in the late dry season when browsed feedstuff usually lost some quality so that marginal protein-energy under-nutrition and imbalance in supply of plant lipids were probable despite the availability of feed supplements. The lipid and protein contents of erythrocyte membrane can be modified by diet; and browsing capabilities of animals will vary even in a semi-intensive management regime. In protein-energy malnutrition, erythrocytes were reported to show increased resistance to osmotic lysis as a result of decreased erythrocyte cholesterol-phospholipid molar ratio and increased membrane contents of cholesterol and phospholipids (Ramanadham & Kaplay, 1982; Kaplay, 1984). Physiologic availability of some vitamins (Kual *et al.*, 1995; Adenkola *et al.*, 2010; Minka & Ayo, 2010; Wahab *et al.*, 2010; Azeez *et al.*, 2011; Marar 2011), minerals (O'Dell *et al.*, 1987; Johannig & O'Dell, 1989; Tongyai *et al.*, 1989; Kraus *et al.*, 1997) and lipids (Hagve *et al.*, 1991; Vajreswari and Narayanareddy 1992) have also been reported to stabilize erythrocyte membranes and it is uncertain whether nutritional factors within the flock contributed to the variability of MEF.

In conclusion, the report has presented the baseline MEF in Sahel goats (during the late dry season) relevant as reference interval for conditions affecting osmotic membrane stability; and has identified the phenotypic drift in EOF occurring as a physiological variation without influence by erythrocyte size and PCV.

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