Erythrocyte Morphology and Haemoglobin Types of Neonatal Roan Antelopes (*Hippotragus equinus*) with Hypochromic Poikilocytic Anaemia

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Summary

Neonatal, poikilocytic anaemia in some members of the Hippotragini has previously been documented but not fully investigated. This study was undertaken to describe the erythrocyte morphology of roan antelopes (*Hippotragus equinus*) during the first 4 weeks after birth and to identify aspects of haemoglobin (Hb) production that might be implicated in this syndrome. Twenty-nine roan antelope calves were sampled on, or close to, 1, 7, 14 and 28 days after birth. Erythrocyte morphology was characterized, and microhaematocrit values and Hb parameters determined, for each sampling occasion. Findings indicated a significant change in erythrocyte morphology during the neonatal period and two haemoglobin types, fetal and adult, were identified. The perinatal onset of adult Hb synthesis was delayed relative to the termination of fetal Hb production, resulting in the observed anaemia. Haemoglobin concentration and erythrocyte morphology were significantly correlated. These findings suggest an intimate relationship between Hb synthesis and the observed poikilocytosis. An imbalance in the synthesis of the α- and ß-globin chains of Hb (a thalassaemia) may prove to be the underlying pathophysiology of this syndrome.

Article Outline

1. Introduction

Neonatal anaemia, often in association with poikilocytosis, has been described in numerous ruminant species and is especially pronounced amongst members of the Hippotragini (Bush *et al*., 1976; Hawkey and Hart, 1984; Hawkey and Dennett, 1989). Goats (*Capra hircus*) also display a neonatal poikilocytic anaemia (Holman and Drew, 1964). In that species, poikilocytosis occurring in adult-onset anaemia has been associated with the production of haemoglobin C (HbC) (Jain *et al*., 1980), which is also produced during the neonatal period (Huisman *et al*., 1969). Steyl *et al*. (2003) reported on this syndrome in the roan antelope (*Hippotragus equinus*), speculating on the relationship between anaemia and the high mortality from theileriosis in neonatal animals of this valuable species.

In this study, the hypochromic, poikilocytic anaemia of neonatal roan antelopes was investigated. Poikilocytosis is associated with erythrocyte membrane abnormalities, while hypochromasia is an indication of impaired haemoglobin synthesis (Bell, 1998). The aims of the study were (1) to characterize, by electrophoresis and chromatography, the occurrence of haemoglobin variants during the neonatal period, (2) to describe the changes in morphology of erythrocytes during this period, and (3) to seek a possible association between haemoglobin synthesis and the observed poikilocytosis.

2. Materials and Methods

2.1. Animals

Animals used in this study comprised the first 29 calves born to a privately owned captive population of West African roan antelope on Mauricedale Game Ranch, near Malelane, Mpumalanga Province, South Africa (25°30′00″S 31°30′00″E). Calves were hand-reared from the age of 1 day and fed a cow milk formula at approximately 10% body weight per day. During this investigation, a rotavirus outbreak in the calves resulted in treatment of those showing haemorrhagic diarrhoea with a potentiated sulphonamide antibiotic (Trivetrin; Schering-Plough Animal Health, Johannesburg, South Africa) for 3–9 days (15 mg/kg daily). These animals displayed significant increases in microhaematocrit (Hct) and haemoglobin (Hb) concentration during the treatment periods, suggesting that parameters measured in this study were not significantly affected by this episode. When possible, blood (4 ml) was collected from each animal, on *ca* 1, 7, 14 and 28 days after birth, and transferred to a tube containing potassium ethylenediamine-tetraacetate (EDTA) (Vacutainer; Becton Dickinson Vacutainer Systems, Preanalytical Solutions, Plymouth, England).

2.2. Haemoglobin Analysis

Haemolysates were prepared within 36 h of sampling (Beckman Paragon Hemolyzing Reagent; Beckman Coulter, Inc., Fullerton, CA, USA) and stored at −20 °C. Samples were analysed for Hb variants with a commercial Cation Exchange High Performance Liquid Chromatography (CE-HPLC) system (VARIANT™ Hemoglobin Testing System and VARIANT™ Hemoglobinopathy Program; Bio-Rad Laboratories, Hercules, CA, USA). This system was capable of separating fetal, neonatal and adult haemoglobins of goats (unpublished data). Analysis was performed according to the manufacturer's instructions except for initial sample preparation. As a result of the low Hb concentrations of the samples tested, for each sample 1.0 ml of haemolysis reagent supplied with the system was added to 25 µl of thawed haemolysate rather than to 20 µl (as used for human neonatal samples).

