Hormonal correlates of 'masculinization' in female spotted hyaenas (*Crocuta crocuta*). 2. Maternal and fetal steroids

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Summary. Concentrations of androgens (androstenedione, testosterone, 5α-dihydrotestosterone), oestrogen and progesterone were measured in relation to pregnancy in the spotted hyaena (*Crocuta crocuta*). The gestation period was estimated to be about 110 days. There was a marked progressive rise in all the steroids starting in the first third of gestation. Chromatographic separation of plasma showed that much of the oestrogen is not oestradiol (only 12% of total measured) and that a significant fraction of the 'testosterone' may be dihydrotestosterone. In the final third of pregnancy, concentrations of androgen (especially testosterone plus dihydrotestosterone) in the female circulation reached the maximal values of adult males; the percentage of dihydrotestosterone relative to total testosterone plus dihydrotestosterone was higher in females (44 ± 3·9%, *n* = 20) than in males (29·5 ± 3·5%, *n* = 17). Plasma androstenedione was also significantly higher in females, but the increment was less than for oestrogen, testosterone and progesterone, and the temporal pattern was less clear. Samples from the maternal uterine and ovarian circulation showed that androstenedione is largely of ovarian origin and metabolized by the placenta, while testosterone, progesterone and oestrogen are primarily of placental or uterine origin. Fetal samples were taken from two mixed-sex sets of twins and one male singleton. Gradients across the placenta measured in the fetal circulation confirmed that the placenta metabolizes androstenedione and is a source of testosterone for the female fetus; there were no consistent differences in androgens between male and female fetuses. It is suggested that the conspicuous masculinization of the female spotted hyaena, especially evident in the external genitalia at birth, is a result, at least in part, of high placental production of testosterone or dihydrotestosterone derived from the metabolism of high maternal androstenedione.

Keywords: hyaena; steroids; pregnancy; masculinization; placenta

Introduction

Androgens administered to female mammals during organizational periods of fetal and postnatal life masculinize the external genitalia (Jost, 1985), increase body weight (Wade, 1976), and promote the emergence of masculine reproductive (Phoenix *et al.*, 1959), playful (Meaney *et al.*, 1981) or aggressive (Beatty, 1979) behaviour. Female spotted hyaenas exhibit an array of morphological and behavioural characteristics that parallel such effects. The genitals of the female spotted hyaena are highly masculinized (Matthews, 1939; Davis & Story, 1949; Neaves *et al.*, 1980; Frank *et al.*, 1990). Female hyaenas are also heavier than male hyaenas (Matthews, 1939; Frank *et al.*, 1989; Glickman *et al.*, 1992), engage in as much rough-and-tumble play as male hyaenas (Pedersen *et al.*,

1990) and are more aggressive than males (Frank *et al.*, 1989; Baker, 1990), dominating them in competitive situations (Kruuk, 1972; Frank, 1986). In the absence of an external vagina, they receive the male and give birth through the clitoral meatus. In other respects, they display normal female reproductive behaviour.

In several papers (Glickman *et al.*, 1987, 1992), we noted that the primary circulating androgen in adult, non-pregnant female hyaenas is androstenedione and that testosterone is lower than in males. The present report is concerned with shifts in steroid concentrations that accompany pregnancy. Increments in androgens and other steroids during pregnancy are common in many mammalian species. During our studies, there were marked increments in several androgens, oestrogen and progesterone during pregnancy, which were not fully appreciated by earlier more limited measurements on hyaenas (Racey & Skinner, 1979; Lindeque & Skinner, 1982). Androgens, in particular, could have important effects on the developing fetus, since female spotted hyaenas have conspicuously masculinized genitalia at birth.

Racey & Skinner (1979) and later Lindeque & Skinner (1982) concluded that the fetal ovaries are the primary source of androgens and masculinize the fetal hyaena. To examine further the contributions of the placenta to circulating steroid concentrations, we analysed steroids in plasma from several compartments of the maternal and fetal circulations.

Methods

Animals

Five female and six male spotted hyaenas, collected as infants in south-west Kenya, were studied. They were members of two cohorts (ten hyaenas per cohort) assembled in January and December 1985. All animals were reared in social groups maintained at the Field Station for Behavioral Research adjacent to the Berkeley Campus in California.

Blood sampling

Adults were immobilized (with blowdarts) with intramuscular injections of ketamine (4–6 mg kg^{-1}) and xylazine (1 mg kg^{-1}). Blood was drawn from the jugular vein using a 22-gauge needle–vacutainer system. The blood was then centrifuged at 1000 g for 5 min and the plasma drawn and frozen for subsequent radioimmunoassay.

