CHAPTER 2:

MATERIAL AND METHODS

Study animals

Trapping and maintenance

Mole-rats were trapped using modified Hickman live traps (Hickman 1979b), baited with sweet potato. Cape mole-rats were captured during August 2002 (breeding season/winter) and February 2003 (non-breeding season/summer) in the Darling area, Western Cape Province (33°22'S, 15°25'E) (Plate 2.1), while Natal mole-rats were obtained from Glengarry, KwaZulu Natal (29°19'S, 29°43'E) (Plate 2.2) during six trapping expeditions spread every second month throughout one year. The mole-rats were housed in plastic containers (40 cm x 30cm x 30cm) provided with wood shavings, and were fed on sweet potato, carrots and gem squash. No free water was provided as moisture is obtained from the food. Urine and blood samples were taken no longer than two weeks after the animals were captured.

Body mass of the Cape mole-rat

The mean body mass (±SE) of male Cape mole-rats captured in the breeding season was 168±16.3g (n=6) and ranged between 121g and 222g. Out of the breeding season, the mean body mass of males was 110±17g (n=4) and ranged between 75g and 154g. In breeding season, the mean body mass of female Cape mole-rats was 142±7.8g (n=25) and ranged between 86g and 232g. The mean body mass out of the breeding season was 177±12.1g (n=17) and a range between 104g and 272g (Figure 2.1). In the population studied, male animals were encountered much less frequently than females.

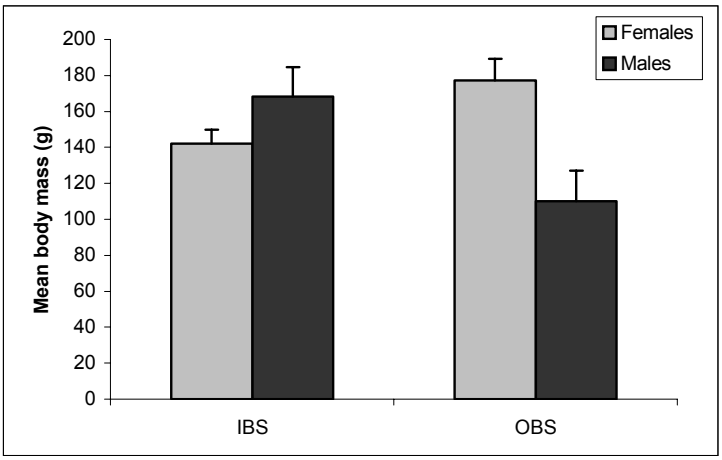

Figure 2.1: Mean (±SE) body mass (g) of the Cape mole-rat in and out of the breeding season.

Body mass of the Natal mole-rat

Breeding animals were typically heavier than non-breeding individuals in the Natal mole-rat. The mean body mass of female Natal mole-rats was 97g (n=23) for breeding animals with a range between 73g and 127g; 71g (n=72) for non-breeding animals, ranging between 47g and 111g. Breeding males had a mean body mass of 133g (n=45), ranging between 106g and 162g, and the mean body mass of the non-reproductive males was 91g (n=61) with a range between 40g and 119g (Figure 2.2).

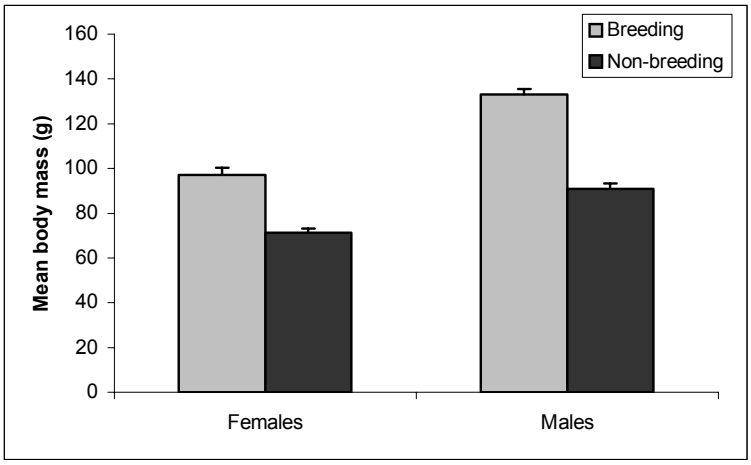

Figure 2.2: Mean (±se) body mass (g) of breeding and non-breeding/subordinate Natal mole-rats.

