objective being to gain an understanding of possible factors in captivity that might influence male fertility through effects on testicular function.

STUDY AREA

The study was carried out at the De Wildt Cheetah Research Centre which is situated some 20 km west of Pretoria in the foothills of the Magaliesberg (25°40'S 27°52'E). For details of rainfall, climate and veld type vide Degenaar (1977).

MATERIAL & METHODS

Management

Cheetahs are fed on 6 days of the week and fast on Sundays. Each receives about 2 kg of meat per day to which a vitamin and mineral mixture is added. Beef or mutton supplied by the National Zoological Gardens and poultry from the neighbouring poultry farm are the staple diets. All meat is inspected prior to feeding and excess fat removed.

Cheetahs born on the farm are vaccinated at 3 months of age with a combined attenuated live vaccine against feline panleucopenia infectious rhinotracheitis and calici virus (Felocell CVR, Norden Labs., Lincoln). Adult cheetahs are vaccinated once a year with the same vaccine.

Cheetah cubs are numbered by ear notching using the Versfeld method as adopted by the South African Studbook Association.

Adult cheetahs are identified by Miss Van Dyk who recognizes and names them individually.

All animals are sprayed regularly with a 1:2000 solution of quintiofos (Bacdip,Bayer, Isando) for the control of ticks.

Breeding management

The cheetah enclosures were designed and erected taking cognisance of the reported reproductive behaviour of this species. Eaton (1974) indicated that the wild cheetah female spent most of her life as a solitary individual; made brief contact with males during oestrus and cared for her litter until the cubs reached approximately 15 months of age. It was suggested that a group of up to 5 cheetah males should be put with a captive female when she was in oestrus and that competition and aggression between males would stimulate sexual activity (Eaton & York 1970; Herdman 1972; Eaton 1974). Florio & Spinelli (1967, 1968) and Manton (1970,1971) however reported the successful breeding of captive cheetahs using a single male. Cheetahs were bred successfully at Whipsnade Park where the sexes were kept apart for most of the year and the female came into oestrus shortly after the male was released into her enclosure (Manton, 1970,1971).

Prior to the commencement of the present study a group of 5 cheetah males were released into a female's enclosure, at the Centre, when she was thought to be in oestrus. She was attacked, severely mauled and died as a result of her injuries.

A plan of the cheetah enclosure, about 45 ha in extent, is shown in figure 1. The perimeter is fenced with 50 mm diamond mesh to a height of 3 m with a 150 cm overhang at the top. Internal enclosures are fenced to a height of 2 m with the same diamond mesh.