During the first year of study with the first cohort, we sampled hormones monthly. However, concern with the possible effects of frequent immobilization and sampling on concurrent or long-term behavioural assays led us to restrict sampling to intervals of 2–4 months for subsequent phases of this research (Glickman *et al.*, 1992). When we had identified pregnancy in a female, we attempted to obtain at least two samples during gestation.

Three pregnancies were terminated during the last 30 days of a 110-day gestation and concurrent samples of plasma were obtained from various sites in the fetal and maternal circulations. Fetal age was estimated from ultrasonic determinations of crown–rump length in a set of pregnancies allowed to carry to term, supplemented by assessments of tooth development and body weight. Repetitive blood samples were obtained, before and after removal of the fetus and placenta, from maternal peripheral, uterine and ovarian veins to evaluate placental contributions to plasma steroids. Measurements were also obtained from the fetal umbilical and peripheral circulation to examine potential androgenic influence on the fetus of maternal or placental activity and to evaluate the claim (Lindeque & Skinner, 1982) that there was no significant maternal androgenic contribution to the fetus.

Measurements of plasma steroids

Plasma samples were extracted with diethyl ether (Mallinkrodt, Boston, MA) for radioimmunoassay of steroids, by procedures detailed previously, using ^{3}H-labelled steroids (purchased from New England Nuclear, Boston, MA) and charcoal to separate free from bound hormone (Licht *et al.*, 1982).

Chromatographic analysis on mini columns of celite (Wingfield & Farner, 1975) were used to resolve further the identity of each steroid, especially testosterone versus dihydrotestosterone and oestrogen versus oestrone because of nonspecificity of the antisera. Ether extracts of plasma were applied to columns, dissolved in iso-octane (2,2,4,-trimethylpentane, Nanograde) and fractionated by application of a solution of iso-octane containing increasing concentrations (0–50%) of ethyl acetate. Separate fractions containing oestrone and oestradiol were assayed with the 'oestrogen' antiserum with [^{3}H]oestradiol as tracer; oestrone or oestradiol were used as standards. A few tests were also done with an antiserum to oestradiol that did not show appreciable crossreaction with oestrone. Separate

fractions containing testosterone and dihydrotestosterone (based on authentic samples and internal samples) were assayed with the 'testosterone' antiserum using either testosterone [1,2,6,7-^{3}H] or dihydrotestosterone [1,2,4,5,6,7-^{3}H(N)], with the corresponding cold steroid as standard. Recoveries of steroids were calculated from recoveries of added internal tracers; values ranged from 60–85%. Interassay and intra-assay variations were <12 and 10%, respectively, for all assays. Minimal detectable limits for these assays were 0·075 ng testosterone ml^{-1}, 0·05 ng androstenedione ml^{-1}, 0·03 ng oestradiol ml^{-1} and 0·1 ng progesterone ml^{-1}.

Further confirmation of the identities of testosterone and dihydrotestosterone in a pooled sample of plasma from pregnant hyaenas was based on thin-layer chromatography. Labelled testosterone and dihydrotestosterone were added to the sample before extraction in dichloromethane and application to a silica gel GF254 thin-layer plate (Merck). The plate was first developed 2 × in one dimension using toluene:cyclohexane (1:1) followed by 3 × in benzene:ethyl acetate (3:1) and then in a second dimension 1 × in chloroform:methanol:water (90:10:1). Radioactive spots corresponding to authentic internal standards were localized using a Berthold TLC Scanner. These regions were scraped, extracted in ether and radioimmunoassayed.

The results of chromatographic separation and thin-layer chromatography revealed that the radioimmunoassays for testosterone and oestradiol had reacted to significant quantities of dihydrotestosterone and oestrone, respectively (see Results). We, therefore, adopted the conventions of referring to nonchromatographed 'testosterone' and characterizing radioimmunoassays for oestradiol as 'oestrogen'.

Statistical analyses

Analyses were performed with the microcomputer program Systat (SYSTAT, Inc) and included correlations (Pearson) among steroids and Student's *t* tests for comparisons between groups. Since individual patterns for pregnant animals were consistent, even when absolute values varied, we considered it more informative to display individual values to describe changes associated with this important period of activity.

Results

There were significant effects of pregnancy on plasma concentrations of all steroids measured in this study (Fig. 1). To examine these effects as a function of the stage of pregnancy, we estimated gestation at 110 days (Matthews, 1939; Kruuk, 1972); this estimate was confirmed by changes in plasma progesterone (Fig. 1). Since our samples were limited within any given pregnancy, we combined data from successive pregnancies for each female in the study. Individual values for five females of reproductive age obtained at irregular times during two to five pregnancies for each female, representing 15 pregnancies (Figs 1–3), show that first pregnancies (estimated age of conception) occurred at 33·7 ± 2·5 months (mean ± SEM), and final samples were taken until 67 (64·2 ± 3·8) months of age. For concentrations in nonpregnant females, we used 39 samples taken from the same females during the same age period (47 ± 2: range 30–70 months), excluding the 30 days after parturition; these values did not differ significantly from those for three other intact females from the study population that did not become pregnant (cf. Glickman *et al.*, 1992).

