Relationships of foetal haemoglobin level to age of newborn fallow deer (*Dama dama*)

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The relative proportions of foetal and adult haemoglobins were quantified by densitometry of isoelectric focusing patterns in blood samples from 157 fawns from a free-living population of fallow deer (*Dama dama*) in Coto Doñana National Park, southwestern Spain. During the first week of life, the ratio of adult to foetal haemoglobin concentration increases with age. The proportion of foetal haemoglobin to total haemoglobin in peripheral blood proved a better indicator of age than morphological characteristics based on the persistence of natal traits in the fawns' external phenotype. The erythrocytes of even the youngest fawns contained some adult haemoglobin, the mean percentage of adult haemoglobin in offspring less than 24 h of age being 13.7% in the reproductive season of 1989 ($n = 28$) and 7.18% in 1990 ($n = 25$). Half of the fawns whose blood contained less than 3% adult haemoglobin failed to survive past the first weeks of life (even when body mass was not below average), whereas not a single fawn with more than 5% adult haemoglobin on its first day of life was subsequently found dead. Even when the effects of sexual dimorphism in body size are taken into account, the blood of male fawns contained less adult haemoglobin at a given age than that of females, as was evident from significantly lower levels of adult haemoglobin when referenced to several body size covariates such as body length, shoulder height, hind-foot length, and body weight.

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Les proportions relatives d'hémoglobine fœtale et d'hémoglobine adulte ont été déterminées par densitométrie des patterns de focalisation isoélectrique dans des échantillons de sang recueillis chez 157 faons d'une population libre de Daims (*Dama dama*), dans le parc national de Coto Doñana (sud-ouest de l'Espagne). Au cours des 3 premières semaines de la vie, le rapport entre la concentration d'hémoglobine adulte et la concentration d'hémoglobine fœtale augmentait en fonction de l'âge. La proportion d'hémoglobine fœtale par rapport à l'hémoglobine totale dans le sang périphérique s'est avérée un meilleur indicateur de l'âge que les caractéristiques morphologiques basées sur la persistance des caractères naux dans le phénotype externe des faons. Les érythrocytes des faons même les plus jeunes contenaient de l'hémoglobine d'adulte; le pourcentage moyen d'hémoglobine adulte chez les faons de moins de 24 h a été évalué à 13,7% au cours de la saison de reproduction de 1989 ($n = 28$), et à 7,18% en 1990 ($n = 25$). Parmi les faons contenant moins de 3% d'hémoglobine adulte, un sur deux est mort au bout de quelques semaines d'existence (même lorsque la masse de ces faons n'était pas au-dessous de la masse moyenne), alors que les faons contenant plus de 5% d'hémoglobine adulte le jour de leur naissance ont tous survécu. Même en tenant compte des effets du dimorphisme sexuel de la taille, les faons mâles contenaient moins d'hémoglobine adulte à un âge donné que les femelles, comme en témoignent les concentrations significativement plus faibles d'hémoglobine adulte relevées en fonction des diverses covariables reliées à la taille, longueur du corps, hauteur au garrot, longueur du pied arrière, et masse corporelle.

[Traduit par la rédaction]

Introduction

Reports of a number of investigations are available on the ontogenetic development of cervid fawns (Krebs and Cowan 1962; McEwan and Wood 1966; Retfalvi 1969; Nordan et al. 1970; Short 1970; Von Ulmenstein 1985). In all polygynous and sexually dimorphic species of deer where the early ontogeny of male and female fawns was compared, males were found to be heavier at birth and to grow faster than females. It also appears that gestation is longer for male than female offspring of red deer (*Cervus elaphus*) (Clutton-Brock et al. 1982).

No data are available on the growth efficiencies of male or female offspring relative to a given maternal caloric input, but sociobiological interpretations focus on differences in "ontogenetic strategy" between the two sexes in polygynous species. These are considered adaptive traits in populations in

which only few dominant males (but most females) will reproduce (Clutton-Brock et al. 1982). During previous investigations (Braza et al. 1988) we found that in a free-living population of fallow deer (*Dama dama*) at Doñana National Park male offspring are heavier at birth than female offspring, and some of their body measurements are larger.