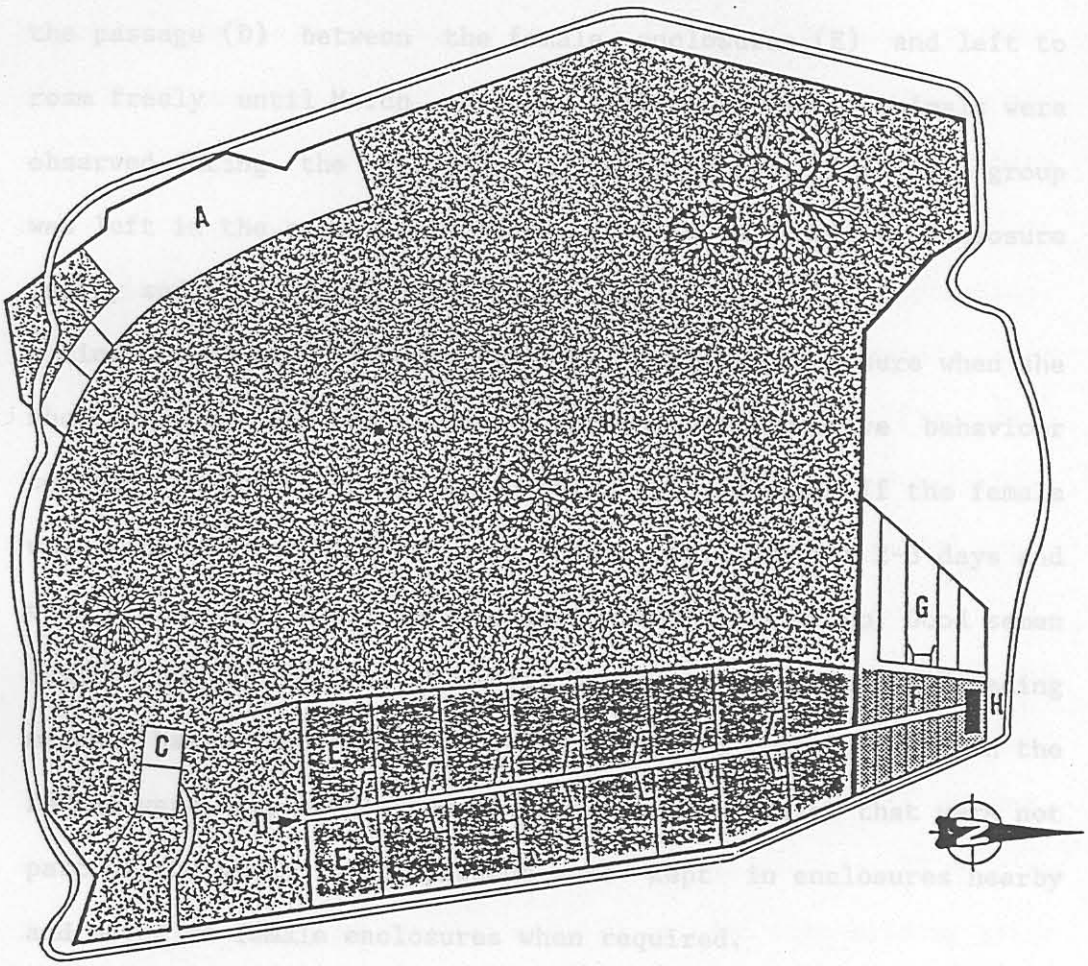

Figure 1: Plan of the cheetah enclosures at the De Wildt Cheetah Breeding and Research Centre of the National Zoological Gardens of South Africa.

Female cheetahs were kept in individual enclosures (E) about 0,5 ha in size. Compatible males were kept in groups of 2 - 10 individuals in 0,5 - 5 ha enclosures situated out of sight and sound of the females for most of the year (A).

Between November and December a group of males was introduced to the passage (D) between the female enclosures (E) and left to roam freely until March of the following year. Animals were observed during the early morning and evenings. The male group was left in the area for 10 days, removed to a holding enclosure nearby and then brought back again a week later.

A single male was introduced into a female's enclosure when she showed signs of behavioural oestrus; interactive behaviour between the cheetah pair was observed and recorded. If the female was receptive the male was left in the enclosure for 2-3 days and then coaxed out into the passage to rejoin his group. Good semen quality and indications of libido were used in selecting breeding males. Males that did not show an immediate keen interest in the female were replaced after 30 min. Cheetah males that were not part of the group in the passage were kept in enclosures nearby and moved to female enclosures when required.

Females observed to copulate or which were believed to be pregnant, were transferred to maternity enclosures (F) where they were kept until parturition. Females that raised their cubs remained in the maternity enclosure until the litter was weaned

at about 4 months of age. Thereafter cubs were removed to rearing enclosures (G) in groups of a maximum of 10 animals and females were returned to their previous individual enclosures.

Immobilization

Males free in their enclosures were darted using a Palmer Capchur pistol to deliver a drug mixture of 6-8 mg/kg ketamine hydrochloride (Ketalar,Parke Davis,Isando) and 0,5 mg/kg xylazine hydrochloride (Rompun,Bayer,Isando), this dosage being sufficient to induce a state of hypnosis that made safe handling possible. Thereafter the level of anaesthesia was increased, when necessary, using intravenous thiopentone sodium (Intraval, Maybaker,Halfway House) or by the administration of halothane (Fluothane,ICI,Isando) after intubation with a cuffed endotracheal tube.

An alternative capture technique was later developed in which the animal was caught in a crush, pinned down with wooden poles, and given 2 mg/kg intravenous CT1341 (Saffan, Milvet, Pietermaritzburg). Crushes were constructed out of a 25 mm pipe frame 175 cm long, 75 cm wide and 120 cm high with sliding gates at each end. The sides of the crush were closed with 10 mm iron rods welded to form a square mesh with 150 mm X 150 mm openings. These made it possible for handlers to get their hands into and out of the crush freely. A limb of the captured animal could be drawn through the mesh, a vein located and an intravenous injection given.

Semen collection & evaluation

Semen was collected by electroejaculation using an electro-ejaculator described by Van Rensburg & De Vos (1957) as modified by Van Tonder, Bolton, Roberson & Greeff (1973). The power source, a bicycle dynamo, is driven by a 120 mm rubber disc turned by hand. The dynamo used produced a sine wave AC current with a voltage that varied from 8 to 10 volts.

