Plasma androgen concentrations in initial samples from spotted hyaenas immobilized with Zoletil (CI-744) reflect hormonal status estimated by GnRH challenge and immobilization stress response

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The use of single samples to assess the androgen status of animals can be problematic owing to extensive short-term fluctuations in the concentrations of these hormones. Thus, this study evaluated a number of estimates for both testosterone and androstenedione status in the spotted hyaena (*Crocuta crocuta*), namely, initial samples, the immobilization stress response and the response to exogenous GnRH administration, for their mutual consistency. This study found that in animals immobilized with Zoletil and maintained on halothane, all three estimates show a good consistency. Moreover, the large variance in androgen status reported here within the sexes explains earlier contradictory results, and implies that the debate concerning the sex-specific differences in plasma androgens is confounded by other variables such as the reproductive and social status of the individuals included in the analysis.

Die gebruik van enkele bloedmonsters om die androgeenstatus van diere te bepaal kan probleme oplewer, hoofsaaklilk as gevolg van grootskaalse korttermynskommelings in die konsentrasies van hierdie hormone. Dus is 'n studie wat 'n aantal skattings van beide testosteroon- en androsteendioonstatus in die gevlekte hiëna (*Crocuta crocuta*) naamlik, die oorspronklike monsters, die immobilisasie stressreaksie en die reaksie op uitwendige gonadotrofiese hormoontoediening, ge-evalueer vir onderlinge konstantheid. In hierdie studie is gevind dat in diere wat met Zoletil ge-immobiliseer is, en met halothane onder verdowing gehou is, al drie skattings 'n merkwaardige konstante patroon openbaar. Die groot variasie in androgeenstatus wat hier binne die geslagte gevind is, verklaar ook vroëere teenstrydige resultate, en impliseer dat die debat aangaande die geslagspesifieke verskille in plasma-androgeenstatus deur ander faktore soos die voortplanting- en sosiale status van die individue wat in die analise ingesluit is, verdoesel word.

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The role played by androgens during the establishment of both genital monomorphism, and female dominance in the spotted hyaena (*Crocuta crocuta*) has been the subject of extensive enquiry (Racey & Skinner 1979; Gould & Vrba 1982; Lindeque & Skinner 1982; Hamilton, Tilson, & Frank 1986; Lindeque, Skinner & Millar 1986; Glickman, Frank, Davidson, Smith & Siiteri 1987; Van Jaarsveld & Skinner 1987). Moreover, it has become increasingly clear that the unusual hormonal manifestations in this species could enhance present understanding about the role played by androgens during sexual differentiation and the establishment of behavioural dominance in other mammals. However, estimates of plasma androgens in animals can be unreliable owing to the marked fluctuations that occur over short periods of time. In domestic species this problem may be circumvented by taking serial blood samples at short intervals. On the other hand, in animals in the wild state serial sampling is only feasible after chemical capture and prolonged anaesthesia (Illius, Haynes, Lamming, Howles, Fairall & Millar 1983; Lindeque *et al.* 1986; Brown, Goodrowe, Simmons, Armstrong & Wildt 1988).

It is known that chemical capture and anaesthetic procedures may modify endocrine function, and consequently 'provocation' tests in which hormonal responses to the administration of exogenous hormones or releasing factors were suggested as estimates of androgen status in captured narcotized wild animals (Illius *et al.* 1983; Brown *et al.* 1988). However, these procedures are laborious, expensive and may be difficult to perform on animals of all ages and sizes, thus precluding comparative analyses. The use of 'provocation' tests as indicators of hormonal status in spotted hyaenas has been the subject of a single preliminary investigation by Lindeque *et al.* (1986), who illustrated that the administration of GnRH, hCG and ACTH is an effective way of evaluating both adrenal and gonadal contributions to the hormonal status of individuals in this species. Although positive responses were recorded by Lindeque *et al.* (1986), it has not been established whether estimates of androgen status obtained by provocation tests provide more accurate estimates than single samples collected after chemical capture. As comparative basal levels from non-narcotized animals are unavailable, the accuracy of estimates can only be evaluated by comparing various estimates with one another. Different results obtained using the various estimates would then indicate biases in any one or more of the hormonal status estimates. In order to evaluate the necessity of performing a pituitary challenge to estimate both testosterone and androstenedione status in the spotted hyaena, different estimates obtained under halothane anaesthesia following Zoletil (CI-744) immobilization were investigated for inconsistencies.