Many of the parameters necessary for a comprehensive evolutionary interpretation of perinatal sex dimorphism are difficult to investigate in wildlife species, particularly the growth efficiency of animals of both sexes and the energetic investment required for certain ontogenetic processes, both pre- and post-natally. We also encountered these limitations when studying the perinatal sex dimorphism of fallow deer solely on the basis of body weight and biometry.

We therefore tried to determine if the relative proportions of foetal (HbF) to adult (HbA) haemoglobin can serve as an indicator of age and development of fallow deer fawns under field conditions. Human foetal haemoglobin level is useful in identifying pre- or post-maturely born human infants (Abrahamov

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et al. 1956; Ponxis and Visser 1956; Cook et al. 1957; Rooth and Sjöstedt 1957; Bard et al. 1970; Smith and Miller 1972). Since the switch of transcriptional activity from γ -globin to β -globin chains in sheep and goats is essentially completed perinatally (Barker et al. 1977), the rate of replacement of foetal haemoglobin by adult haemoglobin in the peripheral blood could also serve as an indicator of the rate of early post-natal development. Published knowledge about ontogenetic globins in cervids is limited to the electrophoretic work of Kitchen et al. (1964, 1967) and Seal and Erickson (1969), who demonstrated the presence of two foetal haemoglobins in young white-tailed deer (*Odocoileus virginianus*).

This study presents quantitative data on the persistence of foetal haemoglobin in a free-ranging population of fallow deer, to evaluate whether it can serve as a rate marker of early ontogeny and contribute to the still limited knowledge concerning cervid foetal haemoglobin.

Methods

The birth season for fallow deer at Doñana National Park in southwestern Spain is concentrated between 25th of May and 15th of June. A total of 95 (46 males, 49 females) and 62 (38 males, 24 females) fawns were captured by hand during their hiding phase (first week of life) in the reproductive seasons of 1989 and 1990, respectively. Sixteen of these fawns were recaptured a few days later (2 males and 8 females in 1989, 4 males and 2 females in 1990).

Deer were captured by searching the study area on foot, sometimes with the assistance of a vehicle or of a ranger on horseback. For comparison, 11 adult deer (5 males, 6 females) were captured in the autumn of 1989 with the aid of nets and horses (San José 1991). Blood samples were collected into tubes containing EDTA from either the jugular or femoral vein of physically restrained deer. Subsequently, the animals were ear-tagged, weighed, and sexed, and body length, shoulder height, hind-foot length, tail and ear lengths, and chest girth were recorded.

In Doñana National Park, fallow deer inhabit an ecotone area between Mediterranean scrubland and marsh, which consists predominantly of various kinds of meadows and areas of rushes. Females seek the cover of the rushes during parturition (Braza 1975; Braza et al. 1988; San José and Braza 1992).

Since parturition is difficult to observe, the fawns' ages were estimated on the basis of morphological characteristics (e.g., hardening of the hooves, degree of umbilical healing), behaviour, and mobility. These criteria were deduced from 41 cases in which the exact time of parturition was discerned from an observation tower (San José and Braza 1992). The following eight development classes were defined on the basis of these criteria.

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- Class 1. Fur moist
 - Class 2. Fur dry, umbilicus bloody
 - Class 3. Fur dry, cord dry, curled-up body posture retained during manipulation
 - Class 4. Membranes at margins of hooves soft and complete
 - Class 5. Membranes at margins of hooves dry; umbilical filament retained
 - Class 6. Membranes at margins of hooves dry; no umbilical filament
 - Class 7. No traces of parturition
 - Class 8. Exhibits escape behaviour at the approach of a person and during manipulation
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Preparation of samples

After the blood samples were stored on ice in the field, pellets of blood cells were frozen (within 2 days) in liquid nitrogen and transported to the laboratory on dry ice. The purification of haemolysates and isoelectric focusing in polyacrylamide slab gels ($T = 7.5\%$, $C =$