Electrophoresis of haemolysates was performed with a commercial electrophoresis system (Sebia MG 300 electrophoresis system and Sebia Hydragel Hemoglobin K (20) agarose gel kits; Sebia, Issy-les-Moulineaux, France). Densitometry of stained electrophoregrams was performed with a yellow filter (Sebia DVSE densitometer; Sebia).

2.3. Haematology

Microhaematocrit was determined manually following microcentrifugation (Hema-C; Jouan, Winchester, VA, USA). Haemoglobin concentrations were determined by automated spectrophotometry (Cell-dyne 3500; Abbott Laboratories, Abbott Park, IL, USA). Absolute concentrations of Hb fractions were calculated by multiplying relative values as determined by chromatography by total Hb concentration. For each sampling occasion, mean cell Hb concentrations were determined by dividing Hb concentration by the microhaematocrit value.

2.4. Erythrocyte Morphology

Immediately after sampling, 3 blood smears were made by hand and stained as follows: one with Romanowsky stain (Rapidiff; Clinical Sciences Diagnostics, Johannesburg, South Africa), one with New Methylene Blue stain (Reticulocyte Stain; Clinical Sciences Diagnostics), and one with Prussian Blue stain as described by Swirsky and Bain (2001). From each smear, digital photomicrographs were taken (Olympus Camedia C-3040 ZOOM; Olympus Corporation, Tokyo, Japan) of 10 fields from the front part of the smear (excluding the feathered edge), in which cells had maintained a biconcave morphology and as few cells as possible were touching one another. Five digital photomicrographs from each smear were examined. Erythrocytes were categorized according to morphology (Romanowsky-stained smears) and inclusions present (Table 1) and the number of each cell type was recorded. Cells not completely included by the borders of the image, those deformed by neighbouring cells or smear artefacts and those categorized as echinocytes were excluded from the study. Mean cell surface area (MCA) was quantified with ImageJ, an image processing and analysis programme in the public domain, obtained from the National Institutes of Health, USA. Anisocytosis, a measure of

the variation in erythrocyte surface area, was quantified as the coefficient of variation of the MCA.

Table 1.

Terms describing erythrocyte types based on morphology and inclusions present (compiled from Bessis, 1977 and Jain, 1986)

Cell type	**Morphological description**
Acanthocyte	A spheroidal cell with 3–12 often club-like spicules of uneven length, irregularly distributed over the cell surface
Elliptocyte	An oval-shaped cell ranging from that slightly more oval than the discocyte to almost rod-shaped
Keratocyte	A cell displaying one or more notches. Horn-like projections extend from one or more of the borders of the notch
Schizocyte	A fragment of a cell following damage to it. These fragments may resemble portions of the original cell or occur as bizarre forms
Dacryocyte	A tear-shaped cell with an extended tail
Echinocyte	A cell with 10–30 spicules distributed regularly over the surface
Spherocyte	A densely staining spherical, globular or acanthocytic cell with no central pallor
Siderocyte	An erythocyte containing increased numbers of ferritin granules which stain blue with Prussian Blue stain
Reticulocyte	A cell displaying two or more well-defined blue-staining aggregates of irregular shape and distribution when supra-vitally stained with New Methylene Blue stain
Howell-Jolly body cell	An erythocyte containing a Howell–Jolly body: a small, dark, round inclusion, often occurring eccentrically

2.5. Statistical Analysis

This was performed with STATA, Release 8 (StataCorp LP, College Station, TX, USA). To describe changes in erythrocyte and Hb parameters within individuals over time, cross-sectional time-series regression models were employed to regress each erythrocyte and Hb parameter on calf age. To investigate relationships between Hb synthesis and

erythrocyte morphology, each erythrocyte parameter was regressed on each Hb parameter as above.

3. Results

3.1. Haemoglobin Analysis by HPLC

Chromatograms derived from the CE-HPLC of neonatal roan antelope haemoglobins revealed five distinct peaks (Fig. 1). The second peak occasionally separated into two peaks of equal area (Fig. 1a, c); however, this division was interpreted as spurious. The first three peaks occurred consistently in ratios of approximately 1:4:5 and decreased in relative size with increasing age of the calves. These were identified as fetal haemoglobin variants. The fourth and fifth peaks occurred consistently in a ratio of approximately 1:3.5, increased in relative size with calf age, and were identified as adult haemoglobin variants.

