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# The haemoglobins of wild cattle (Bovini Simpson, 1945)

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## Abstract

The haemoglobins of nine bovines (anoa, Cape buffalo, European and American bisons, banteng, gaur, domestic yak, dwarf zebu, and taurine domestic cattle) were investigated by isoelectric focusing (and starch gel electrophoresis). The focusing patterns obtained were characteristic for each wild cattle species, with the exception of the two forms of the genus *Bison,* the haemoglobin of which could not be differentiated. Despite very small sample sizes, intraspecific variation of the red cell pigments were found in gaur, anoa and dwarf zebu. The genetic nature of this variability could be rendered likely by finding heterozygous individuals, and by densitometry which suggested dosage effects of genetic expression.

## Introduction

In his description of a new haemoglobin polymorphism in Norwegian red cattle, Braend (1988) provided a short review of recent work about the population genetics of the bovine red cell pigment. So far, thirteen alleles of this protein have been detected in domestic taurine and zebu cattle. Haemoglobin alleles as genetic markers have served to reconstruct the phylogenetic descendance and breeding history of cattle races and population lines (Baker and Manwell 1980; Graml et al. 1986), as well as to analyse unknown pedigrees (e.g. Buschmann and Schmid 1968; Schleger and Soos 1967; Braend 1972). The haemoglobins of domesticated non-taurine bovines, such as the domestic water buffalo (Vella 1958), Bali cattle (Vella 1958; Namikawa and Widodo 1978), and the gayal or mithun (Winter et al. 1984) received less attention, with the exception of the Indian zebu (e.g. Naik et al. 1969; Manwell and Baker 1980). The study of wild bovine species has been largely neglected due to the difficulties implied in blood sampling, and only few studies are available on the haemoglobins of selected wild Bovinae, usually applying starch gel electrophoretic methods (Braend and Stormont 1963; Braend and Gasparski 1967; Davis and Read 1985; Davis et al. 1988; Hartl et al. 1988; Read et al. 1987). At the same time, the decrease in population numbers of several bovines in the wild and stricter laws concerning the trade in endangered wildlife imply that new blood is increasingly difficult to obtain for zoological gardens, and a better organization of the breeding management of the frequently very small captive populations is required for their long-term conservation. Stud-books and species management plans kept by zoos include those for the gaur *(Bos gaurus)*, the European bison *(Bison bonasus)*, and the wood bison *(B. bison athabascae)*. A stud-book for the anoa (*Bubalus depressicornis* ssp.) is being compiled (Seifert and Nötzold 1989). Major tasks for genetic research to support the captive breeding of wild cattle species include the necessity of finding genetic markers for the differentiation of the various described (or suggested) forms of anoa (which cannot be unambiguously determined by means of their phenotype), and an investigation of the alleged hybridization of captive bantends with either Bali or taurine cattle (Davis et al. 1988). Current efforts to bring founder animals of two critically endangered wild bovines into captivity, the

kouprey *(Bos sauveli)* and the tamaraw *(Bubalus mindorensis)*, as a safeguard against extinction, increase the necessity of knowing genetic markers, in order to optimize breeding plans for the small initial populations.

For these purposes, blood samples were collected (and screened for their haemoglobins) from the wild cattle stock of the Berlin Zoological Gardens, when a transfer to new enclosures required the tranquilization of the whole population. Moreover, the fairly complete bovine collection of the Berlin Zoological Gardens permitted a comparison of electrophoretic patterns of bovine haemoglobins across the species boundaries.