3%) have been described previously (Schreiber and Göltenboth 1990). Polyacrylamide-gel electrophoresis was performed as described by Schreiber and Matern (1988). Because differential osmofragility of erythrocytes containing foetal and adult haemoglobin was observed during washing of the blood cells with 0.9% NaCl, the centrifuged cells were later frozen without previous washing. Linear focusing gradients in the required pH range (7–8) were achieved by blending two volumes of Servalyte (pH 7–8) with one volume of Servalyte (pH 4–9). The foetal haemoglobin (HbF) was quantified by densitometry (Chromoscan 3, Joyce Loeb) of the focusing gels. The weakness of some haemoglobin bands necessitated artificial staining of the protein to increase scanning precision. Referencing against patterns of unstained haemoglobin bands in lanes overloaded with proteins ensured that no bands traceable to additional loci were scanned. A solution of 0.2% bromophenol blue in ethanol – acetic acid – water (10:1:9) was found to yield less nonspecific background stain than protocols based on Coomassie Blue, which rendered densitometry difficult. The scans were integrated by Chromoscan software, and the HbF profiles were identified by reference to the adult patterns.

Denaturing electrophoresis in urea starch followed Chernoff and Petit (1964). After haemoglobins were denatured in 8 M urea and 10% β -mercaptoethanol, the peptides were separated in an alkaline starch gel (Veronal buffer, pH 8.0) containing 6 M urea.

The enzyme immunoassays used to determine the presence of foetal proteins in fallow deer plasma were Enzygnost-AFP (Behring Diagnostika, Frankfurt) for α -foetoprotein, and CEA-EIA (Roche, Grenzach) for carcinoembryonic antigen.

ANCOVA was performed to compare the proportion of HbA in male and female fawns relative to age (P1V, BMDP series; Dixon 1985). To compare the percentages of HbA in newborns of both sexes and in the eight development classes, a two-way ANOVA was performed (P7D, BMDP series; Dixon 1985). Percentages were subjected to arcsine square root transformation prior to analyses. We never used more than one datum for each individual.

Results

One haemoglobin allele (HbA) was found in all 168 free-ranging Doñana fallow deer, including 157 fawns and 11 adults. In adult deer, this protein represented all erythrocyte pigment (Fig. 1). It was resolved into seven bands by isoelectric focusing in the pH range 7–8, and consisted of one band after electrophoresis in an alkaline polyacrylamide gel (Tris-borate, pH 8.6). In the fawns, a more prominent haemoglobin accompanied HbA. Its focusing pattern consisted of two major bands and its isoelectric point was less acidic than that of HbA (Fig. 1). It migrated more cathodally than HbA in polyacrylamide-gel electrophoresis. Without exception the fawns contained this second haemoglobin, while it could not be found in any adult. There was no indication of allelic segregation between the two proteins that would indicate a genetic haemoglobin polymorphism.

Since the molecular structure of the red-cell pigment in cervids can be very complicated owing to a combination of genetic polymorphism and the facultative expression of duplicated globin genes, we resolved fallow deer haemoglobin by denaturing electrophoresis in alkaline urea starch (pH 8.0). This assay revealed one globin chain migrating towards the cathode in both adults and fawns. By analogy with patterns of denatured haemoglobin from humans and white-tailed deer (*Odocoileus*) (Kitchen et al. 1967) we presume that this peptide represents an α -chain. The adult haemoglobin of adult deer revealed one anodic fraction, which was complemented in fawns by a prominent second band migrating towards the anode. The latter exhibited a slower mobility in our electrophoretic system, and is more plausibly interpreted as being

FIG. 1. (a) Focusing patterns (pH 7–8) of fallow deer haemoglobin in ultrathin polyacrylamide gels ($T = 7.5\%$, $C = 3\%$). First lane: haemoglobin from adult fallow deer. Second lane: haemoglobin of a female fawn sampled within the first 24 h after birth. Third, fourth, and fifth lanes: increase of HbA in a young male sampled at 1.5, 4, and 6 days of age, respectively. (b and c) Densitogram of focused (pH 7–8) haemoglobins of a female fawn sampled at 3 days (b) and 12 days of age (c). Obvious HbA peaks are indicated by arrowheads. (d) Scanned focusing pattern (pH 7–8) of HbA from an adult fallow deer.

another member of the β -globin family of proteins, like the fetal γ -chain from man and *Odocoileus*. From these observations we conclude that the second haemoglobin we found in fallow deer was a foetal haemoglobin.