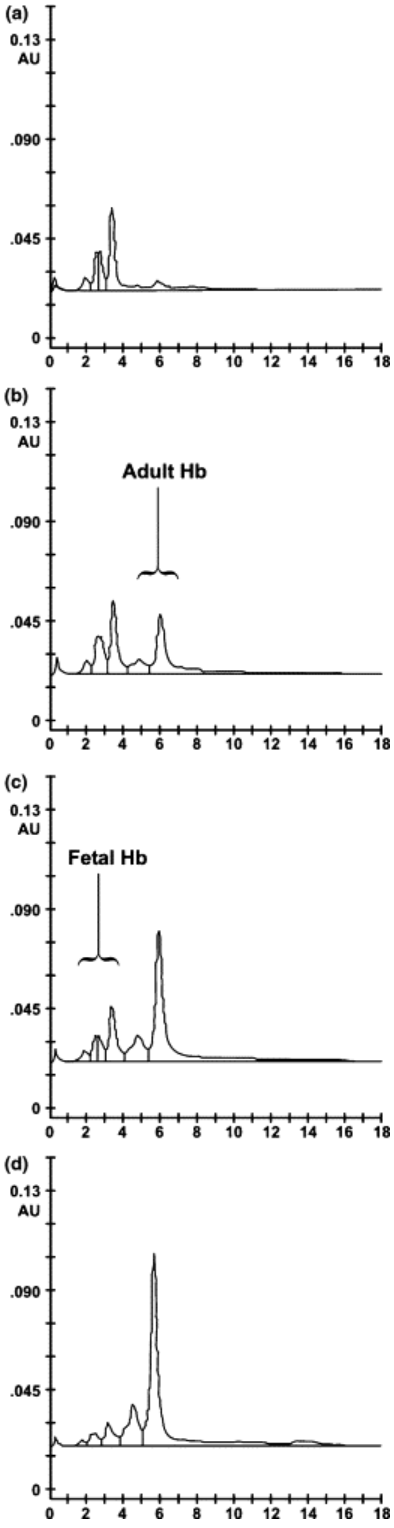

Fig. 1. Bio-Rad VARIANT™ printouts of chromatograms of roan antelope haemoglobins (calf 308) at (a) 1, (b) 8, (c) 15 and (d) 29 days after birth (*x*-axis, time (minutes); *y*-axis, absorbance (arbitrary units)).

3.2. Haemoglobin Analysis by Electrophoresis

Electrophoregrams of neonatal roan antelope haemoglobins revealed two distinct bands (Fig. 2). The faster band decreased in relative size with increasing age of the calves and was identified as fetal haemoglobin. The slower band increased in relative size with increasing calf age and was identified as adult haemoglobin.

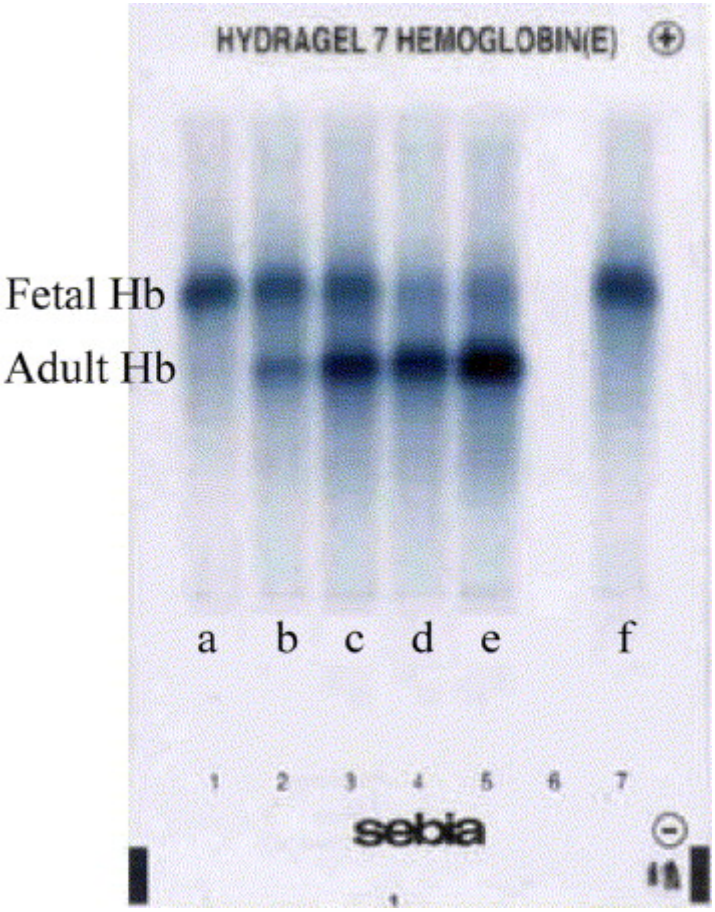

Fig. 2. Electrophoregrams of roan antelope haemoglobins (calf 308) at (a) 1, (b) 8, (c) 15, (d) 21, and (e) 29 days after birth; and (f) calf 325 haemoglobins at 1 day after birth.