Materials and Methods

Eighteen spotted hyaenas (> 2 years) were sampled between July 1984 and July 1987 from the Kruger National Park and the Kalahari Gemsbok National Park, South Africa. The entire procedure described below was performed on the sample of six males and twelve females, sexed by

scrotal palpation and nipple morphology (Matthews 1939).

Free-ranging hyaenas were immobilized with Zoletil (~ 4mg/kg, CI-744: Anchorpharm Pty. (Ltd) Bramley, S.A.) following Van Jaarsveld (1988). Extended anaesthesia (4 h) for serial blood sampling was achieved by administering halothane (Fluothane: I.C.I. Pharmaceuticals Ltd, Johannesburg, S.A.) using a circle absorber machine. Induction was achieved with a 10% mixture of halothane in oxygen and the animals were maintained using 2–4% halothane in a closed circuit. This method was preferred as it provided a more stable plane of anaesthesia than other chemical immobilizing agents. Clinical procedures followed Van Jaarsveld, McKenzie & Meltzer (1984).

All animals were subjected to serial blood sampling. Blood samples (10 ml) were collected from the cephalic or saphenous veins using multi-sample needles and heparinized venoject evacuated tubes. Samples were stored upright at 4°C until centrifuged for 10 min at 3000 r.p.m.. Plasma was stored at –20°C until assayed. Serial blood sampling in the field was started as soon as possible following immobilization. Animals were weighed, intubated and attached to a circle absorber machine, and subsequently left to stabilize for 90 min. A Ringer-lactate drip (Keagrams Ltd, Johannesburg, S.A.) was inserted in the jugular vein to replace fluids lost during blood sampling. After 90 min the animals were administered 1 μg GnRH (LH–RH; Hoechst, Frankfurt, West Germany) per kg body weight through intravenous injection. Administered doses were in accordance with those previously found effective in wild ungulates (Illius *et al.* 1983). Mass dependent doses were administered to compensate for peripheral dilution in animals of different body sizes. Serial blood samples were collected at approximately 15-min intervals for the total duration of anaesthesia (4 h). Furthermore, rectal temperature was monitored intermittently and, if declining, arrested with the aid of hot water bottles and solar blankets.

Plasma testosterone was assayed using the same chemical reagents, and following identical procedures previously described by van Aarde & Skinner (1986). Duplicate plasma sample (0,05–0,5 ml) were assayed using antisera raised in rabbits against testosterone-3-carboxymethyl-oxine conjugated to bovine serum albumin (Millar & Kewley 1976). Testosterone antiserum specificity was described by Millar & Kewley (1976). Cross-reaction with all major steroids was < 0,1% except for dihydrotestosterone for which it was 5,1%. Assay sensitivity ranged from 40 to 290 pg/ml ($\bar{x}$ = 140,30 ± 96,81 *s.d.*; n = 12). Recovery estimates for a plasma pool ranged from 85–98% (91,18; n = 12) for 100 μl samples and was 85% (n = 1) for 500 μl samples. Intra- and inter-assay coefficients of variation were 7,8% and 9,8% respectively. Buffer blanks contained 12,17 ± 0,11 pg/ml (n = 12) testosterone equiv./ml. Parallelism was demonstrated over the whole range of the standard curve by serial double dilution of plasma samples. Accuracy was estimated by the addition of 250, 500, 1000, 2000, 2500, 3750 and 5000 pg testosterone/ml (Δ^4-androsten-17α-ol-3-one; Sigma Chemical Co., Dorset, U.K.) to a plasma pool, and resulted in recoveries of 105% (n = 7) over the range.