A bipolar brass electrode consisting of a 250 mm brass rod, 150 mm in diameter, with an isolated 20 mm tip was used during the initial stages of the study. This was later replaced by a teflon probe of similar dimensions but with two 4 mm brass ring electrodes 10 mm apart 20 mm from the tip. In the latter stages of the study a probe with 3 ventral, 50 mm longitudinal electrodes was used (Ball & Furman 1972, in Ball 1976). The polarity of the central electrode being opposite to that of the two lateral ones. The probe was inserted 200 mm into the rectum and inclined ventrally onto the floor of the pelvis in the region of the symphysis.

The standard electrical stimulus applied consisted of a cycle of three to five 1 sec bursts of current each followed by a short rest period of similar interval. This was followed by a continuous stimulus for 6-8 sec and a rest period of two seconds. Thereafter the cycle was repeated. After 2-3 cycles had been

applied the rectal probe was withdrawn in the rectum until the tip slipped behind caudal ridge of the pubic bone. It was then inserted again.

Ejaculates were collected in graduated glass or plastic test tubes. The volume, colour and an estimate of density recorded and the pH measured using test tape (Spezial-Indikatorpapier, Merck,Darmstadt). Immediately after collection a fresh sample was placed on a glass slide that had been heated to a temperature of 32°C on a warm stage, covered with a coverslip and examined microscopically using phase contrast and a 400 X magnification. An estimate of sperm concentration, % live spermatozoa and % showing progressive motility was made. Forward progression of spermatozoa was scored on a scale of 0 - 5 (Carroll, Ball & Scott, 1963, Ball 1976). At the same time an estimate of the proportion of morphological abnormal spermatozoa present was made.

Fresh semen was classified being of good, fair, or poor quality after an assessment of the above parameters had been made. Examples of the characteristics of these categories of semen quality are presented in table 1.

Table 1.: Characteristics of fresh semen samples classified as good, fair and poor immediately after collection.

	Density x 10^6 ml	% Live Sperm	% Motile Sperm	Forward progression
Good	> 30	> 70	> 70	4 - 5
Fair	20 - 30	50 - 70	40 - 60	3
Poor	< 20	< 50	< 40	3

Semen smears were prepared for microscopical examination using either phase contrast or after modified Karras staining (Bosman, 1975, in Van Tonder,1977). Two hundred individual spermatozoa per smear were examined for morphological abnormalities using a 1000 X magnification. Morphological abnormalities were classified as either major or minor using criteria as suggested by Blom (1972). Spermatozoal concentration (spermatozoal numbers/ml of ejaculate) was calculated using a standard haemocytometer counting procedure.

Samples of the ejaculate were prepared for electron microscopic examination; 0,25-0,5 ml was immediately fixed in 2% glutaraldehyde in Millonig's phosphate buffer (pH 7,3) (Millonig, 1961) for 1 h at 40°C. Using a process of gentle centrifugation and resuspension, the sperm were washed once in phosphate buffer

and then post-fixed at room temperature for 1 h in 2% osmium tetroxide in the same buffer. After two more buffer rinses sperm were drawn into micro-capillary tubes and centrifuged in a MSE Minor centrifuge equipped with a micro haematocrit head assembly. The resultant pellets were removed from the capillary tubes using a fine glass piston, diced into small blocks dehydrated through a graded ethanol series, cleared in propylene oxide and embedded in Epon 812. Thin sections were cut with glass and diamond knives on a Reichert Om U4 ultramicrotome, stained for 5 min. each with a saturated aqueous solution of uranyl acetate (Watson,1958) and 0,2% lead citrate (Reynolds, 1963) and examined with a Phillips EM301 transmission electron microscope operated at 80 kv.

Initially semen evaluations were conducted to establish a rational breeding approach at the centre. Males were selected for inclusion in the breeding program after an assessment had been made of their semen quality (Meltzer, Coubrough, Van Dyk & Brand 1974). Thereafter 16 cheetah males from two distinct groups were electroejaculated at regular intervals for a period of one year. The first group of seven animals consisted of cheetah males that had arrived at the centre as two pairs and a group of three and had been kept separately. The second group, nine animals, were selected from 12 cheetah males that had been captured as adults in South West Africa - Namibia - and kept together ever since. The remaining three animals were used for the purposes of a separate study (Degenaar, 1977). These animals arrived at the centre in 1973, in poor condition, having been confiscated by the

Transvaal Department of Nature Conservation. By the commencement of the present study, in 1975, they were in good condition and were being kept in an enclosure approximately 4 ha in extent. The conditions in which they were kept differed markedly from those of the first group in that all of these animals were subjected to hierarchical pressures within the group and fighting broke out from time to time. Animals kept as pairs or small groups were well adapted to one another and no such problems occurred in their case.