3.3. Microhaematocrit and Haemoglobin Parameters

During the first 4 weeks after birth, roan antelope calves showed a significant increase in haematocrit, total Hb concentration, mean cell Hb concentration, adult Hb concentration and mean cell adult Hb concentration. Concurrently, a significant decrease in fetal Hb concentration and mean cell fetal Hb concentration occurred (Table 2, Fig. 3).

Table 2.

Changes in microhaematocrit and haemoglobin parameters of roan antelope calves during the first 4 weeks after birth

Parameter	**Results (mean±SD)**				**Regression on calf age**		
	Day 1	**Day 7**	**Day 14**	**Day 28**	**Summary**	***P***	r^2
	(*n*=29)	(*n*=23)	(*n*=18)	(*n*=15)			
Microhae-matocrit (%)	21.0±3.2	26.6±3.1	29.3±3.5	33.0±3.1	*y*=20.0+0.44(d)	*	0.6
Total Hb (g/dl)	5.9±1.0	7.4±1.1	8.3±1.2	10.2±1.3	*y*=5.9+0.16(d)	*	0.65
Mean cell Hb (g/dl)	27.9±2.5	27.7±1.5	28.6±1.8	30.9±1.5	*y*=27.4+0.11(d)	*	0.24
	(*n*=17)	(*n*=22)	(*n*=13)	(*n*=14)			
Total HbF (g/dl)	4.8±0.9	4.3±1.2	3.4±0.9	1.8±0.5	*y*=4.8−0.11(d)	*	0.59
Mean cell HbF (g/dl)	22.1±2.2	15.9±2.5	11.3±3.2	5.6±1.9	*y*=20.8−0.57(d)	*	0.78
Total HbA (g/dl)	1.2±0.4	3.1±0.8	5.2±1.4	8.5±1.8	*y*=1.2+0.26(d)	*	0.84
Mean cell HbA (g/dl)	5.5±1.6	11.8±2.8	17.4±3.9	25.4±3.3	*y*=6.3+0.7(d)	*	0.84

Hb, Hb concentration; HbA, adult Hb concentration; HbF, fetal Hb concentration; d, age in days; *=*P*<0.01.

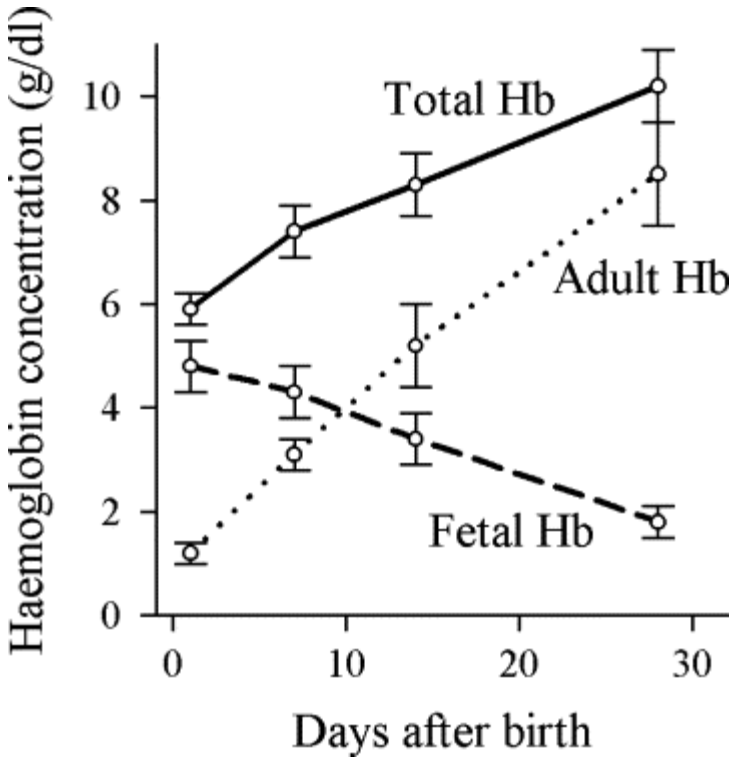

Fig. 3. Neonatal Hb switching pattern of the roan antelope. Mean Hb concentrations (○) and 95% confidence limits (⊤) on days 1, 7, 14 and 28 after birth.