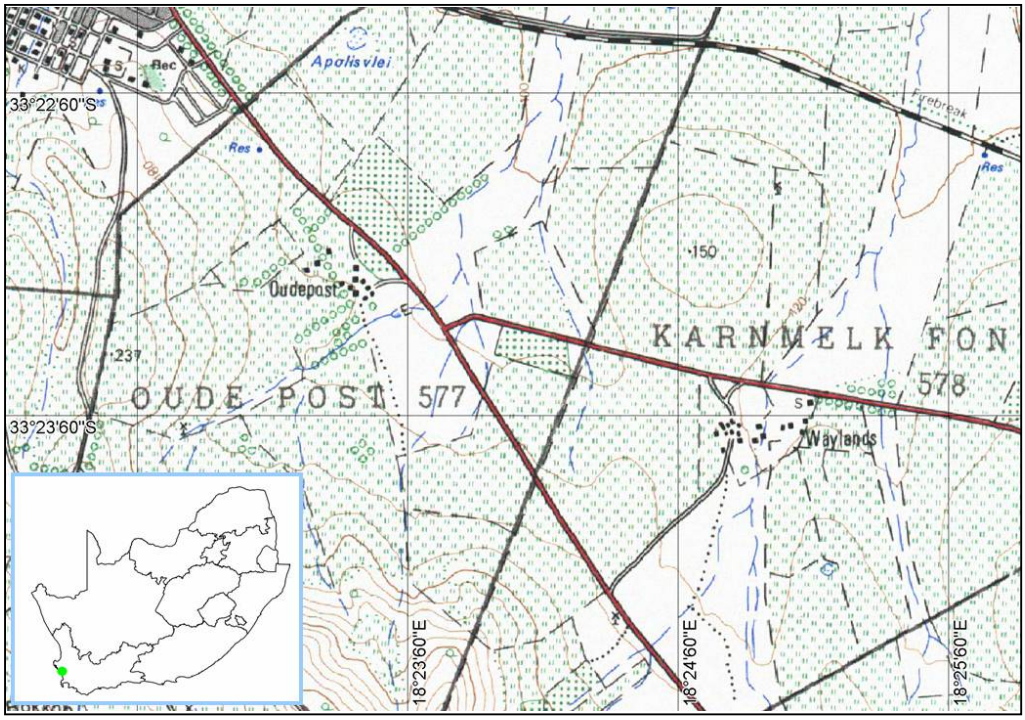

Plate 2.1: Study area near Darling, Western Cape Province, where the Cape mole-rats for this study were captured.