Blood sampling for plasma hormone determinations

Peripheral blood samples, for hormone assays, were collected from the saphenous or recurrent cephalic veins into 10 ml heparinized vacuum tubes (Venoject, Terumo, Tokyo) and centrifuged immediately thereafter. Plasma was harvested into three separate portions and stored at -20°C until assayed.

Radioimmunoassays

Testosterone

Serial plasma samples from individual animals were assayed together, in duplicate. Pre-GnRH and maximum post-GnRH plasma testosterone samples were selected after the first round of assays and reassayed using a single assay kit. Data on plasma

testosterone levels analysed statistically were from this single radioimmunoassay performed at the end of the study. Control sera were included in all assays.

Plasma testosterone levels were measured using a RSL NOSOLVEX I^{125} assay kit (Radioassay Systems Laboratories, Carson, California. USA.) which was later replaced by a COAT-A-COUNT I^{125} kit (Diagnostics Products Corporation, Los Angeles, USA). The latter assay was less time consuming and easier to perform.

RSL NOSOLVEX I^{125} testosterone kit is a non-extraction double antibody radioimmunoassay using testosterone I^{125}, rabbit anti-testosterone and goat anti-rabbit gamma globulin. Cross reaction at 50% binding, as determined by the supplier, was 10,3% for dihydrotestosterone, 0,56% for androstenedione and less than 0,1% for progesterone, corticosterone, de-oxycorticosterone and oestradiol-17ß. Recovery of exogenous testosterone added to female plasma was 96,8-98 %. Interassay and intra-assay coefficients of variation were 12,3% and 7% respectively. The mean sensitivity was 0,37 nmol/l (n=10).

The COAT-A-COUNT assay does not require extraction and the anti-body is bound to the wall of polypropylene tubes supplied by the manufacturer. One hundred microlitres of plasma was added to the tube together with I^{125} testosterone and decanting of the supernatant after 3 h incubation terminated competitive binding. Standard solutions of testosterone ranged from 1,0 - 104 nmol/l. Maximum binding was approximately 50%. Cross reaction with dihydrotestosterone and androstenedione, as determined by the manufacturer, was less than 10% and 0,1% respectively. Recovery

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of exogenous testosterone ranged from 96-103%. Interassay and intra-assay coefficients of variation were 8,2% and 2,7% respectively. The mean sensitivity was 0,87 nmol/l (n = 8)

Cortisol

The concentration of serum cortisol was measured using an I^{125} RIA kit (RIANEN^{TM}, New England Nuclear, No. Billerica, MA. USA). This assay employed a cortisol antiserum complex solution containing rabbit cortisol antibody prereacted with an antiserum to rabbit gamma globulin in phosphate buffer. As determined by the manufacturer the rabbit cortisol antibody had the following crossreactions: 100% with cortisol; 38,9% with prednisolone; 26,4% with corticosterone; 7,4% with aldosterone; 5,9% with 11-deoxycortisol; 3,5% with 17α-hydroxyprogesterone and 2% or less with progesterone, testosterone, dihydrotestosterone and oestradiol-17ß. Interassay and intra-assay coefficients of variation were 6,2% (n = 6) and 2,7% (n = 10), respectively, and minimum assay sensitivity was 27,6 nmol/l.

Plasma testosterone concentrations in relation to time, the rate of testosterone production, were estimated from graphs drawn on standard metric graph paper. The rate of testosterone production was estimated by measuring the area under the curve using a Kontron Image intensifer and calculating the rate of production as follows: nmol/l/h = $\frac{\text{Total area under the curve}}{\text{area of 1 hour at 1 nmol/l}}$

Luteinizing hormone

A heterologous antibody radioimmunoassay was used to measure serum LH. This assay orignally developed by Chakraborty, Wildt & Seager (1979) for the domestic cat (Felis catus) was validated for use in cheetah (Wildt, Chakraborty, Meltzer & Bush 1983). The isotope was I^{125} ovine LH (LER-1056-C), the first antibody was a bovine LH antiserum (JJR-5: dilution, 1:80000) and the results were analysed on the basis of a canine pituitary standard (LER-1685-1). Interassay and intra-assay coefficients of variation were 11,7% (n=5) and 11,1% (n=9) respectively, with a minimum assay sensitivity of 0,3 µg/l.