## Material and methods

Stabilized whole blood (EDTA) was mailed from Berlin Zoo to Heidelberg immediately after sample collection had been completed, and erythrocyte lysates could be frozen in liquid nitrogen within two days (and stored at −70 °C until analysis). The methods of preparation and purification of the haemolysates, and the details of isoelectric focusing and starch gel electrophoresis were essentially as described by Schreiber and Matern (1989). Prior to the runs, the haemoglobin concentrations of different haemolysates were brought to identical molarity by comparing their photometric extinctions at 410 nm, which corresponds to the maximum range of absorbance of taurine haemoglobin A. Isoelectric focusing was performed in thin (0.3 mm) polyacrylamide slab gels (4 % acrylamide, 0.16 % bis-acylamide, 5 % ampholines) polymerized on film foils (LKB). A LKB 2117 Multiphor II electrophoresis chamber, connected to a constant power supply (ECPS 3000/15, LKB), was used for electrophoretic separation. Approximately 5 µl of haemolysate (diluted 1:20) were applied to the gel, using a perforated rubber band as sample applicator. After a prerun of 10 minutes of 15 W (at maximal voltage and limiting current of 15 mA), focusing was performed at 20 W, 50 mA, and free voltage. When coloured isoelectric point markers (purchased from Promochem, Wesel) had reached their final position, the power was increased by 50 % to sharpen the bands. The ampholine gadients were tested after the runs by measuring the pH values of eluates from small successive gel portions cut from along the gradient. Stable and even ph-gradients in the banding region of haemoglobin were obtained by blending ampholines of different pH-ranges. The gels were photographed immediately after electrophoresis without artificial staining. For densitometric quantitation, a Chromoscan 3 (Joyce Loebl) was used. The scanning profiles are reproduced without any background correction.

The study of plasma proteins shortly mentioned was performed in an alkaline polyacrylamide gel as detailed by Schreiber and Matern (1989).

## Results

### Intraspecific variation

Individuals from nine bovines were included in the present investigation (see Table for their scientific names and specimen numbers). Three species exhibited more than one haemoglobin phenotype: three band patterns were descernible in the four gaurs (Fig. 1), and two in the three anoas (Fig. 2) and the two dwarf zebus.

**Overview of the wild cattle species and specimen numbers sampled, and the number of haemoglobin phenotypes encountered**

| Species | Individuals sampled | Haemoglobin phenotypes |
|---|---|---|
| Lowland (?) anoa (*Bubalus depressicornis* ssp.) | 3 | 2 |
| Cape buffalo *(Synceros caffer)* | 4 | 1 |
| American bison *(Bison bison)* | 3 | 1 |
| European bison *(Bison bonasus)* | 4 | 1 |
| Banteng *(Bon javanicus)* | 3 | 1 |
| Gaurs *(Bos gaurus)* | 4 | 3 |
| Domestic yak (*Bos mutus* f. grunniens) | 1 | 1 |
| Dwarf zebu | 2 | 2 |
| Domestic taurine cattle (Buntvieh) | 10 | 1 |

In the gaurs (Fig. 1a), the two less complex phenotypes are simultaneously present in two of the four individuals, suggesting a heterozygous condition. The densitometric analysis of the focusing patterns revealed that the bands of the presumably homozygous individuals contained approximately twice as much pigment (and hence proteid) each than the corresponding fractions of the heterozygous pattern (Fig. 1b). This suggests a simple gene dosage effect due to the expression of two codominant alleles. Winter et al. (1984) suggested a haemoglobin polymorphism consisting of two alleles in the mithun, which is the domesticated form of the gaur. These authors included samples from three gaurs from Schönbrunn Zoo, Vienna, in their study, which lacked individual variation.

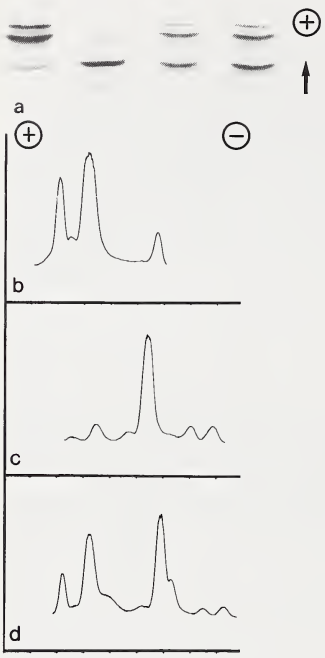


*Fig. 1.* Variability of gaur haemoglobin. a: Three phenotypes are evident after isoelectric focusing (pH 6–8, 4 % polyacrylamide). The first and second lanes depict the two alleles in homozygous patterns, while the remaining two patterns are interpreted as heterozygous. b–d: Densitograms of the gaur haemoglobins; b: Anodic allele (first lane in Fig. 1a), c: Cathodic allele (second lane in Fig. 1a), d: Suggested heterozygous pattern

In the three anoas, two animals showed a composed pattern which is interpreted as a heterozygous phenotype of two alleles (Fig. 2a). The anoa haemoglobin with faster mobility in starch gel, and the fraction with an isoelectric point shifted towards the acidic, is the only one found in the third specimen. An individual which is homozygous for the putative cathodal allele is lacking. The integrals of the densitometic peaks of the anoas again suggest a model of two codominant alleles with similar rates of genetic expression (Fig. 2b). The present study also suggested a postalbumin polymorphism in the Berlin anoas (which is not illustrated), but their transferrins are monomorphic.