Plasma androstenedione was assayed by radio-immunoassay using duplicate plasma aliquots (0,1 ml) following extraction with 4 ml of a 4 : 1 (v/v) mixture of hexane (Merck, Analar; Darmstadt, F.R.G.) and diethyl ether (Merck, Analar; Darmstadt, F.R.G.). Dried extracts were dissolved in 0,1 ml phosphate buffer containing 1% gelatine and 1% azide (pH 7,0). Standards ranging from 7,8 to 1000 pg androstenedione (Δ^4-androstene-3,17-dione; Sigma Chemical Co., Dorset, U.K.) per 0,1 ml phosphate buffer and buffer blanks were included in duplicate in each assay. Antiserum raised against androstenedione-7-hemisuccinate-bovine serum albumin (Miles Yeda, Kiryat Weizman, Rehovot, Israel) in rabbits, and diluted to 13 ml in phosphate buffer was added (0,1 ml) to standards, reagent blanks and plasma extracts. To this was added tritiated androstenedione (TRK 454 ([1,2,6,7-^{3}H] androst-4-ene-3,19-dione: Radiochemical Centre, Amersham, Bucks, U.K.) dissolved in assay buffer in 0,1 ml (~ 10 000 c.p.m.) aliquots. The contents of each tube were mixed and incubated overnight at 4°C. Separation of antibody-bound and free androstenedione was carried out at 4°C by adding 0,75 ml dextran-coated charcoal consisting of a suspension of charcoal (0,156%; Aktivole; Merck, Darmstadt, F.R.G.) in assay buffer containing 0,0156% Dextran T-40 (Pharmacia, Uppsala, Sweden) to each tube. These solutions were mixed gently for 30 s, incubated at 4°C for 12 min and centrifuged at the same temperature at 1500 g for 10 min. The supernatants were decanted into scintillation vials and scintillation fluid (4,0 ml; Scintillator 299™; Packard Instrument Co., Illinois, USA) was added to each vial. The contents of the vials were mixed well and radioactivity was measured at least 4 h later for 2 min, using a Packard 1500 Tri-Carb scintillation counter (Packard Instrument Co., Illinois, USA). Mathematical interpolation of recorded sample values against a standard curve was carried out using Securia™ Plus RIA/QC software (Packard Instrument Co., Downers Grove, UK) over the range 7,8–1000 pg/tube. Recovery of known amounts of [1,2,6,7-^{3}H] androst-4-ene-3,17-dione (~ 10 000 c.p.m.) in phosphate buffer to which pooled plasma were added, served to determine procedural losses incurred during extraction. Extraction efficiency and the original volume of plasma (0,1 ml) extracted were taken into account when calculating the concentrations of androstenedione in plasma samples.

Androstenedione antiserum specificity was determined by the suppliers (Bio-Yeda). Cross-reaction with other steroids was as follows: 5-androstane-3,17-dione 32%, testosterone and dehydroepiandrosterone 3%, 11-deoxycorticosterone and progesterone 0,6%, oestrone 0,2% and oestradiol-17β, < 0,01%. Sensitivity of the assays ranged from 3,92 to 16,70 pg/ml ($\bar{x}$ = 5,98 ± 3,63 *s.d.*; n = 7). Extraction efficiency ranged from 87,66–96,63% ($\bar{x}$ = 90,31; n = 7) and was unaffected by plasma volume (50–500 μl). Buffer blanks contained 14,81 ± 29,22 pg/ml androstenedione equiv./ml. The addition of 250, 500, and 1000 pg androstenedione / tube to a plasma pool, resulted in recoveries of 99% (n = 3) over the range. Parallelism was evident over the entire range of the standard curve, and intra- and inter-assay coefficients of variation were 7,31% and 5,28% respectively.

A number of frequently used estimates of hormonal status (Brown *et al.* 1988) were compared to determine if any showed biases in testosterone or androstenedione status values, namely:

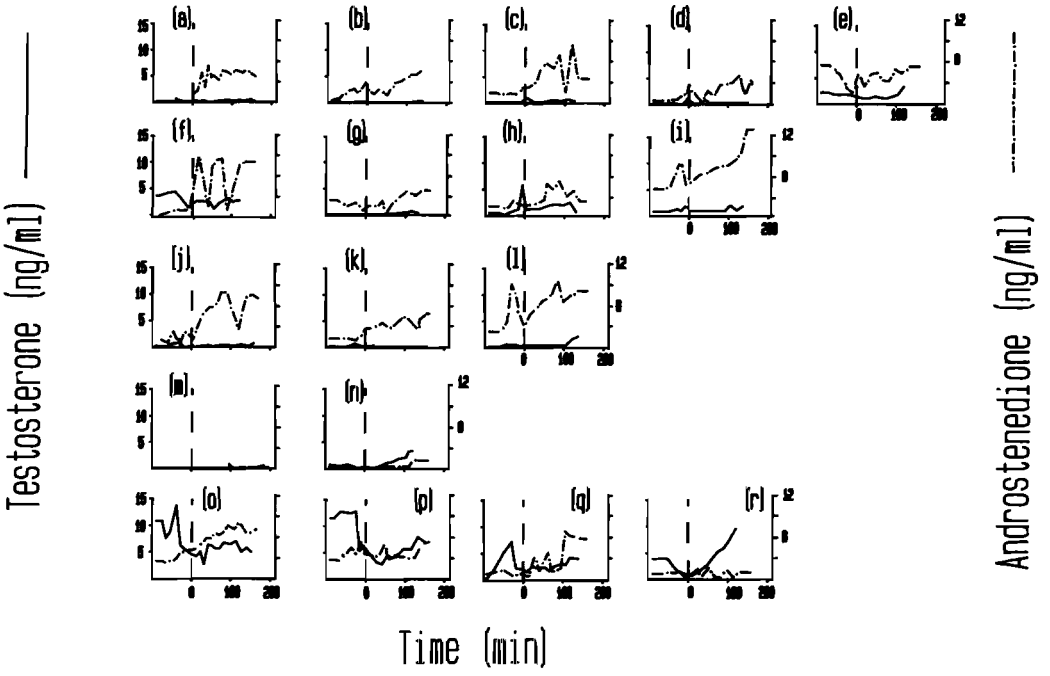

Figure 1 Combined testosterone and androstenedione profiles for 18 spotted hyaenas, including nine parous females (a–i), three nulliparous females (j–l), four adult males (o–r) and two sub-adult males (m,n) anaesthetised with halothane following Zoletil immobilization. The three androgen status estimates, namely, the initial sample (•), immobilization stress response (–90 to 0 min) and GnRH response (0 to 150 min) are indicated. GnRH was administered at time zero.

(a) *Initial sample* — which was represented by the first blood sample collected from an immobilized hyaena, irrespective of the elapsed time since darting.

(b) *Immobilization response* — which was represented by the area beneath the response curve for the first 90 min of anaesthesia (Figure 1). This represents the stress response of the animal to immobilization as a controlled stressor (Sapolsky 1982). Areas were measured with a graphic tablet and using a CADDIE graphics system (Letter Graphics, Johannesburg).

(c) *GnRH response* — the procedure used to estimate the extent of hormone secretion in response to exogenous GnRH stimulation followed the procedure described by Brown *et al.* (1988). The area beneath the curve was measured from the time of GnRH administration to the termination of anaesthesia: 90–240 min after immobilization (Figure 1).

All statistical procedures used were in accordance with the statistical principles formulated by Sokal & Rohlf (1980). Statistical manipulations were carried out on the University based mainframe computer using SAS Institute Inc. (Illinois, USA) software. The relevant procedures used are specified together with the results.

Results

The testosterone and androstenedione profiles obtained for all animals investigated are given in Figure 1. There is a tendency for males (Figure 1 m–r) to show a larger testosterone response than females (Figure 1 a–l) during both the immobilization stress response and following GnRH stimulation. However, the two sub-adult males (Figure 1 m,n) showed weaker testosterone responses than the four adult males (Figure 1 o–r) investigated. Also, a total lack of testosterone response was recorded in the nulliparous females (Figure 1 j–l), whereas the parous females (Figure 1 a–i) showed a large variation in testosterone responsiveness. Some parous female testosterone responses were comparable with those found in males (Figure 1 e,f,h,i), however, most females did not respond at all. In contrast with testosterone, females (Figure 1 a–l) generally seem to have higher androstenedione responses than males (Figure 1 m–r). Both parous (Figure 1 a–i) and nulliparous (Figure 1 j–l) females showed significant responses, while adult males (Figure 1 o–r) were comparable with females and the two sub-adult males (Figure 1 m,n) showed very little response.

The various estimates of testosterone and androstenedione status evaluated, namely, the initial sample, immobilization stress response and GnRH stimulation, all revealed similar relative values of hormonal status. This is apparent from the high rank correlations (r_s) obtained when comparing these different estimates (Table 1). These results suggest that the estimates show good consistency, and there is no clear advantage associated with laborious 'provocation' procedures performed under prolonged anaesthesia. This suggests that initial samples are as effective as 'provocation tests' in estimating androgen status under this particular anaesthetic regime.