Crossreactivity with CT1341

CT1341 consists of two pregnanedione derivatives, 3∝-hydroxy-5∝-pregnane-11, 20-dione (alphaxalone) and 21-acetoxy 3∝-hydroxy-5∝-pregnane-11, 20 dione (alphadolone acetate). Due to the steroidal nature and configuration of this drug, it was considered necessary to determine its potential crossreaction with the antisera used in the cortisol and testosterone assays. Five, 10, 20 and 50 µl of a diluted CT1341 solution (1,2 µg/ml) were subjected to standard procedures used in each of the radioimmunoassays. Quantities of cortisol and testosterone measured were below the detectable limits of the assays used ensuring that the crossreactivity of the CT1341 with the antiserum for either steroid was negligible and nonsignificant.

Diurnal variation in plasma testosterone

Diurnal variations in plasma testosterone concentration were determined in five cheetah males kept for 24h in crushes as described above. Blood samples were collected without drug restraint every two hours during this period.

Plasma testosterone after increasing doses of GnRH

The effects of different intramuscular doses of gonadotrophin releasing hormone (GnRH) (Gonadorelin, Abbott Laboratories, Chicago, Illinois, USA) on plasma testosterone concentrations were measured in cheetah males anaesthetized with CT1341. Four males were selected at random from a group of animals aged between 4-5 years (m34, m45, m49 and Swa). On day 1 m34, the control, received an equivalent volume of saline, m45 was given 50 µg, m49 100 µg and Swa 250 µg GnRH. At two weekly intervals thereafter animals were rotated each receiving a different dose of GnRH or acting as a control so that at the completion of the experiment each had received in turn 0 µg, 50 µg, 100 µg or 250 µg GnRH. Blood was collected prior to GnRH or saline injection, one hour later and thereafter at 30 min intervals until 300 min after GnRH injection. Animals were kept in a state of anaesthesia sufficient to allow safe handling throughout the period by repeated intravenous injections of CT1341 as required.

Plasma LH & testosterone after 50 µg GnRH

This study was conducted in January during the breeding period at the research centre. Fourteen sexually mature male cheetahs with a mean age of 3,7 ±0,4 years were used. Animals were caught and anaesthetized with CT1341. Immediately after the induction of anaesthesia a blood sample was collected (pre-stimulation plasma sample) the animals were divided at random into two groups, one of which was given the saline vehicle, the other 50 µg GnRH (Gonadorelin, Abbott Laboratories, Chicago, Illinois, USA) by intramuscular injection. Blood was collected at 15 min intervals for the first hour and thereafter every 30 min. Cheetah males given saline were bled for 120 min and the animals that received GnRH were bled until 180 min post injection.

Plasma testosterone after 50 µg GnRH in different age groups

Groups of six to eight cheetah males from different age groups were anaesthetized with CT1341 and gathered under a suitable shady tree. Blood was collected prior to the injection of a standard dose of 50 µg GnRH, one hour later and thereafter at 30 min intervals until 300 min after GnRH injection. This sampling regimen was followed until radioimmunoassays indicated that plasma testosterone concentrations reached a peak between 150 and 180 min after GnRH injection. Sampling was then limited to a

maximum post-injection period of 240 min. Controls where animals were given an equivalent volume of saline in place of GnRH were included, at random, from time to time.

Plasma testosterone in July & November

Five cheetah males were selected at random out of a group of 12 animals aged two years. These animals were examined as described above after 50 µg GnRH on two occasions during July and November of the same year

The effect of anaesthesia on the plasma testosterone response

Eight cheetah males of similar ages were selected at random and divided into two groups. One group was anaesthetized with 2 mg/kg CT1341, the other with thiopentone sodium. Thiopentone sodium was given as a single knock-down bolus of 400 mg by rapid intravenous injection. CT1341 was given in the standard manner. Intermittent intravenous injections of each of the drugs were given as required to maintain a state of hypnosis sufficient to allow safe handling. Animals caught first were maintained in this state until the entire group of eight males had been brought together at a central point. Blood was collected prior to anaesthesia and then at 30 min intervals for two hours until 50 µg GnRH was

given. Two control animals from each group were given an equivalent volume of saline. Thereafter blood was collected at intervals as above.