3.4. Erythrocyte Morphology

Blood smears made from roan antelope calves during the first 4 weeks after birth showed a predictable change in composition with regard to erythrocyte morphology (Fig. 4, Table 3). During this time there was a significant decrease in the number of elliptocytes, schizocytes, keratocytes and dacryocytes and a significant increase in that of acanthocytes and spherocytes. A peak in reticulocytosis occurred 7 days after birth, and in cells containing Howell–Jolly bodies, 14 days after birth, with MCA and anisocytosis peaking in conjunction with reticulocytosis.

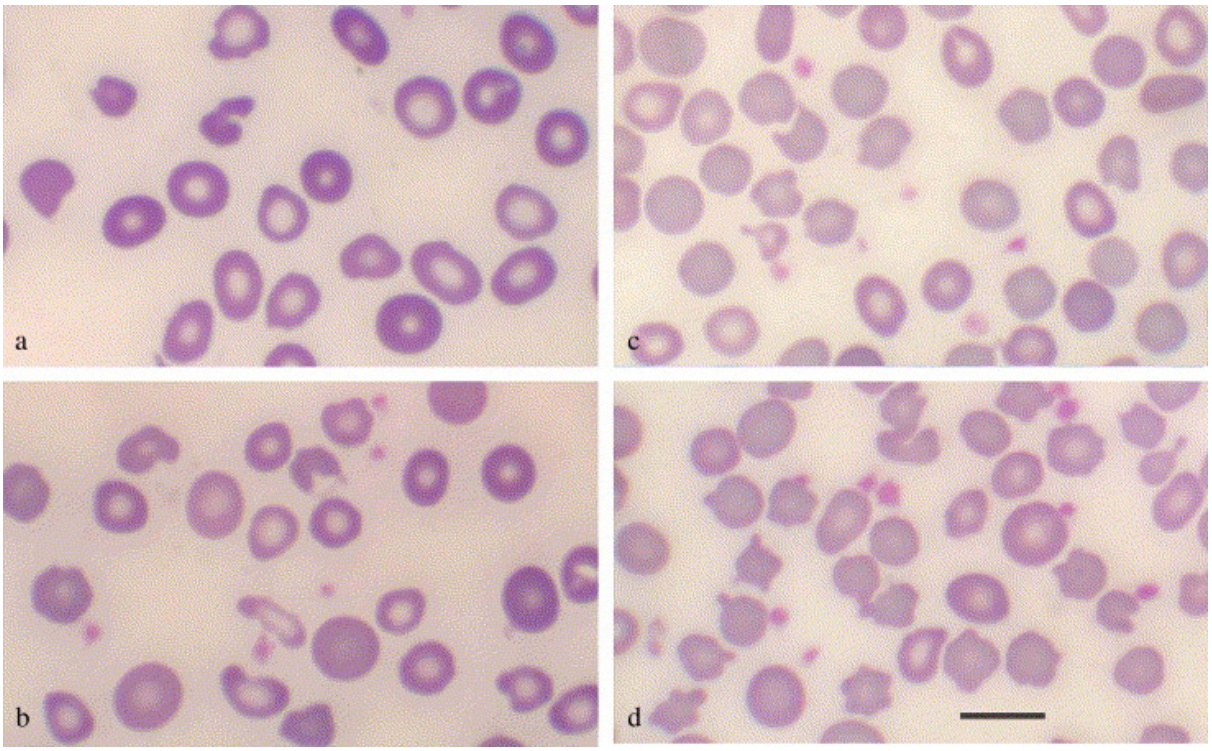

Fig. 4a–d. Erythrocytes from blood smears made from a roan antelope (calf 308) at (a) 1, (b) 8, (c) 15 and (d) 29 days after birth. Romanowsky stain. Bar, 20 µm.

Table 3.

Mean cell area, anisocytosis and the percentages of erythrocyte types in blood smears from neonatal roan antelopes

Parameter	**Results (mean±SD)**				**Regression on calf age**		
	Day 1	**Day 7**	**Day 14**	**Day 28**	**Summary**	***P***	r^2
	(*n*=22)	(*n*=20)	(*n*=16)	(*n*=15)			
Mean cell area (μm^2)	75.5±3.8	78.8±5.36	73.5±4.5	68.8±4.0	*y*=77.8−0.31(d)	*	0.7
Anisocytosis (%)	22.4±2.4	27.1±7.0	24.8±4.6	24±3.7	…	n.s.	…
Elliptocytosis (%)	71.7±6.9	61.3±14.1	45.9±12.1	38.7±16.9	*y*=68.3−1.06(d)	*	0.48