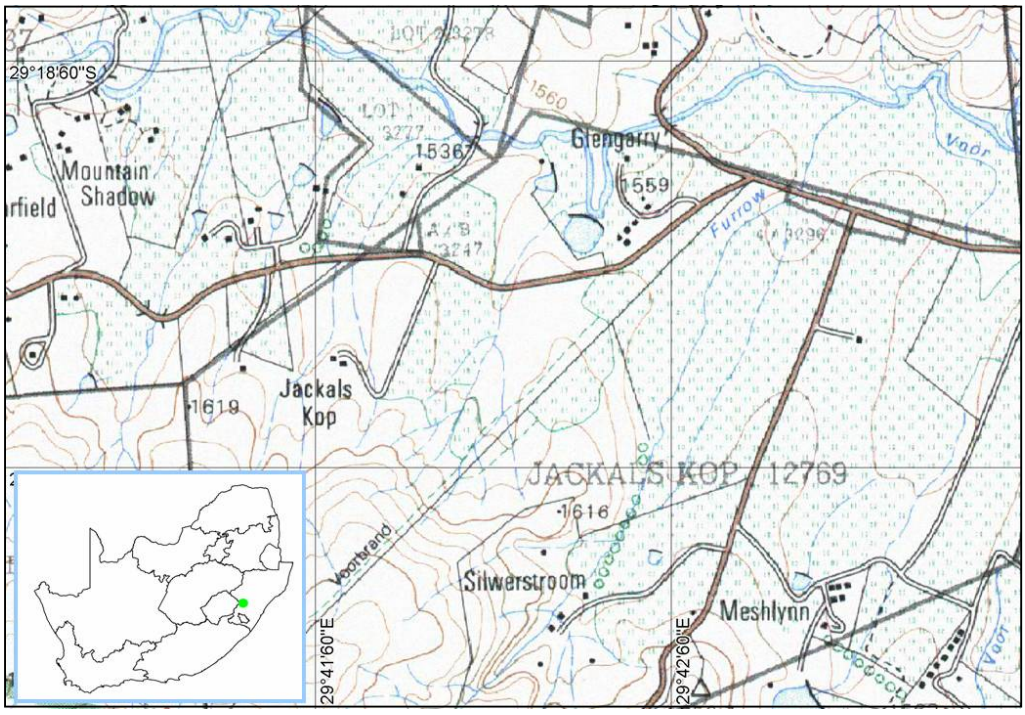

Plate 2.2: Study area near Mooiriver, KwaZulu Natal, where the Natal mole-rats for this study were captured.

Chapter 3

Radioimmunoassays

Testosterone determination

Plasma testosterone concentrations were determined using a Coat-A-Count total testosterone kit (Diagnostic Products Corporation, Los Angeles, California, USA). Extraction or chromatography was not required for this procedure. A 50µl sample in duplicate was used for the assay. The procedure entails solid-phase radioimmunoassay based on hormone specific antibody immobilised to the wall of a polypropylene tube. ^{125}I-labeled testosterone competes for a fixed time with the specific hormone in the given sample for antibody sites. The tube is then decanted to separate bound from free and is then counted in a Cobra gamma counter.

The antiserum is highly specific for testosterone and has a low cross reactivity with other naturally occurring steroids except dihydrotestosterone, which is less than 5%.

The assay was validated by testing for parallelism using serial doubling dilutions of un-extracted plasma over the dilution range (1:1 to 1:64). The slope of the lines were compared and found not to differ significantly (ANCOVA $F_{(1,6)}$=4.3 P>0.05) following a log-logit data transformation (Chard 1987). The sensitivity of the assay (90% binding) was 2.2 nmols/l. The intra-assay coefficient of variation was 2.5% (n=6).

Oestrogen determination

Oestradiol-17β was determined in mole-rat urine using a Coat-A-Count Oestradiol-17β kit (Diagnostic Products Corporation, Los Angeles, California,

USA). A 100µl sample in duplicate was used for this assay. The method is a solid-phase radioimmunoassay that does not require purification of steroids or separation by chromatography. The antiserum is highly specific for oestradiol-17β, with a low cross reactivity with any other steroids present in the urine. The assay was validated by testing for parallelism using serial doubling dilutions of un-extracted urine over the dilution range (1:1 to 1:64) following log-logit transformation of the data (Chard 1987). The slope of the lines were compared and found not to differ significantly (ANCOVA $F_{(1,6)}$=0.09, P>0.05). The sensitivity of the assay was 2 pmols/l. The intra-assay coefficient of variation was 9% (n=8).

Progesterone determination

Urinary progesterone concentrations were determined using a Coat-A-Count progesterone radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, California, USA), as described by Bennett *et al.* (1996). A volume of 100:1 of urine in duplicate was assayed without extraction.

The antiserum is highly specific for progesterone with a low cross reactivity to all other naturally occurring steroids except 20-α- dihydroprogesterone and 11-deoxycortisol with a cross reactivity of 2% and 2.4% respectively. A pooled urine sample (one with expected high concentrations from a pregnant queen) was double diluted from 1:1 to 1:64 and assayed.

In the assay were also included 6 samples at a dilution of 1:64 from a pool of low concentration progesterone. To these samples 100µl of progesterone in increasing concentrations (0.3, 1.6, 6.4, 31.8, 63.6 and 120 nmols/l) was added in duplicate. The curve was perfectly parallel to the standard curve. The assay was validated for the test species by comparing the slope of the curve produced using serial doubling dilutions of un-extracted mole-rat urine

(over the range 1:1 to 1:64) against the standard curve (ANCOVA $F_{(1,6)}$=4.9, P>0.05). The intra and inter assay coefficients of variation were 7 and 11% respectively. The sensitivity of the assay at 90% binding was 0.4nmols/l.