One of the two dwarf zebus possessed a second haemoglobin (in addition to Hb A), which, on account of its close vicinity to Hb A, could have been Hb C (or the Hb X described by Naik et al. 1969; review: Schwellnus and Guérin 1977). However, since the search in a small series of domestic cattle (Buntvieh) failed to discover Hb B which could have served as a reference marker, we cannot be certain as to the identity of the second, anodal dwarf zebu haemoglobin.

Neither the four Cape buffaloes, nor the three American and four European bisons, nor the three bantengs exhibited individual variation in the banding patterns of their haemoglobins; only one yak could be sampled.

The results concerning intraspecific variation must be interpreted against the back-

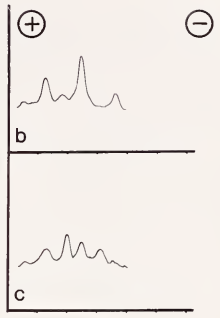


*Fig. 2.* Haemoglobin variation in the anoa. a: Both variants after focusing at pH 5–8 (4 % acrylamide). The first and third lanes are thought to show heterozygous haemoglobin patterns, and the central lane the anodic allele. b and c: Densitograms of these banding patterns; b: Anodic allele, c: Suggested heterozygous combination

ground of the small specimen numbers available. Furthermore, the lack of detailed data about the origin, and past breeding history of most individuals, renders certain conclusions difficult. Additional work about the bovine populations of other collections should follow soon, so that a more comprehensive understanding of the haemoglobin polymorphisms in wild cattle will emerge before the variation is lost in the tiny populations. Isoelectric focusing is suggested as the method of diagnosis, since it facilitates comparisons between the various, and unavoidably small, population samples available for study.

## Comparative aspects

Most of the investigated cattle species (see Table) exhibited a distinctive haemoglobin band pattern both after isoelectric focusing (Fig. 3) and, albeit less conspicuously, starch gel electrophoresis (Fig. 4). The only exceptions were the American and European bisons, the


*Fig. 3.* Bovine haemoglobins after electrofocusing at pH 6–8 in a polyacrylamide gel (4 % acrylamide, 0.16 % bis). a: Domestic yak. b: European bison. c: American bison. d: Dwarf zebu. e: Banteng. f: Gaur (anodic allele, see Fig. 1). g: Cape buffalo. h: Anoa. (anodic allele, see Fig. 2). For explanation, see text

haemoglobins of which displayed identical electrophoretic mobilities and patterns (small differences in the focusing pattern of the minor haemoglobins seem possible, but this assumption requires confirmation in more specimens). Moreover, the cathodic allele in the

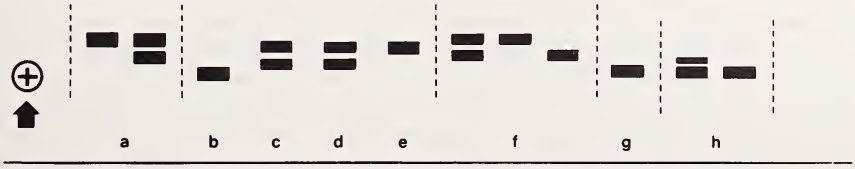


*Fig. 4.* Diagrammatic comparison of haemoglobin patterns from wild cattle after alkaline starch gel electrophoresis. a: Anoa. b: Cape buffalo. c: European bison. d: American bison. e: Banteng. f: Gaur. g: Taurine domestic cattle (haemoglobin A). h: Dwarf zebu

dwarf zebus (in starch gel) was indistinguishable from taurine Hb A. The high resolution properties of isoelectric focusing in thin-layer slab gels with species-specific patterns is noteworthy in view of the need to recognize the introgression of feral domestic cattle into some wild species. The resolution of the focused protein tetrameres into several bands indicates multiple haemoglobins, as in frequent in Artiodactyla (Butcher and Hawkey 1977). Figure 5 presents densitograms to facilitate interspecific comparisons of the protein fractions. The redundant peaks in the scanning profiles of the bisons and the yak might be