Hormone studies in anaesthetized & electroejaculated males

Two groups of cheetah males were examined. Both groups were captured and anaesthetized with CT1341. Fourteen males were electroejaculated using a regimented stimulation protocol and bled serially. Seven control animals were bled at similar time intervals to the electroejaculated group. The electroejaculator used (P-T Electronics, College Station, Texas, USA) permitted controlling and monitoring of voltage and amperage. A total of 80 stimulations was given in three series consisting of 30, 30 and 20 stimuli each. The stimulation in the first series consisted of 10 stimuli at 4 V followed by two sets of 10 stimuli at 5 and 6 volts respectively. The animal was rested for 2-3 min and then stimulated again. The first 10 stimuli of this series given at 5 V were followed by two sets of 10 stimuli each at 5 and 6 volts respectively. After a similar rest interval, two final sets of 10 stimulations each were given at 6 and 7 volts.

Blood samples were collected from both groups within 5 min of capture and prior to anaesthesia. Subsequent samples were collected from the electroejaculated group following anaesthesia, immediately after each series of electroejaculation stimuli, 30 min after electroejaculation and 60 min later. Mean time inter-

vals between blood collections were as follows: from pre-anaesthetic to post anaesthetic (pre-ejaculation), 25 min; post anaesthetic to the end of series 1, 12 min; from the end of series 1 to the end of series 2, 9 min and from the end of series 2 to the end of series 3, 9 min. A similar bleeding schedule was followed in the non-ejaculated anaesthetized control cheetah males. Blood samples were cooled immediately after collection, kept at 4 °C for 2-3 h, and serum collected after centrifugation. Sera were kept at -20°C and shipped to the USA packed in dry ice, where they were assayed by for cortisol, testosterone and luteinizing hormone in the Department of Obstetrics and Gynaecology, Uniformed Services University of the Health Sciences, Bethesda, Maryland. U.S.A.

Adrenal function tests.

Two adult cheetah males were anaesthetized with CT1341 in the usual way and bled serially. Following a zero time sample each male was given 25 I.U. ACTH by intramuscular injection. They were then bled at 15 min intervals for the next hour and two samples taken at 30 min intervals thereafter. Two cheetah males were caught, kept in crushes for 2 h, and treated similarly without drug restraint. Samples were assayed for cortisol, testosterone and luteinizing hormone.

Plasma testosterone and semen quality

Eight cheetah males were immobilized, electroejaculated and then given 50 µg GnRH and serially bled for the next 4 h. Semen quality was assessed in fresh semen samples immediately after collection. The animals were classified into groups having good, fair and poor quality semen using criteria as summarized in Table 1. The pre-stimulation and maximum plasma testosterone concentrations were measured and the plasma testosterone response after GnRH was calculated. The means of each of these parameters measured in each of the groups were compared using an analysis of variance.

Plasma testosterone after intravenous & intramuscular GnRH

Six Cheetah males aged from 4-5 y that had previously been given 50 µg intramuscularly and serially bled were chosen at random and anaesthetized.

They were bled prior to being given 50 µg GnRH by intravenous injection and bled thereafter at 30 min intervals for 180 min. Plasma was harvested and stored until assayed as described above.

Plasma testosterone after HCG injection

Four males, Harry, Gouws, Boytjie and m46, were selected at

random from a group of adult cheetahs. The mean age of this group was 6,9 ±1,6 years. Each of the animals was anaesthetized, and restrained for the 3 h period with CT1341. On day 1 of the trial Harry received the equivalent of 2,5 mg LH in the form of an intramuscular injection of HCG (APL, Ayerst Labs), m46 was given 5 mg, Gouws 10 mg and Boytjie 20 mg. At two weekly intervals thereafter animals were rotated each receiving a different dose of HCG so that at the completion of the experiment each had received at random the equivalent of 2,5 mg, 5 mg, 10 mg or 20 LH. Blood was collected prior to HCG injection, one hour later and thereafter at 30 min intervals until 300 min after HCG.

RESULTS & DISCUSSION

Management

Females came into oestrus soon after males were introduced into the passage between their enclosures for the second time. This became apparent when males congregated at a particular female's enclosure. The female was seen close by on the other side of the fence, chirping at the males, submissive and rolling. The males were excited, several developed an erection and short fights broke out amongst them. It was not uncommon to find two or three females in oestrus at the same time. When overt signs of oestrus were observed the animals were often seen to copulate soon after