When cubs were successfully reared, the mean interval between litters (*n* = 5) was 14·6 ± 1·4 months. After stillbirths during initial pregnancies (*n* = 2), or failure of maternal behaviour (*n* = 4), the interlitter interval was reduced to 8·4 ± 1·1 months.

Plasma steroid concentrations

Oestrogen, progesterone (Fig. 1a, b) and 'testosterone' (Fig. 1c) showed progressive parallel increases during pregnancy. Progesterone was the first to show a dramatic increase (by about 10 days after conception); oestrogen and 'testosterone' began to increase at about 30 days. (This early increase in progesterone proved a valuable indicator of pregnancy in our later studies; the lowest pregnancy value (11 ng ml^{-1}) was higher than all but one value for nonpregnant periods (<1 ng ml^{-1})). Maximal values of all three steroids were recorded during the last 3 weeks of gestation. Oestrogen and 'testosterone' were highly and positively correlated across times and individual samples (*r* = 0·84, *P* < 0·0001). Weaker correlations were observed between progesterone and oestrogen (*r* = 0·55, *P* = 0·001) and 'testosterone' (*r* = 0·46, *P* = 0·001).
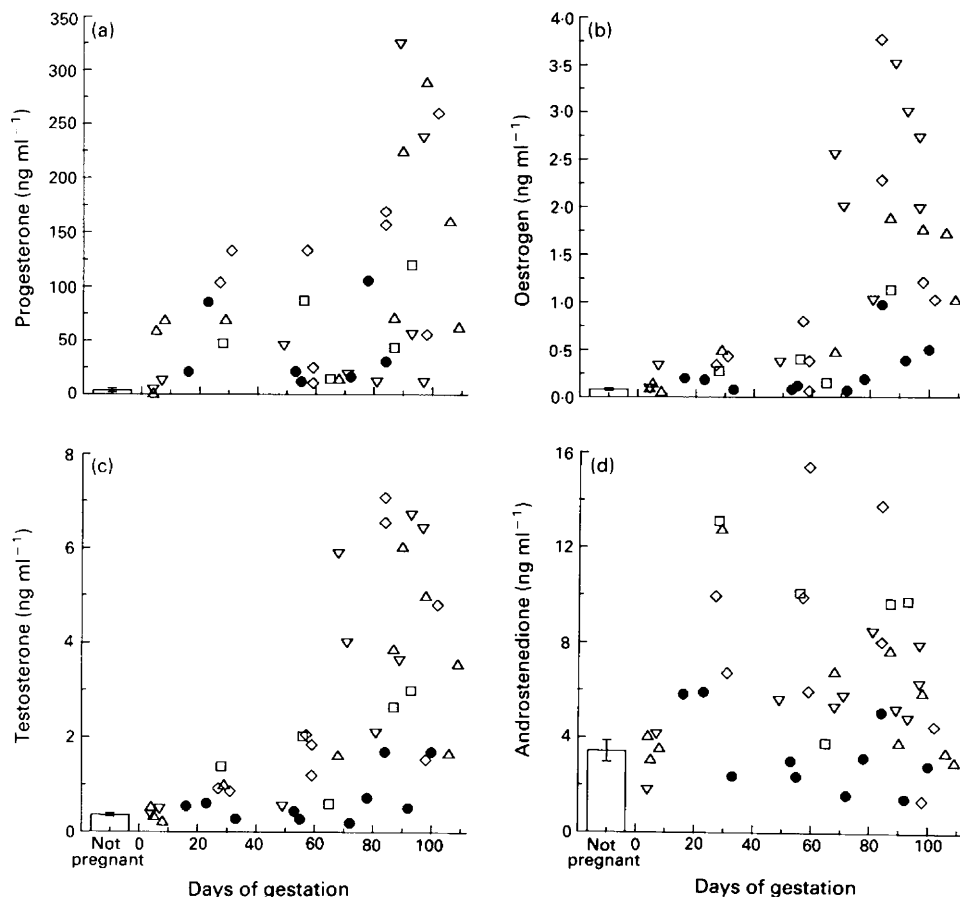
Fig. 1. Concentrations of plasma (a) progesterone, (b) oestrogen, (c) 'testosterone' and (d) androstenedione during pregnancy in five spotted hyaenas; 15 pregnancies are represented. Different symbols represent individual animals during pregnancy and correspond to the same individuals in each graph. The bar at the left indicates the average value of the steroid in 36 samples taken from the same animals when not pregnant (vertical bar shows 95% confidence limits for the mean); (●) represent a single female (no. 16), which showed aberrant parental behaviour (infanticide). All values were above minimal detectable limits of the assay.