Parameter	Results (mean±SD)				Regression on calf age		
	Day 1	**Day 7**	**Day 14**	**Day 28**	**Summary**	***P***	r^2
Acanthocytosis (%)	6.6±3.9	3.1±3.5	12.6±8.3	28.1±13.1	*y*=1.9+0.86(d)	*	0.52
Schizocytosis (%)	6.7±3.3	4.3±2.4	4.4±3.5	1.7±1.3	*y*=6.5−0.18(d)	*	0.26
Keratocytosis (%)	1.1±0.9	0.7±1.0	0.8±0.5	0.3±0.6	*y*=1.2−0.03(d)	*	0.1
Dacryocytosis (%)	0.5±0.6	0.3±0.6	0.5±0.7	0	*y*=0.5−0.02(d)	*	0.07
Spherocytosis (%)	18.3±12.4	28.5±13.1	39.8±19.0	70.1±17.4	*y*=15.3+1.91(d)	*	0.61
	(*n*=22)	(*n*=20)	(*n*=16)	(*n*=14)			
Reticulocytosis (%)	0.4±0.3	0.8±1.4	0.2±0.3	0.2±0.3	…	n.s.	…
Cells containing Howell–Jolly bodies (%)	0.1±0.2	0.2±0.2	0.2±0.2	0.1±0.2	…	n.s.	…
	(*n*=19)	(*n*=19)	(*n*=16)	(*n*=15)			
Siderocytosis (%)	0.1±0.1	0.1±0.2	0.1±0.1	0.1±0.1	…	n.s.	…

d, age in days; *=*P*<0.01; n.s., not significant; …, no entry.

3.5. Regression of Erythrocyte Types on Hb Parameters

A significant increase in acanthocytosis and a decrease in elliptocytosis occurred with increasing adult Hb concentration. In addition, a significant increase in spherocytosis and decrease in schizocytosis occurred with increasing total Hb concentration (Table 4).

Table 4.

Regression of erythrocyte types on Hb parameters of neonatal roan antelopes

Parameter (%)	**Regression analysis**	
	Summary	r^2
Elliptocytosis	y=100−5.7 (HbA)*	0.56
Acanthocytosis	y=−0.8+2.95 (HbA)*	0.48
Spherocytosis	y=−44.6+10.4 (Hb)*	0.67
Schizocytosis	y=12.9−1.1 (Hb)*	0.38

Hb, Hb concentration; HbA, adult Hb concentration; *=P<0.01.

4. Discussion

The neonatal anaemia of roan antelopes is associated with low and decreasing concentrations of fetal haemoglobins and the onset of adult Hb production (Fig. 3). The possibility that this scenario results from compromised intra-uterine Hb production is not excluded, but a number of studies suggest that the Hb "switching pattern" described above, indicates an imbalance between the conclusion of fetal Hb synthesis and the initiation of adult Hb synthesis, and that this pattern is normal in the roan antelope.

Numerous reports of neonatal anaemia, often in conjunction with anisocytosis, poikilocytosis and reticulocytosis, in various ruminant species, including those other than the Hippotragini, suggest that this picture represents a non-pathological occurrence (Karesh *et al*., 1986; Roeder *et al*., 1990).

Domestic goats, A-haplotype sheep (*Ovis aries*) and mouflon (*Ovis musimon*), shown to be closely related to roan antelopes (Hassanin and Douzery, 1999), display a perinatal Hb switching pattern in which the onset of adult Hb synthesis is delayed relative to the conclusion of fetal Hb synthesis (Huisman *et al*., 1969; Blunt and Huisman, 1975; Masala *et al*., 1991). These species, however, produce a third Hb, HbC, during this period and switch from adult Hb synthesis to that of HbC in response to adult-onset anaemia and erythropoeitin treatment. The fetal to adult Hb switching pattern of the roan antelope most closely resembles that of the mouflon (Fig. 5).