Creatinine determination

Urine concentration varies with fluid consumption; therefore creatinine is used to standardize samples. Creatinine is a breakdown product from tissue proteins and is excreted at a relatively constant rate (Schmidt-Nielsen 1997).
A modified Jaffe reaction was used to calculate the creatinine concentration for urine samples (Folin 1914).

The samples are assayed in duplicate. Ten microlitres of standard or sample were added to the wells of a micro plate, leaving two wells empty as a blank control. 200µl of picric reagent was added to all the wells, including the blanks. The picric reagent consists of saturated picric acid solution, alkaline triton solution (4.2 ml triton X-100, 12.5 ml 1N NaOH and 66.0 ml distilled, deionised water) and distilled deionised water in the proportion of 1:1:10. The alkaline triton can only be used once the product is homogenous. The microplate is then placed in the dark for a period of 1,5 hours, at room temperature, to allow colour development to occur. A standard curve (R^2>0.99) was used to determine all sample values.

Chapter 4

Blood sampling

Prior to sampling, the animals were placed into a temperature regulated chamber at 36°C for 20 minutes to bring about vasodilatation, thus facilitating blood collection. Mole-rats were hand restrained while blood was taken from the saphenic vein in the foot, prior to and 20 minutes after the administration

of a saline injection or a single GnRH challenge. Heparinised capillary tubes were used to collect between 300 and 400µl of whole blood, whereafter the blood was centrifuged to separate the plasma from the cellular component of the sample. Plasma was stored at -40°C until being assayed. This method has been successfully used to investigate pituitary sensitivity and secretion in naked mole-rats (Faulkes *et al.* 1990b, 1991), Damaraland and Mashona mole-rats (Bennett *et al.* 1993, 1996, 1997) and suricates (O'Riain *et al*. 2000).

GnRH administration

A chimaeric analogue of mammalian GnRH produced in the laboratory of R.P. Millar (Chemical Pathology, University of Cape Town), was administered to the mole-rats. The hormone was synthesized using solid phase methodology and had a purity of >98% homogeneity (Millar *et al*. 1989). A dose of 2µg in 100µl of sterile physiological saline was used to challenge the pituitary. Control animals were injected with 0.2ml sterile physiological saline.

LH bioassay

LH concentrations were determined using an *in vitro* bioassay based on the production of testosterone by dispersed mouse Leydig cells (Van Damme *et al.* 1974). The incubation medium (12ml Eagle's basal medium, 2.1ml 7.5% sodium hydrogen carbonate 2ml foetal calf serum and 100ml distilled water) was placed on ice and gassed slowly under Carbogen 5 (95% O_2: 5% CO_2). A six week old male mouse was killed by cervical dislocation, the testes removed and decapsulated in 5ml incubation medium. The cell suspension was stirred on a magnetic stirrer for 5 minutes, filtered through fine nylon mesh and incubated under Carbogen 5 gas for 1 hour in a shaking water bath at 34ºC. Subsequently the incubated cell suspension was washed and centrifuged at 2500 r.p.m for 5 min at 4ºC. The supernatant was decanted and

the cells resuspended in the incubation medium. The process was repeated after which the cell suspension was slowly stirred on the magnetic stirrer for 5 minutes. A haemacytometer was used to count the number of cells. Incubation medium was added until the number of cells counted corresponded to the final cell suspension volume (in ml). The medium was stirred for 5 minutes.

The LH buffer (2.9g disodium hydrogen orthophosphate dodecahydrate, 0.29g sodium dihydrogen orthophosphate dehydrate and 4.38g sodium chloride) was made up to 1 litre in distilled water with 0.1% BSA. The mole-rat plasma samples were prepared at a 1:20 dilution in LH buffer. A standard curve was obtained by serially double diluting the mammalian LH in LH buffer, within the range of 360-1.4μIU ml/100 μl. 100μl of either plasma sample, standard, quality control or LH buffer (to obtain an estimate of total binding), was added to the bioassay tubes. Standards and total binding were assayed in triplicate while samples and quality controls were assayed in duplicate. The mammalian LH standard (2nd International standard 1988, Code 80/552, Hertfordshire, U.K.) was provided by the National Institute of Biological Standards and Controls (Storring & Gaines 1993).