Using initial samples as an index of testosterone status, males (4,52 ± 2,18 s.e.m. ng/ml; n = 6) in this sample had significantly higher ($t_{0,05[16]}$ = 2,42) testosterone titres than females 0,75 ± 0,32 s.e.m. ng/ml; n = 12), whereas females (1,67 ± 0,48 s.e.m ng/ml; n = 12) had significantly higher ($t_{0,001[16]}$ = 7,26) plasma androstenedione titres than males (1,14 ± 0,47 s.e.m. ng/ml; n = 6). A dis-

Table 1 Calculated relationships between three estimates of androgen status in the spotted hyaena ($n = 18$). All estimates were collected from the same animals during a single immobilization. SAS PROC CORR SPEARMAN (r_s) was used

Estimates	Spearman's coefficient (r_s)	Significance level
Testosterone		
1. Initial sample *vs* immobilization response	0,82	$p < 0,001$
2. Initial sample *vs* GnRH response	0,77	$p < 0,001$
3. Immobilization response *vs* GnRH response	0,91	$p < 0,001$
Androstenedione		
1. Initial sample *vs* immobilization response	0,91	$p < 0,001$
2. Initial sample *vs* GnRH response	0,55	$p < 0,05$
3. Immobilization response *vs* GnRH response	0,66	$p < 0,05$

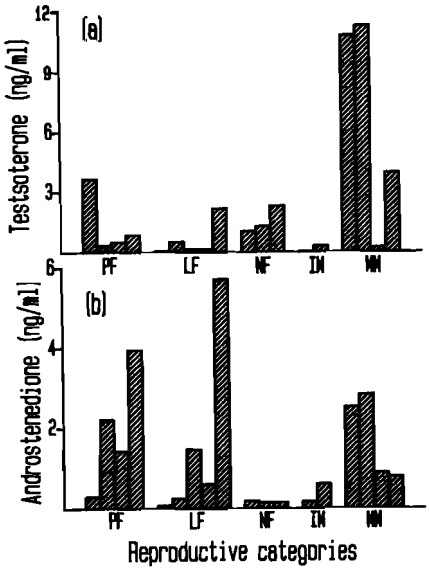

Figure 2 A distribution diagram illustrating the variance in measured androgen concentrations recorded within, and between, the various reproductive categories of spotted hyaenas. PF = parous females; LF = lactating females; NF = nulliparous females; IM = immature females; MM = mature males.

tribution diagram (Figure 2) illustrates the large variance in initial testosterone and androstenedione concentrations recorded in the different reproductive classes, and within the sexes. Although there is a clear tendency for mature males to show elevated testosterone concentrations, and for nulliparous females and immature males to have reduced androstenedione concentrations, statistical treatment cannot be justified for these small sample sizes, considering the large variance within the respective categories. A more comprehensive investigation would require an extended data base.

Discussion

The variance in testosterone status observed in male and female spotted hyaenas, with sub-adults of both sexes showing a near total lack of response to both immobilization stress and GnRH stimulation (Figure 1), seems to indicate that developmental aspects play a significant role in regulating the ability to secrete testosterone. On the other hand, the differences in the responses of the parous females may be related to cyclic ovarian activity. The higher initial testosterone concentrations recorded in males *cf.* females support the findings by Glickman *et al.* (1987) but differ from those of Racey & Skinner (1979) and Lindeque *et al.* (1986) who found no significant differences between the sexes. Both the androstenedione profiles, and the mean plasma levels recorded using initial samples, showed that females have higher androstenedione concentrations than males. Again this supports Glickman *et al.* (1987) and differs from Racey & Skinner (1979) and Lindeque *et al.* (1986), although the latter study found significantly lower androstenedione concentrations in sub-adult males when compared with sub-adult females. Therefore, the large variance in androgen status recorded within the sexes explains the contradictory results concerning sex-specific differences of circulating androgens published to date. Clearly a sex-specific analysis of circulating androgens in the spotted hyaena is confounded by other factors, such as the social and/or reproductive status of the particular individuals included in the analysis (Van Jaarsveld 1990). Moreover, the limited comparative data presented in this study suggest that the gross reproductive categories originally defined by Matthews (1939) give no clear insight into the androgen dynamics of the spotted hyaena.