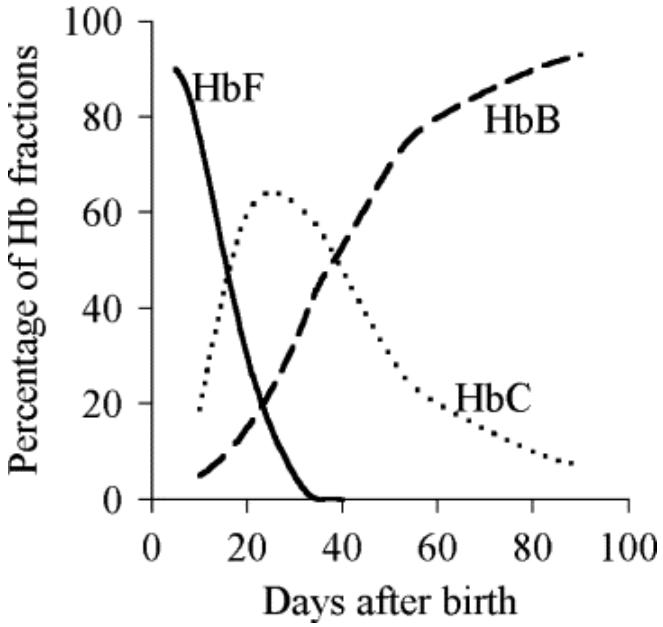

Fig. 5. Neonatal haemoglobin (Hb) switching pattern of the mouflon (Reproduced from Masala *et al*., 1991). Haemoglobin B (HbB) is the adult Hb of the mouflon.

The peak in reticulocytosis in roan antelope calves 7 days after birth suggests a recent and acute drop in Hb concentration in these animals (Jain, 1993), as might be expected with the conclusion of fetal Hb synthesis (Fig. 5).

Accepting that the Hb switching pattern described above is non-pathological, extrapolation of these data provides for the perinatal switching pattern illustrated in Fig. 6. In this case, total Hb production during this period would be represented by the curve in Fig. 7 and an explanation is provided for the profound anaemia present during this time. Those members of the Caprini that produce HbC during the perinatal period, in contrast to the roan antelope, display a neonatal anaemia which is far milder.

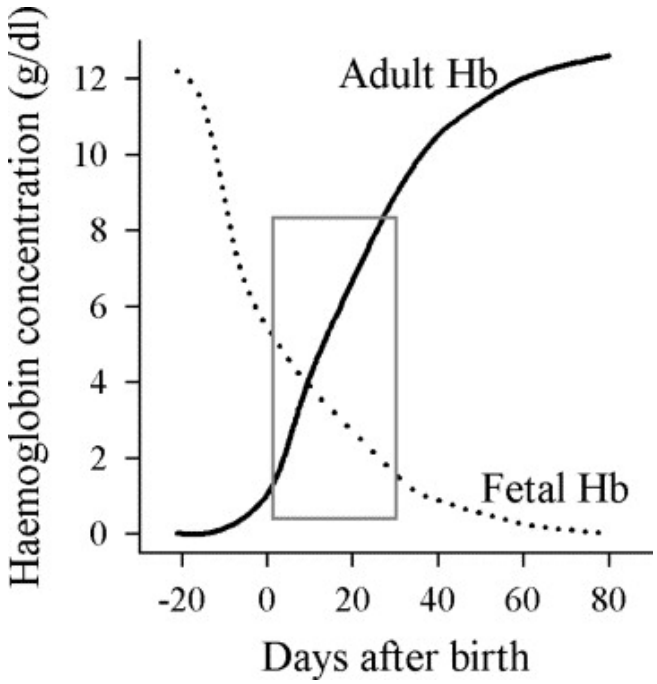

Fig. 6. Perinatal Hb switching pattern of the roan antelope, extrapolated from the neonatal Hb switching pattern (inside "box").

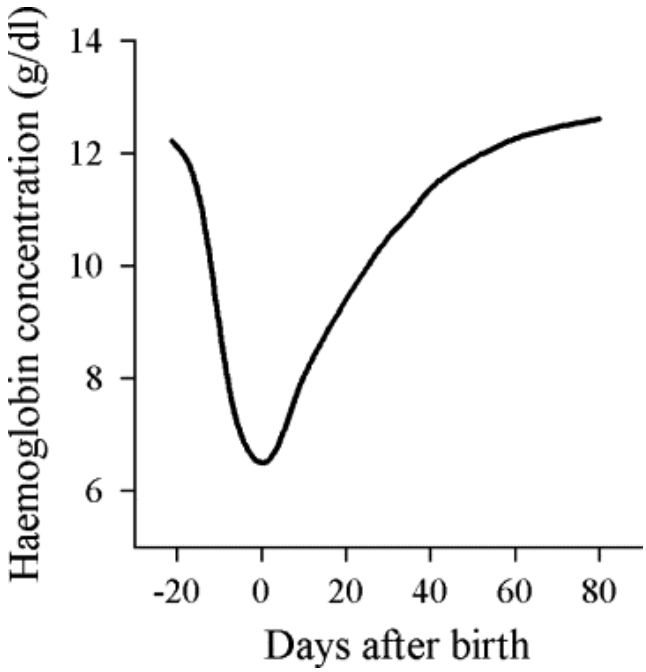

Fig. 7. Perinatal Hb production in the roan antelope, extrapolated from Fig. 6.