After 200μl of diluted cell suspension was added to each assay tube, the tubes were incubated in a shaking water bath at 34°C for 3h under Carbogen 5 gas. Further testosterone production by the Leydig cells was inhibited by boiling the tubes containing the cell preparation in a water bath at 100°C for 15 minutes. Subsequently the tubes were placed on ice and 0.3ml phosphate buffered saline with 0.1% gelatine was added. Testosterone production during the incubation period was determined by radioimmunoassay (Bennett 1994).

Radioimmunoassay

Concentrations of testosterone were determined by radioimmunoassay of duplicate sample aliquots. Testosterone antiserum in phosphate buffer (0.1 ml) at a working dilution of 1: 800 was added to standards and reagent blanks. The contents were mixed and subsequently [1,2,6,7–^{3}H] testosterone TRK 402 (sp. Act. 80-105 Ci/mmol; Radiochemical Centre Amersham, Bucks. UK) in 0.1ml assay buffer (~10,000 cpm) was added. The contents of each tube were mixed and incubated overnight at 4^{o}C.

The tubes were cooled at 4^{o}C and separation of antibody bound and free testosterone was carried out by adding 0.5ml dextran coated charcoal (Norit A charcoal 1.0g and 0.1g Dextran T-40 in 400ml assay buffer), incubating at 4^{o}C for 12 minutes and then centrifuging at 3000 rpm for 20 minutes at 4^{o}C. The supernatant was decanted into scintillation vials and scintillation fluid (10ml) (Ready-Solve CP, Beckman Instruments (Pty) Ltd, Johannesburg, South Africa) was added to each vial. The contents of the vial were mixed, left for 1 h and finally counted for 2 min using a Tricarb Scintillation counter.

Cross-reaction with all major naturally occurring steroids was <0.1%, except for dihydrotestosterone for which it was 5.1%. The sensitivity of the assays, defined as twice the standard deviation of values obtained from the buffer blank was 0.5 miu/ml.

The inter-assay variation was 14%. Serial dilutions of plasma obtained following GnRH administration paralleled the standard curve over the dilution 1:0 to 1:64 (Cape mole-rat: ANCOVA $F_{(1,6)}$=0.883, p=0.379; Natal mole-rat: ANCOVA $F_{(1,6)}$=0.069, p=0.800).

Chapter 5

GnRH immunocytochemistry

Animals were weighed and deeply anaesthetized with an overdose of fluorothane anaesthetic (Zeneca, RSA). They were perfused intracardially with 0.9% saline at 37°C, followed by 4% paraformaldehyde (PFA) (Saarchem) in 0.1M phosphate buffer (pH 7.4) (Sigma) at 4°C. The heads were removed and the brains sectioned out. The brains were stored in 2% PFA until further treatment. Prior to sectioning, brains were placed in 30% sucrose until saturated for cryoprotection. When saturated, the brains were quick frozen with dry ice, and 25μm thick coronal sections were cut on a cryostat (Bright Cryostats, UK), and every sixth section was used.

The sections were pre-treated in 0.5%-X100 triton which increased the permeability of the cell membrane. Endogenous peroxidase was suppressed using 0.02% H_2O_2. The sections were briefly rinsed in PBS and incubated in 2% normal donkey serum for an hour after which the sections were incubated in GnRH primary antibody (manufactured in rabbits, INCSTAR) for 48 hours at 4°C (dilution 1:20 000). After a brief rinse, sections were incubated in secondary biotin-SP conjugated AffiniPure Donkey anti-rabbit IgG antibody for two hours (dilution 1:200, Jackson Immunoresearch, West Grove, PA). After rinsing in PBS the tissue was incubated in an avidin-biotin peroxidase complex (1:1000, Elite Kit, Vector Laboratories, Petersborough, United Kingdom). Following a rinse in TRIS buffer they were incubated in 0.05% diaminobenzidine (DAB) with 0.15% ammonium nickel sulphate and 0.005% H_2O_2 to visualise GnRH immunoreactivity. All the sections for a given comparative group were processed in parallel.