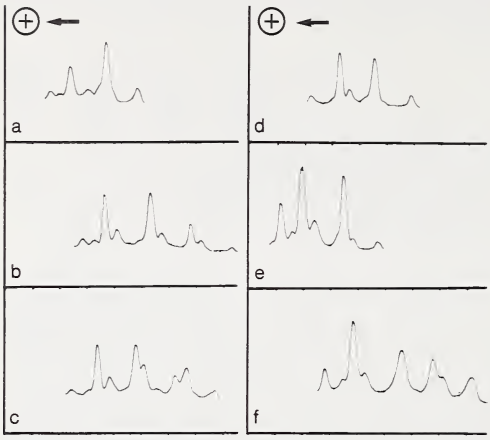


*Fig. 5.* Densitograms of focusing patterns of bovine haemoglobins (pH 6–8, 4% polyacrylamide). The anodic front is always oriented to the left. a: Anoa (anodic allele, see Fig. 2). b: Cape buffalo. c: European and American bisons. d: Banteng. e: Gaur (anodic allele, see Fig. 1). f: Domestic yak. Note: the bison and yak samples contained traces of methaemoglobin

due to the presence of partially oxidized haemoglobin: in these species, haemolytic plasma had to be used for electrophoresis instead of haemolysate, since the samples had been frozen prior to mailing (charged compounds of low-molecular masses, and salt present in blood plasma were removed by gel filtration in Sephadex columns, to prevent possible distortions of the electrophoretic results). Some of these minor bison haemoglobins grew weaker (or disappeared completely) after incubation of the proteins with 5 mM potassium cyanide for a few minutes (compare Bunn and Drysdale 1971). In starch gel, the bison haemoglobin was resolved into two bands. This must be due to gene duplication within the genome of each individual (and not to heterozygosity), because each specimen displayed the same pattern, and segregation was not observed. In ruminants, such multiple haemoglobins are frequently the result of a duplication of the alpha-globin chains. Like in the present study, no haemoglobin polymorphism could be detected in the 113 wild-living American bisons investigated by Braend and Stormont (1963), the largest population of any wild bovine species studied so far. The banding patterns obtained in that study (by starch gel electrophoresis) resembled those that were found in the Berlin bisons. Mazur et al. (1986) investigated one European bison from Munich Zoo which separated into two bands in a polyacrylamide gel. An identical pattern was observed with the Berlin samples when haemolytic plasma was separated in an alkaline polyacrylamide gel. Braend and Gasparski (1967) also resolved the haemoglobin of four European bisons into two bands (in starch gel). Butcher and Hawkey (1977) electrofocused the haemoglobins of one American and 6 European bisons; their micrograph does not show these details, but they listed two major bands (identical in both species), and 5 *(B. bison)* or 4–6 *(B. bonasus)* minor haemoglobins. The pattern of the single yak was complex. Lalthantluanga et al. (1985) described two variants in a sample of three (adult) yaks with the suggested heterozygous haemoglobin resolving into three, and the homozygous tetramere into two bands (in alkaline polyacrylamide gel electrophoresis). Yak occurs at elevated altitudes in the Central Asian mountain ranges, and it is capable of heavy work in an atmosphere of

very low oxygen pressure. Physiological adaptations can be expected to facilitate oxygen transport, and its supply to the tissues.

## Discussion

Since the current efforts to compile a stud-book for the anoa, in order to improve the coordination of its conservation in captivity, is hampered by taxonomic uncertainties, the search for informative genetic markers is a priority in this small and phylogenetically interesting bovine (Seifert and Nötzold 1989): the breeding lines of its two morphologically distinctive subspecies, which are generally recognized, the lowland *(Bubalus d. depressicornis)* and the mountain anoa *(B. depressicornis quarlesi)*, cannot be separated merely on the basis of phenotypical evidence, such as body size, horn form, coat colour, and the texture of their fur. Indeed, the validity of (a) further subspecies has been suggested. The Berlin animals were at times thought to be mountain anoas, but this is now doubted. We preliminarily list them here as lowland anoas, according to recent karyotypic findings (Schreiber and Held, unpubl.). The occurrence of intermediate morphological types between both forms requires a comprehensive molecular investigation to improve the taxonomic basis for a sound conservation strategy. It remains unknown whether the existence of intermediate types is the result of a natural cline between two gradually differentiated subspecies, or is due to hybridization. Hardly anything has been published about the natural history of anoas in Sulawesi, and recent references treat the anoa forms as either ecologically separated subspecies, or, alternatively, suggest their true specific separation on account of a reported regional sympatric occurrence of both forms (e.g. Groves 1969; Honacki et al. 1982; review: Frädrich 1973; Seifert and Nötzold 1989).