The neonatal Hb switching pattern of the common ancestor of the goat and roan antelope cannot be predicted with certainty from current evidence. The β^C-globin gene probably evolved about 12 million years ago (Li and Gojobori, 1983), while the divergence of the subfamily Antilopinae into distinct tribes is estimated to have occurred between 13.6 and 15.3 million years ago (Hassanin and Douzery, 1999). It seems probable, therefore, that HbC appeared in the goat and sheep lineage following divergence from the *Hippotragus* lineage and that the Hb switching pattern of the roan antelope may be indicative of that of the common ancestral ruminant.

It is believed, however, that the Caprini and Hippotragini belong to a single clade which probably diverged more recently than did other antelope tribes. This, and difficulties in accurately dating evolutionary events (Hassanin and Douzery, 1999), suggest that the possibility that these tribes diverged following the evolution of HbC cannot be excluded. The neonatal Hb switching patterns of goats, A-haplotype sheep and mouflon differ markedly and it is possible that the production of HbC has allowed the onset of adult Hb synthesis to drift forward to different degrees in the ontogeny of these species. If a common ancestral ruminant of the Caprini and Hippotragini produced HbC during the perinatal period, this may have allowed the delay in onset of adult Hb to evolve. The Hb switching pattern of the roan antelope might then be explained as the result of the deletion or dysfunction of the β^C-globin gene. Such a deletion is believed to have occurred in B-haplotype sheep (Garner and Lingrel, 1988).

Whichever evolutionary scenario proves to be correct, the drop and subsequent rise in Hb synthesis during the perinatal period provides for a possible explanation for the profound poikilocytosis, schizocytosis and reduced haematocrit observed in the roan antelope during this period. The predictable, progressive change in the numbers of various erythrocyte morphological types during the first 4 weeks after birth points to a progressive change in the underlying membrane pathology of these cells. More specifically, changes in the numbers of certain morphological cell types are related to increasing Hb concentrations and therefore, by extension, to increasing synthesis of adult ß-globin chains.

In ß-thalassaemia, a human disease characterized by a hypochromic, poikilocytic anaemia, abnormalities in the production of adult ß-globin chains of Hb result in a build-up of excess intracellular α-globin chains. Excess globin chains have been shown to aggregate and adhere to erythrocyte membrane and membrane skeleton proteins, resulting in intramedullary cell death, cell shape alterations, increased fragility, and reduced lifespan (Rachmilewitz and Schrier, 2001). If the synthesis of β-globin is reduced in the neonatal roan antelope and that of α-globin is not, a thalassaemic episode may prove to be the underlying pathology of the poikilocytic erythrocytes. As the production of adult β-globin increases over time, the α-globin chain excess decreases, resulting in changes in erythrocyte membrane pathology. An imbalance between β^C- and α-globin synthesis may also prove to be the cause of the HbC-associated poikilocytosis of goats.

The consequence of a possible β^C-thalassaemia in the roan antelope makes for interesting speculation. Haemoglobin C would not be produced in adult animals under anaemic

conditions, but the mechanism for "switching off" adult Hb synthesis might still be functional. In effect, the onset of anaemia in the adult roan antelope might trigger a thalassaemic episode. This possibility is supported by the observation of poikilocytosis in an anaemic 18-month-old roan antelope (M. du Plessis, pers. com.).

Speculating on the adaptive advantage of neonatal anaemia, Steyl *et al*. (2003) suggested that it may enhance the "freezing" and hiding behaviour known to occur in calves of the Hippotragini (Skinner and Smithers, 1990). In addition, gestation length may be influenced by Hb switching. This switching is regulated by a "molecular clock" in that factors specific to fetal developmental stages are believed to control the sequential production of different haemoglobins (Baron, 1997). In the case of the roan antelope, decreasing Hb concentrations during the fetal/adult Hb switching period will therefore, at a specific gestational age, stimulate a fetal hypoxic stress response that may contribute to the initiation of partus (Liggins *et al*., 1973). By synchronizing gestation length, this mechanism would complement oestrus synchronization, shown to occur in the sable antelope (*Hippotragus niger*) (Thompson, 1995), resulting in synchronization of calving within a herd.

The possible link between the increased susceptibility to theileriosis and the anaemia of neonatal roan antelope was not specifically investigated in this study. However, it would seem unlikely that such anaemia is related to a toxicosis or nutritional deficiency. This suggests a direct relationship between the low Hb levels and the increased susceptibility to haemoprotozoal disease. Further investigation into the role of Hb, and specifically HbC of members of the Caprini, in resistance to *Theileria* and other haemoprotozoa might prove rewarding.

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