The temporal pattern for androstenedione was distinct from those of the other three steroids (Fig. 1d). While androstenedione during pregnancy (6.06 ± 0.52 ng ml^{-1}) was on average significantly higher ($P < 0.001$) than during nonpregnancy (3.42 ± 0.45 ng ml^{-1}), there was not a clear temporal trend. Plasma androstenedione was not correlated with oestrogen ($P > 0.4$), 'testosterone' ($P > 0.12$) or progesterone ($P > 0.3$). The magnitude of the change between samples from pregnant and nonpregnant animals was considerably less than for the other steroids. For example, even the single maximal pregnancy androstenedione values recorded for each female (11.1 ± 1.7 ng ml^{-1}; range 5.8–13.1) were only about three times greater than values for nonpregnant animals compared with the order of magnitude increases in the other three steroids.

In one female sampled 48 h after parturition, values of all steroids were at pre-conception levels. Similar results were obtained for several samples taken at 2–4 weeks after parturition.

One of the five females (no. 16) was unusual in killing her young: they were eaten shortly after birth in three of four pregnancies (fetuses were removed by Caesarean section near term in the fifth). Concentrations of all four steroids for this female during pregnancy tended to be lower than
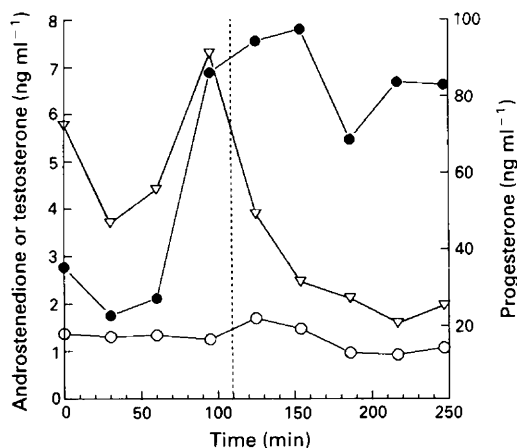
Fig. 2. Steroids in the cephalic vein of an anaesthetized pregnant spotted hyaena (no. 16) at about 96 days of gestation. Removal of the fetal–placenta unit (male) was completed at the time shown by the vertical dotted line. Corresponding values for fetal blood samples are shown in Table 1; ($\triangledown$) progesterone, ($\bullet$) androstenedione and ($\bigcirc$) 'testosterone'.

for other individuals (see shaded circles in figures). The other four females did not show consistent differences. No differences among these five females were evident during nonpregnant periods.

There was little overlap in plasma 'testosterone' between pregnant and nonpregnant hyaenas after the first month of gestation (except in the female that had aberrant maternal behaviour). During the final third of pregnancy, the highest values recorded for individual females ($4 \cdot 9 \pm 1 \cdot 07$ ng ml^{-1}) did not differ significantly from the highest values in males ($4 \cdot 1 \pm 0 \cdot 78$ ng ml^{-1}) (Glickman *et al.*, 1992). In contrast, maximal individual 'testosterone' values in nonpregnant females were consistently lower than in males (Glickman *et al.*, 1992).

Radioimmunoassay after chromatographic separation

All progesterone and androstenedione immunoreactivity was recovered in appropriate chromatographic fractions; correlations between values obtained with and without chromatography were high for both ($r > 0 \cdot 95$). Chromatographic separation of oestrogens and androgens was focused on plasma samples from pregnant females with high concentrations and on samples from males with > 1 ng 'testosterone' ml^{-1} as shown by radioimmunoassay.

Oestrogen immunoreactivity recovered in the oestradiol fraction accounted for only 12% ($n = 30$) of the total 'oestrogen' measured by the same antiserum without chromatography, but estimates of oestradiol from chromatographed samples correlated positively with total 'oestrogen' estimated without chromatography ($r = 0 \cdot 5$; $P < 0 \cdot 05$). Most of the remainder of the oestrogen activity co-chromatographed with oestrone, but about 20% crossreactivity also occurred in other fractions. Limited tests of nonchromatographed plasma samples, with an antiserum specific for oestradiol, agreed with values obtained from oestradiol chromatographic fractions using the less specific antiserum. Plasma samples from nonpregnant females ($n = 6$) also showed significant activity in the oestrone fraction, but the activity in the oestradiol fraction accounted for 46% of the total.