Davis and Read (1985) screened the haemoglobins of 14 bantengs living in North American zoos. Other than in the present study, they found two haemoglobin variants (which they called B and C). Namikawa and Widodo (cited by Davis and Read 1985) found only one haemoglobin allele in bantengs sampled in Indonesia (the number of specimens is not cited). Davis and Read (1985) conclude from their finding that Hb A of domestic cattle was not present in American zoo bantengs, and that there were mutually exclusive alleles of acid phosphatase, that there is no reason to doubt the genetic purity of the American zoo population of *Bos javanicus* (against the view that it could contain cattle blood). Moreover, Davis et al. (1988) published a statistical model (on the basis of four blood proteins) suggesting a high probability that no significant introgression of taurine cattle have affected the North American zoo bantengs. The monomorphism of the Berlin bantengs coincides with the results by Namikawa and Widodo (although it appears from other publications that these authors do not always clearly differentiate between wild bantengs and Bali cattle). The bantengs in Berlin Zoo belong to the nominate subspecies *(Bos j. javanicus)*, and descend from founder specimens caught in Ujung Kulon National Park, Java. Ujung Kulon is one of the remote reserves in Java, and, lying in the moist western half of this island, mainly water buffaloes, and few Bali cattle are kept in the surroundings, thus minimizing the possibilities of hybridization. Unfortunately, the degree of inbreeding of the Berlin animals since their import in 1956 is unknown, and not all specimens could be included in the investigation. Follow-up studies in additional European zoo populations are desirable to determine if banteng haemoglobins are polymorphic. Given the insufficient knowledge of allele frequencies in Bali cattle, hybridization of zoo bantengs with their domestic conspecific will be very difficult to exclude. The Berlin bantengs display a strong sexual dimorphism in colour, and are therefore thought to be pure bred (Frädrich, pers. comm. 1989).

By now, the bisons are the best studied wild bovines in terms of protein elec-

trophoresis, and the results emerging are consistently showing very similar (or identical), multiple but monomorphic haemoglobins in both taxa. Both bisons experienced bottlenecks in their recent history, particularly the European form, which is reported to descend from 13 individuals (Slatis 1960; Pucek 1986). The investigation of one (or a few) loci prohibits taxonomic interpretations, and the small individual numbers investigated pose additional incertainties as to the homology of proteins (due to the possibility of the fixation of different alleles by founder effect). Still, the identity of the haemoglobin of both *Bison* forms is both remarkable and plausible, since they are closely related to each other and became separated only comparatively recently by the flooding of the Bering Strait. Indeed, there have been discussions by taxonomists as to their proper classification as distinct valid species (Bohlken 1967; review: Pucek 1986).

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## Zusammenfassung

*Isoelektrische Fokussierung von Rinderhämoglobinen*

Die Hämoglobine von sechs Wildrindarten (Anoa, Kaffernbüffel, Wisent, Bison, Banteng, und Gaur) und drei domestizierten Rinderformen (Hausyak, Zwergzebu, und Bunvieh) wurden in Polyacrylamid-Dünnschichtgelen elektrofokussiert (sowie stärkegelelektrophoretisch aufgetrennt). Mit Ausnahme von identischen Proteinbanden bei Wisent und Bison und dem Übereinstimmen der kathodischen Hämoglobinallele von Zwergzebu und taurinem Hausrind konnten die Fokussierungsmuster aller Arten eindeutig differenziert werden. Bei Anoa, Gaur und Zwergzebu wurden Hämoglobinvarianten nachgewiesen. Das Auftreten (heterozygoter) Kombinationsmuster dieser Varianten legt ebenso die Annahme eines genetischen Polymorphismus mit zwei Allelen nahe wie eine quantitative densitometrische Auswertung der Proteinbanden. Die Möglichkeit der Anwendung dieser Befunde für die Erhaltungszucht der Arten wird angesprochen.

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