Assessment of the relative concentrations of the two haemoglobins in all young deer showed that a high percentage of the presumed foetal haemoglobin molecule always coincided with

FIG. 2. Percentage of adult haemoglobin (HbA) in male and female fallow deer fawns of known age. Sample size (male/female): Aged 0 days, 18/15; aged 1 day, 8/11; aged 2 days, 0/2; aged 3 days, 0/3; aged 4 days, 0/0; aged 5 days, 1/0; aged 13 days, 0/1. In each case the horizontal line represents the mean value, the vertical line the range, and the rectangle the standard deviation.

a small amount of adult protein, and vice versa. This observation is completely compatible with the typical haemoglobin ontogeny of those mammals that possess a foetal haemoglobin, which is gradually replaced by adult haemoglobin during the perinatal period. Accordingly, we will henceforth express the amount of HbF found in each specimen as a percentage of the total haemoglobin (HbF + HbA), the difference from 100% (total) haemoglobin therefore representing the percentage of HbA. We measured only the relative proportions of the two types of haemoglobin because the absolute haemoglobin concentration would show much more individual variation.

The blood of even the youngest fawns, which were born only a few hours prior to sampling, contained some HbA. The mean percentage of HbA in fawns of less than 1 day old was 13.7% in 1989 ($n = 28$) and 7.2% in 1990 ($n = 25$). The mean body weight of newborn fawns was 5728 g (SD = 906) for males ($n = 45$), and 4994 g (SD = 868) for females ($n = 45$) in 1989, and 5458 g (SD = 1017) for males ($n = 38$) and 4904 g (SD = 1037) for females ($n = 24$) in 1990. In 1989, only 1 female fawn out of 28 fawns caught within 24 h after birth had less than 5% HbA. In 1990, this number rose to 12 (9 males, 3 females) out of 25. In the 1990 reproductive season, the percentage of HbA was as low as 0.5–1% of total haemoglobin. However, 5 out of 10 fawns whose erythrocytes contained 3% HbA or less were found dead within a few days after blood sampling, even though body masses were not particularly low. Only animals with less than 5% HbA at birth were found dead in the days following capture. Percentages of HbA in erythrocytes sampled from 24-h-old fawns were exceptionally high: >33% of total haemoglobin.

The percentage of HbA increased with age ($R^2 = 0.116$, $HbA = 0.269 + 0.046 \times \text{age}$ for males, $n = 27$; $R^2 = 0.401$, $HbA = 0.346 + 0.033 \times \text{age}$ for females, $n = 32$; $R^2 = 0.273$, $HbA = 0.309 + 0.038 \times \text{age}$ for both sexes, $n = 59$; comparing the slopes assumed to be equal to 0, there is a significant difference: $F_{[1,56]} = 18.6354$; $p < 0.001$). The increase of HbA in the peripheral blood was directly corroborated in the few animals that were recaptured (Fig. 1).

Densitometric assessment of the focusing patterns of both

FIG. 3. Percentage of adult haemoglobin (HbA) in male and female fallow deer fawns in various development classes (see Methods). Sample sizes are as follows (male/female): class 1, 4/4; class 2, 1/3; class 3, 6/7; class 4, 8/4; class 5, 19/17; class 6, 13/5; class 7, 7/7. For details see Fig. 2.

HbF and HbA in fallow deer fawns revealed a higher proportion of HbA in female than in male fawns (ANCOVA, $p < 0.05$) (Fig. 2). This holds also for the adjusted means of the values, while the slopes did not differ significantly between the sexes. Only those fawns whose exact birth dates were known were included in this statistical evaluation.

To increase the sample size beyond individuals of known age, we assigned each captured fawn to a development class (Fig. 3). There was a significant effect of age ($p = 0.0001$) but not of sex ($p = 0.5654$) (two-way ANOVA).