Chromatographic analysis of samples from adult males ($n = 17$ from five individuals) and pregnant females ($n = 20$ from five individuals) with high 'testosterone' revealed that dihydrotestosterone comprised a significant proportion of the total (testosterone plus dihydrotestosterone), but the percentage of dihydrotestosterone in females ($44 \cdot 1 \pm 3 \cdot 9\%$, $n = 20$) was on average significantly higher ($P < 0 \cdot 01$) than in males ($29 \cdot 5 \pm 3 \cdot 5\%$, $n = 17$). Thin-layer chromatography of the pooled plasma from pregnant females indicated that testosterone and dihydrotestosterone existed in approximately equal proportions.
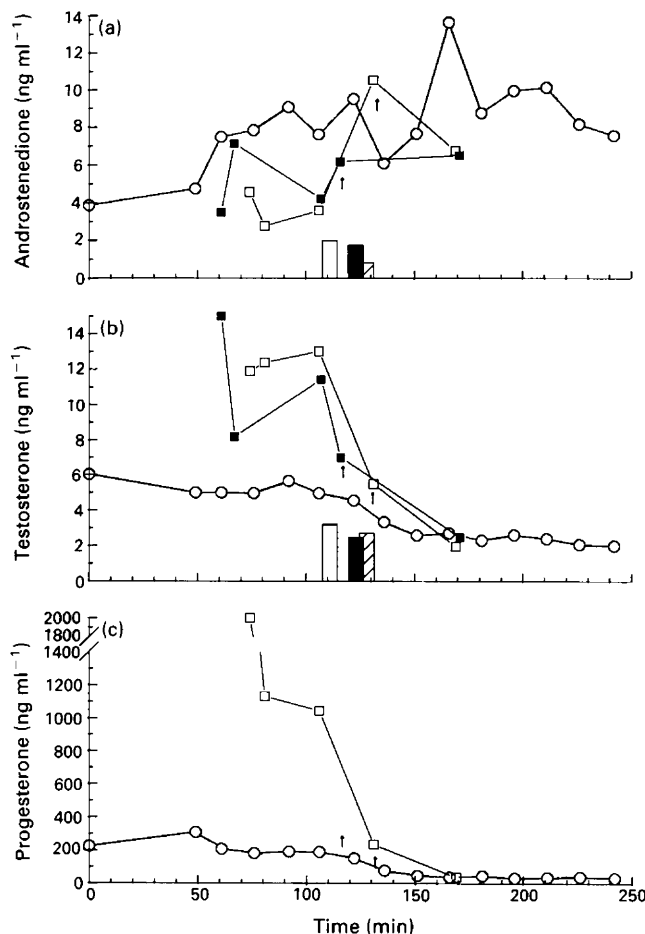
Fig. 3. Profiles of (a) androstenedione, (b) 'testosterone' and (c) progesterone in maternal (no. 7) and fetal circulations in a spotted hyaena with mixed-sex twins at about 90 days of gestation. Curves show maternal samples obtained from cannulae in the jugular vein (○) and in the veins on the uterine horns (□ right and ■ left); the right fetus was a female and the left was a male. Vertical arrows indicate the time during the procedure when placentae were removed from the left and right horns of the uterus. Samples from male fetus umbilical vein (□) and female fetus umbilical vein (■) and artery (▨) are shown by vertical bars. Umbilical cord progesterone (data not shown) were in the same range as for uterine veins.

Individual concentrations of testosterone and dihydrotestosterone correlated highly with total 'testosterone' measured without chromatography (e.g. for testosterone in males, $r = 0.94$, $P < 0.0001$; and females, $r = 0.6$, $P = 0.03$). Overall, these results show that analyses of 'oestrogen' and 'testosterone' without chromatography overestimates actual oestradiol and testosterone, but they accurately reflect trends in the concentrations of these two steroids (with parallel changes in dihydrotestosterone). Ratios of dihydrotestosterone:testosterone in uterine vein samples were similar to those in the general maternal circulation.

Fetal and concurrent maternal plasma steroids

Female no. 1. A single maternal jugular vein sample was taken with concurrent samples from fetal jugular and umbilical veins at 96 days of gestation in mixed-sex twins (Table 1). There was

little difference in androgens between the male and female fetuses and concentrations of both steroids were slightly lower than in the maternal circulation. A striking oestrogen gradient was evident between fetal jugular and umbilical veins, indicating a placental origin for these steroids. The gradient in 'testosterone' was in the same direction, but of lesser magnitude, whereas androstenedione exhibited the opposite gradient.