That 'provocation' tests such as the pituitary challenge technique under prolonged anaesthesia have a higher resolution than single samples is supported by the finding by Illius *et al.* (1983) that they could not distinguish between territorial and bachelor male impala (*Aepyceros melampus*) using testosterone concentrations from single plasma samples, whereas the challenge technique demonstrated significant differences in testosterone status. However, these findings should not be extrapolated uncritically as the relationship between initial samples and 'provocation' test estimates of hormonal status may be dependent on a number of variables, including: the species concerned, the specific immobilizing agent used and the time lapse between darting and the first blood sample being collected. It is therefore essential that the resolution of various estimates of hormonal status be tested for each immobilization regime and species. The findings of the present investigation indicate that the use of 'provocation' tests did not increase resolution in spotted hyaenas immobilized with Zoletil and maintained on halothane. Possibly the use of other narcotic agents for immobilization or prolonged anaesthesia may reveal a higher resolution for 'provocation' tests.

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References

BROWN, J.L., GOODROWE, K.L., SIMMONS, L.G., ARMSTRONG, D.L. & WILDT, D.E. 1988. Evaluation of the pituitary-gonadal response to GnRH, and adrenal status, in the leopard (*Panthera pardus japonensis*) and tiger (*Panthera tigris*). *J. Reprod. Fert.* 82: 227–236.

GLICKMAN, S.E., FRANK, L.G., DAVIDSON, J.M., SMITH, E.K. & SIITERI, P.K. 1987. Androstenedione may organize or activate sex-reversed traits in female spotted hyenas. *Proc. Natl Acad. Sci., USA* 84: 3444–3447.

GOULD, S.J. & VRBA, E.S. 1982. Exaptation — a missing term in the science of form. *Paleobiol.* 8: 4–15.

HAMILTON III, W.J., TILSON, R.L. & FRANK, L.G. 1986. Sexual monomorphism in spotted hyaenas, *Crocuta crocuta*. *Ethol.* 71: 63–73.

ILLIUS, A.W., HAYNES, N.B., LAMMING, G.E., HOWLES, C.M., FAIRALL, N. & MILLAR, R.P. 1983. Evaluation of LH-RH stimulation of testosterone as an index of reproductive status in rams and its application in wild antelope. *J. Reprod. Fert.* 68: 105–112.

LINDEQUE, M. & SKINNER, J.D. 1982. Fetal androgens and sexual mimicry in spotted hyaenas (*Crocuta crocuta*). *J. Reprod. Fert.* 65: 405–410.

LINDEQUE, M., SKINNER, J.D. & MILLAR, R.P. 1986. Adrenal and gonadal contribution to circulating androgens in spotted hyaenas (*Crocuta crocuta*) as revealed by LHRH, hCG and ACTH stimulation. *J. Reprod. Fert.* 78: 211–217.

MATTHEWS, L.H. 1939. Reproduction in the spotted hyaena, *Crocuta crocuta* Erxl. *Phil. Trans. R. Soc.* (B) 230: 1–78.

MILLAR, R.P. & KEWLEY, C. 1976. Production of a specific antiserum for testosterone. *S. Afr. Med. J.* 50: 1021–1022.

RACEY, P.A. & SKINNER, J.D. 1979. Endocrine aspects of sexual mimicry in spotted hyaenas, *Crocuta crocuta*. *J. Zool., Lond.* 187: 315–326.

SAPOLSKY, R.M. 1982. The endocrine stress-response and social status in the wild baboon. *Horm. Behav.* 16: 279–292.

SOKAL, F.J. & RHOLF, R.R. 1981. Biometry, W.H. Freeman and Company, San Francisco.

VAN AARDE, R.J. & SKINNER, J.D. 1986. Reproductive biology of the male Cape porcupine, *Hystrix africaeaustralis*. *J. Reprod. Fert.* 76: 545–552.

VAN JAARSVELD, A.S. 1988. The use of Zoletil for the immobilization of spotted hyaenas. *S. Afr. J. Wildl. Res.* 18: 65–66.

VAN JAARSVELD, A.S. 1990. Endocrine correlates of social development in the spotted hyaena *Crocuta crocuta* (Erxleben). Ph.D. thesis, University of Pretoria, Pretoria.

VAN JAARSVELD, A.S., MCKENZIE, A.A. & MELTZER, D.G.A. 1984. Immobilization and anaesthesia of spotted hyaenas, *Crocuta crocuta*. *S. Afr. J. Wildl. Res.* 14: 120–122.

VAN JAARSVELD, A.S. & SKINNER, J.D. 1987. Spotted hyaena monomorphism: an adaptive 'phallusy'? *S. Afr. J. Sci.* 83: 612–615.