We also looked for differences in the relative amounts of HbA according to sex after controlling for the effect of sexual dimorphism in size. HbA levels were consistently lower for male fawns after the effects of the following size covariates were taken into account: body length ($p < 0.05$), shoulder height ($p < 0.05$), hind-foot length ($p < 0.01$), and body weight ($p < 0.001$). Since the regression slopes of the amount of HbA to chest girth proved significantly ($F_{1,56} = 4.731$, $p < 0.05$) greater for males, the adjusted means in this set of data could not be compared. A correlation analysis, taking both sexes into account, provided the following values: body length (BL) $R^2 = 0.270$, $\text{HbA} = -162.36 + 0.268 \times \text{BL}$, $n = 111$, $p < 0.001$; shoulder height (SH) $R^2 = 0.271$, $\text{HbA} = -189.43 + 0.436 \times \text{SH}$, $n = 112$, $p < 0.001$; hind-foot length (HFL) $R^2 = 0.205$, $\text{HbA} = -182.54 + 0.919 \times \text{HFL}$, $n = 114$, $p < 0.001$; body weight (BW) $R^2 = 0.605$, $\text{HbA} = -45.799 + 0.011 \times \text{BW}$, $n = 116$, $p < 0.001$.

In polyacrylamide gels, sera of fawns displayed an unidentified protein band in the postalbumin region that was not observed in adults. Efforts to determine the presence and concentrations of α -foetoprotein and carcinoembryonic antigen failed. We could not clarify whether this was due to an absence of these proteins in fallow deer plasma or to the inability of the (monoclonal) antibodies we used in the assays to detect determinants in the deer homologues.

Discussion

The results of the present investigation show that fallow deer express an HbF. No indication of a duplicated α -globin gene

was obtained when denatured haemoglobin was resolved, although the resolution power of electrophoresis in urea starch could be too low to guarantee the detection of such variability. In contrast, Kitchen et al. (1967) found two foetal haemoglobins in *Odocoileus*, the only cervid in which foetal proteins have been investigated, the double pattern being due to the duplication of the α -globin locus. In fallow deer, variation in the presence or absence of bands was confined to one minor haemoglobin, the most cathodally migrating one, which could represent either a glycosylated form, methaemoglobin, or a persisting embryonic haemoglobin (Blunt and Huisman 1975). Being present as traces only, this band could have remained undetected by densitometry. The monomorphism of isoelectrically focused haemoglobin in Doñana fallow deer coincides with other reports of very low allozyme and blood protein variability in this species (Randi and Apollonio 1988), in contrast to the complicated haemoglobin polymorphisms of some other cervid species (Schreiber 1991).

Kitchen et al. (1964) estimated that at birth, the blood of white-tailed deer fawns contains some 5–7% adult haemoglobin. Since these authors used starch-gel electrophoresis for the separation and subsequent assessment of haemoglobins, a method that may have found could not resolve the foetal haemoglobins well, their data may not be comparable with our finding of 13.7% HbA within the first day of life in 1989 and 7.18% in 1990.

The percentages of HbF and HbA correlated well with the fawns' ages when the latter could be determined with precision. Therefore, the relative amount of persisting HbF can serve to indicate the age of a fallow deer fawn whose birth date is unknown.

Male fawns are born with less HbA than females, and this sex difference is maintained throughout the first days of life. In view of the mobility of fawns after they leave their birth place and rejoin the herd, it is difficult to capture young deer older than a few days. The exact age when the transition to HbA is completed remains unresolved, as does the question of a sex difference in the rate of replacement of HbF, which may parallel the sex difference in the increase of body mass (Krzyszowski et al. 1984). Two specimens aged 6 months did not retain any HbF, as might be expected in view of the results from few bovid species that have been investigated (Blunt and Huisman 1975).

When the fawns' development classes rather than known ages were considered in analyses, no significant differences in HbF concentration between male and female fawns could be detected. Our phenotypic development classes obviously do not give as precise an indication of age as does the ontogeny of haemoglobins. However, since the morphological markers on which we based our classes were developed after several years of observing fallow deer fawns, this discrepancy could also mean that there is a sex difference in the ontogenetic rate of those phenotypic characteristics that we selected. It might not be appropriate to apply common criteria to both male and female offspring in species with extensive sexual dimorphism, but rather, separate development classes for each sex should be considered.

Sociobiological interpretations of these sex differences focus on a presumed higher maternal pre- or post-natal investment in male offspring in a highly polygamous species, to maximize the chances of producing sons to compete successfully with rivals (cf. Clutton-Brock et al. 1982; Braza et al. 1988). The higher proportion of HbA in female fallow deer

fawns does not fit these interpretations, and suggests that evolutionary inferences from very few biometric or physiological data be postponed until considerably more data are available to provide a holistic description of early ontogeny.

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