Table 1. Steroids (ng ml^{-1}) in maternal (female no. 1) and fetal circulation at about 96 days of gestation in mixed-sex twins in spotted hyaenas

Steroid	Maternal vein	Male fetus		Female fetus	
		Jugular vein	Umbilical vein	Jugular vein	Umbilical vein
Progesterone	11·1	11·02	11·45	14·50	20
Oestrogen	2·0	0·04	1·42	0·07	1·36
Androstenedione	4·7	4·04	1·3	4·7	1·65
Testosterone	9·1	4·01	7·5	5·4	8·2

Female no. 16. Fetal studies on a second pregnancy at the same gestational stage involved the female with atypically low steroid values during pregnancy (shaded circles in Figs 1 and 2) carrying a single male fetus. Temporal profiles in the maternal cephalic vein revealed that removal of the placenta had different effects on each steroid (Fig. 3): 'testosterone' and oestrogen (data not shown) stayed relatively constant, but androstenedione increased and progesterone decreased.

A sharp gradient (approximately tenfold) between maternal uterine and cephalic veins (Table 2) demonstrated the placental output of progesterone, oestrogen and 'testosterone'. In contrast, the reverse gradient in androstenedione indicated that the placenta metabolizes this steroid. Samples from the fetal umbilical and general circulation (Table 2) confirmed the placental origin of oestrogen and progesterone. The fetus (jugular and both umbilical vessels) had slightly higher concentrations of 'testosterone' and lower androstenedione than the mother. There was a small gradient (20%) in 'testosterone' across the placenta, but androstenedione dropped by 50%.

Table 2. Steroids (ng ml^{-1}) in the circulation of a male fetus of spotted hyaena at about 96 days of gestation (female no. 16).

Steroid	Maternal veins		Fetus			
	Cephalic	Uterine	Heart	Jugular vein	Umbilical artery	Umbilical vein
Time (min)[a]	95	92–99	123	118	109	108
Progesterone	91	884	1495	1148	1086	683
Oestrogen	0·53	3·0	<0·04	<0·04	0·18	1·0
Androstenedione	6·9	4·2	5·8	6·2	3·0	2·4
Testosterone	1·25	9·8	4·9	4·0	4·6	2·25

[a]Time after anaesthesia of females (compare with Fig. 2); maternal uterine vein data represent the average for a sample taken at 92 and another at 99 min.

Female no. 7. The most complete set of data came from a pregnancy involving a mixed set of twins estimated at 85–90 days of gestation. The initially high concentrations of progesterone and 'testosterone' in the two uterine veins compared with the jugular, and the precipitous decline in their absolute values and gradients after removal of the placentae support a placental origin for these steroids in the maternal circulation (Fig. 3). Oestrogen showed a similar pattern, but values in

the uterine vein samples were only about 60% higher than in the jugular. In contrast, concentrations of androstenedione in uterine veins were the same as or lower than in the jugular (i.e. reverse gradient) and all tended to increase after removal of the fetal–placental units (Fig. 3). Steroids measured in several samples from the left ovarian arteries were indistinguishable from jugular samples at the same time (data not shown). In a single sample from the ovarian venous drainage (at 90 min), progesterone (184 ng ml^{-1}), oestrogen (3·02 ng ml^{-1}) and 'testosterone' (7·3 ng ml^{-1}) approximated values recorded for the jugular, but androstenedione (313 ng ml^{-1}) was 35 times higher than in jugular or uterine samples.

There was little difference between umbilical samples from the male and female fetuses; concentrations in the umbilical artery and vein in the female were similar. Progesterone concentrations (data not shown) in all fetal vessels were similar and close to those recorded for uterine veins (900–2100 ng ml^{-1}). Jugular concentrations of testosterone in the male fetus 10 min after detachment was comparable to that of the last umbilical vein sample (3·37 versus 3·32 ng ml^{-1}), but androstenedione was higher in the jugular (9·33 versus 1·8 ng ml^{-1}). Similar jugular sampling from the female fetus at 20 min after detachment showed reduced 'testosterone' (1·84 versus 3·08 ng ml^{-1}) combined with high androstenedione (6·9 versus 2 ng ml^{-1}). In both cases, oestrogen was much lower in the jugular, but progesterone was unchanged.

Discussion

Since the initial report by Phoenix *et al.* (1959), it has become customary to distinguish between organizing and activating effects of steroids. Permanent or semi-permanent organizational effects are generally linked with actions during fetal or perinatal periods, while more-transient activational effects are commonly associated with changes in gonadal steroids occurring at or after puberty. Although there are limits to this dichotomous characterization of steroid actions (e.g. Arnold & Breedlove, 1985), high steroid (notably androgen) concentrations during pregnancy and development may have implications for organizational influences on the developing fetus and activational effects in pregnant female spotted hyaenas.

Androgens are commonly high in the maternal plasma during pregnancy, but the timing is variable. For example, in dogs (Concannon & Castracane, 1985), marmosets (Chambers & Hearn, 1979) and baboons (Castracane & Goldzieher, 1983), concentrations of androstenedione and testosterone are high during the early stages of pregnancy and then decline. In cows (270-day pregnancy), increases in these androgens begin shortly after the first third of pregnancy and continue until parturition, as in hyaenas, but peak values (1·4 ng androstenedione ml^{-1}, 0·22 ng testosterone ml^{-1}) (Gaiani *et al.*, 1984) are markedly lower than in hyaenas. Thus, the spotted hyaena presents a unique picture of sustained, high plasma concentrations of androgens during most of gestation; the high dihydrotestosterone is especially striking in the context of masculinization of the external genitalia.

Since maternal steroids can affect the fetus via the placental circulation (Winter *et al.*, 1981), the high maternal concentrations of all androgens suggest a maternal influence on the developing fetus. The female fetus is normally 'protected' from such masculinization by a variety of mechanisms. For example, the presence of high concentrations of aromatase in the placenta and fetal liver promotes the conversion of androgens to oestrogen (Siiteri & Seron-Ferre, 1978). The oestrogens may, in turn, be bound to proteins that do not pass through the placenta, or are bound and inactivated by proteins in the fetal circulation (Siiteri & Seron-Ferre, 1978; Winter *et al.*, 1981). Our data suggest that such placental protection is minimized in the hyaena. Especially noteworthy here is our preliminary finding that placental homogenates from hyaena had a 20–40-fold lower aromatase activity with concomitant increases in testosterone production than in humans, combined with a low concentration of sex-steroid-binding globulin in fetus and neonates (Yalcinkaya *et al.*, 1991). The high proportion of dihydrotestosterone would obviate the possibility of aromatizing all the measured androgen.

The protective mechanisms that normally inactivate androgens before they reach the fetus are of limited effectiveness, since significant masculinization of morphology and behaviour has been achieved by administration of testosterone or androstenedione via the maternal circulation; e.g. in guinea-pigs (Phoenix *et al.*, 1959), dogs (Beach *et al.*, 1972; Beach, 1984) and monkeys (Phoenix *et al.*, 1968; Goy & Resko, 1972). Each of the steroids increased in pregnant spotted hyaenas has been an effective 'masculinizing' agent when administered to a developing female fetus of other species. Dihydrotestosterone has been particularly associated with masculinization of the external genitalia, while testosterone and oestrogen have been linked to masculinization of neural substrates of behaviour in the brain (Ward, 1972; McEwen, 1983). Plasma androstenedione may serve as a substrate for dihydrotestosterone production and mimic its effect on peripheral tissues (e.g. Labrie *et al.*, 1988).

Evidence for the influence of endogenous maternal hormones on fetal development in rats comes from the 'feminization' and 'demasculinization' of males born to stressed mothers (Ward, 1972) and from 'defeminizing' effects of the normal secretions of the maternal ovary during pregnancy on females (Witcher & Clemens, 1987). Stavy (1987) suggests that in African hares (*Lepus capensis syriacus*) high testosterone and oestrogen in the maternal peripheral blood during the final weeks of gestation '. . . may cause a "partial androgenization" of the female fetus, and thus influence development of masculinized characteristics of the adult female hare'. In humans, 'excess' androgen from abnormal maternal adrenocortical activity may cause virilization of the female fetus (Kirk *et al.*, 1990).

A major issue for the spotted hyaena concerns the source of the circulating androgens during pregnancy. Matthews (1939) first suggested that the masculinization of female hyaenas might be caused by androgens secreted by the maternal ovary, but Racey & Skinner (1979) rejected this hypothesis because of the low concentrations of androgens in the maternal ovary and adrenal gland; they proposed that the fetal ovary or adrenal gland was the primary source of these androgens. This conclusion, which tends to diminish the importance of the high androgen in the mother, was reiterated by Lindeque & Skinner (1982) based on comparisons between several maternal and fetal samples. First, they note that maternal 'testosterone' was about six times lower than concentrations in fetal blood (approximately 1·4 versus 5 ng ml^{-1}) in a set of twins estimated to be 31 days of gestational age; values were comparable in presumed male and female fetuses. Second, a cardiac sample from a male fetus (estimated at 80 days of gestation) had about twice as much testosterone as a female twin (approximately 5 versus 2·7 ng ml^{-1}); the male matched the maternal plasma testosterone most closely. Most importantly, samples from the two umbilical veins were virtually devoid of androgens (< 1 ng androstenedione ml^{-1} and $< 0·1$ ng testosterone ml^{-1}). The placenta was also not regarded as a major contributing factor to fetal testosterone concentrations because of its low tissue content and they ruled out the adrenal as a major source of androgen since they found no evidence of fetal adrenal hypertrophy.

While we concur that the maternal ovary is probably not the primary direct source of testosterone, our more extensive results point to the placenta as a major source of steroids, rather than the fetal gonad, especially for the female fetus. Our data differ in several important respects from those presented by others. Concerning maternal values, Racey & Skinner (1979) initially denied the increase in androgen concentrations during pregnancy. This conclusion was later amended (Lindeque *et al.*, 1986), but the magnitude of this rise was not appreciated, perhaps because of the limited data for staged pregnancies. More importantly, we did not observe a pronounced reverse gradient across the placenta in the fetal circulation or between maternal plasma and the umbilical vein. In all five umbilical vein samples, testosterone concentrations were high and close to that of the fetal jugular or umbilical artery. Hormone profiles in the mother (Figs 2 and 3) also point to the placenta as a primary source of the active androgens (testosterone and dihydrotestosterone). Reduced plasma concentrations after ovariectomy showed that the bulk of the androgens ('testosterone' and androstenedione) in nonpregnant hyaenas are of ovarian origin (Glickman *et al.*, 1992). The present data also indicate that the ovary is the primary source of androstenedione

during pregnancy, but probably not of testosterone and dihydrotestosterone. The differences in androgen binding activity between maternal and fetal blood and the complex nature of the two circulations within the placenta complicate interpretations of relative steroid concentrations in mother and fetus. Morphological analysis indicated that the hyaena placenta is distinct from that of other carnivores and suggested that it was highly efficient for transporting substances to the fetus (Wynn & Amoroso, 1964).

Gradients, temporal patterns and the correlations among testosterone or dihydrotestosterone, oestrogen and progesterone, and their decline after removal of fetal–placental units support the conclusion that high 'testosterone', oestrogen and progesterone in the maternal circulation are derived from the placental–uterine complex. A fetal gonadal origin for this 'testosterone' is ruled out by the positive gradient between uterine veins and fetal jugular or umbilical arteries (e.g. Fig. 3 and Table 1). In contrast, ovarian and uterine vein profiles of androstenedione (and lack of correlations with the other steroids) indicate that this androgen is primarily of maternal ovarian origin and that it is metabolized by the placenta. These conclusions are of special interest in the light of our data suggesting that the hyaena placenta produces unusually large amounts of testosterone from conversion of androstenedione (Yalcinkaya *et al.*, 1991); the precise source of the unusually high dihydrotestosterone concentrations remains to be defined. The small increase in jugular concentrations of androstenedione in the pregnant female probably underestimates the actual increase in androstenedione production because of placental metabolism. Results for the fetal circulation are also consistent with these conclusions.

The fetal testes may contribute to fetal androgen titres. For example, 'testosterone' concentrations were slightly higher in the males of mixed-sex twins (Lindeque & Skinner, 1982; this study). Secondly, 'testosterone' concentrations dropped more in a female fetus than in a male fetus after disconnection from the placenta, although the 10 min difference in sampling time complicates interpretation of these data. More importantly, plasma 'testosterone' in female neonates is lower soon after birth and then drops faster than in males in the next few days (Frank *et al.*, 1991).

Although our fetal studies were restricted to late stages of gestation (after substantial virilization had been completed), we suggest that the presence of high fetal androgen concentrations during earlier 'formative' periods (e.g. the entire second two-thirds of gestation) may be inferred from the high concentrations of 'testosterone' in the maternal circulation (see Fig. 1c, d). Thus, although maternal 'testosterone' concentrations may not contribute directly to fetal physiology, they probably indicate the fetal condition. Taken with our evidence for low placental aromatase activity and high testosterone production already mentioned, we suggest that the high maternal ovarian output of androstenedione provides a substrate for production of testosterone (and dihydrotestosterone?) by the placenta and that this serves as a major source of active androgen that may contribute to the masculinization of the female fetus. Extreme female pseudohermaphroditism reported in a newborn human (the mother was also temporarily virilized) and attributed to deficient placental aromatase activity (Shozu *et al.*, 1991); this condition contributed to abnormally low oestrogen and high maternal androgen concentrations (~ 10 ng testosterone ml^{-1} and 2 ng dihydrotestosterone ml^{-1}) that were within the range recorded for pregnant hyaenas (Fig. 1c, d).

We do not have direct evidence for the role of high steroid concentrations in the physiology and behaviour of pregnant hyaenas, but the female (no. 16) characterized by the consistently lowest steroid values was the only one that showed inadequate parental care (infanticide); her one daughter appeared 'normally' virilized. Unfortunately, we lack direct information on fetal androgen concentrations in her female progeny, but her male fetus had higher 'testosterone' than in the maternal circulation (Table 2); this relationship was not observed in the other pregnancies. The high uterine vein values of progesterone, testosterone and oestrogen in this female suggest that the generally low peripheral concentrations of these steroids may reflect 'aberrant' metabolism or clearance rather than low production *per se*. These results emphasize the difficulty in interpreting the source of steroids based solely on absolute concentrations in general maternal and fetal circulations.

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