Appendix I

Morphometric Methods

I.1 Introduction

One of the main objectives of morphometric analysis is to obtain information about cells that can be related to cell function. The cellular components of interest include their number, mean volume, internal organelle volumes, tubule or filament lengths and surface areas (Cruz-Orive and Weibel 1990). Many studies concentrate on ultramicroscopic cellular components, and others are more interested in cellular changes at the light microscope level, with special interest in changes with respect to the whole organ (Bertram and Nurcombe 1992, Bertram *et al.* 1992, Gundersen *et al.* 1988a,b, Mayhew 1991).

Before the early 1980s most morphometric studies used conventional methods, which were useful for estimating volumes for bulk parameters, but often had inbuilt biases that had to be overcome (Weibel 1979). Errors due to fixation shrinkage differences between treatment groups, orientation biases and incomplete randomisation all contributed to the incorrect interpretation of cellular data (Cruz-Orive and Weibel 1990, Mendis-Handagama and Ewing 1990). In the past this meant that to overcome these biases exhaustive counts of numerous sections and use of shrinkage factors such as the Abercrombie or Floderus equations were essential (Barr *et al.* 1971, Mendis-Handagama and Ewing 1990, Tait and Johnson 1982, Weibel 1979). In recent times the reviewing of these methods by several research groups has eliminated many of the laborious methods while optimising the morphometric data obtained (Gundersen et al. 1988a,b, Mayhew 1991). The rest of this review will attempt to summarise the information relevant to the study of cellular changes in the organ systems of *Antechinus stuartii*.

The large physiological changes in *A. stuartii* have been well documented (Barnett 1973, Bradley *et al.* 1980, Lee *et al.* 1977, McDonald *et al.* 1981, Woollard 1971), as have some of the gross and microscopic changes associated with these physiological changes (Barnett 1973, Kerr and Hedger 1983, Moore 1974, Woolley 1966). Most of these studies have documented changes at the light microscope level, although recent studies by Taggart and Temple-Smith (Taggart and Temple-Smith 1989, 1992, Taggart *et al.* 1993) have concentrated

on electron microscopic studies of the epididymis. In the present study on *A. stuartii*, light microscopic studies of the reproductive tract and kidneys will be assessed by morphometric analyses. Thus the recent stereological methods will be reviewed towards this aim.

I.2 Sampling procedures

Selecting the optimum sample size for minimum effort and error while maximising output is the beginning of any stereological study. The variation between animals in a group is the biological variation plus the stereological error variation. If biological variation is large a prohibitive number of animals may be needed to detect differences. If biological variation is less than stereological variation, more work per animal will reduce this variation (Cruz-Orive and Weibel 1990). Five animals per group is considered a good basis for the material of interest as, if some cellular component is found to increase in all five cases, the probability of this occurring by chance alone is $P = (1/2)^5 = 0.03 < 0.05$ (Cruz-Orive and Weibel 1990). When, as in the present study on *A. stuartii*, the groups are unavoidably smaller than five, the biological variability within groups must be very low to produce significant differences between groups.

The initial imprecision of counting on the plane of interest has been shown to be of less overall importance than the variability between fields of view, blocks and animals (Cruz-Orive and Weibel 1990, Gundersen and Østerby 1981). A reasonable estimate of adequate sample size is given by Aherne and Dunhill (1982), where the standard deviation of the sample is used to calculate whether a large enough sample size has been taken. The desired level of precision is $P<0.05$ and standard deviation (**s**), number (n), and mean value from the sample are known (y).

$$\text{Thus } \frac{s}{\sqrt{n}} < 0.05y \text{ and } n > (s/0.05y)^2$$

$$\text{If } y = 15 \text{ and } s = 5, \text{ then } n > (5/0.05 \times 15)^2$$
$$> (5/0.75)^2$$
$$> 44.5$$

Therefore 45 samples need to be taken. Clearly if the standard deviation is smaller, fewer samples are needed.

Another simple method is to make sure that the coefficient of error (CEM) is $P<0.05$ (Gundersen *et al.* 1983b).

$$\text{The CEM} = \frac{\text{standard error of mean (SEM)}}{\text{mean (y)}} < 0.05$$
$$= \frac{\sqrt{(s^2/n)}}{y} < 0.05$$

If the information from our previous example was y = 15, s = 5, but n = 10, then CEM = $\frac{\sqrt{(25/10)}}{15}$ = 0.105 > 0.05.

Thus we would need a larger n, and if s does not decrease with an increase in n, then n = 43 is needed for CEM < 0.05, a result very similar to the previous example.

Using these methods for detecting any untoward errors at each sampling level, sampling biases can be effectively eliminated while minimising sample sizes, and hence work needed (Cruz-Orive and Weibel 1990, Gundersen and Østerby 1981, Gundersen *et al.* 1988b). Moreover, coupled with the point counting method, as few as five fields of view need to be counted and expensive digital analysers can be avoided, as these have been shown not to increase time efficiency or accuracy of area or numerical estimation (Cruz-Orive and Weibel 1990, Gundersen and Østerby 1981, Gundersen *et al.* 1988b). Finally, by keeping the fields of view at the lowest image magnification possible, field variability is considerably reduced, enhancing the efficiency of counting methods (Mayhew 1991).

I.3 Isotropy and section orientation

An isotropic organ is one where the content does not vary with orientation and an homogeneous organ is one where the content does not vary with position (Mayhew 1991). An example of such an organ is the liver, however most biological samples of interest are aniosotropic but homogeneous (e.g. cerebellum, skeletal muscle, gut, where the content varies with orientation but the cytoarchitecture remains unchanging throughout the organ). Isotropy is essential for most stereological procedures, the exceptions are estimation of volume and number (Gundersen *et al.* 1988b). To get a profile that is isotropic, uniform and random (IUR profile), vertical sections are taken (Gundersen *et al.* 1988b). These are especially important for the calculation of surface areas of cellular components of interest (Cruz-Orive and Weibel 1990, Gundersen *et al.* 1988b).

Vertical sections are plane sections taken perpendicularly to the horizontal plane. The horizontal plane is one which need only be a plane of reference, and this defines the orientation of the section.

An example of selection of random vertical sections can be made with a sheep kidney. The kidney is placed on a long side and sliced into sections (either lengthwise or crosswise) 5 mm in width. Every fifth section is taken, the first one is selected randomly (use random number tables). Take each of these slabs and place them on the table and again cut them into 5 mm parallel strips, with

the orientation of the strips selected randomly for each slab. Every fifth strip (again the first is selected randomly) with the vertical axis defined as running longitudinally through the strip. By rotating the strips around this axis as they are embedded, random vertical sections are obtained and are easily identifiable as the long axis of the material in the blocks. Because several blocks are obtained and are rotated differently, a set of sections with a high number of vertical axes necessary for efficient stereological assessment is available (Gundersen *et al.* 1988b).

While this is clearly advantageous for large tissues, in the present study tissues are small and are embedded whole. Moreover precise orientation is necessary for estimation of volume and cell number and for calculation of other renal indices (Relative Medullary Thickness, RMT, Percentage Medullary Thickness, PMT). Parallel planes that are perpendicular to a given horizontal plane do not qualify as vertical planes as they must be allowed to rotate around the vertical axis. The systematic random sections necessary for the Cavalieri method of volume estimation (see below) cannot be used for direct estimation of surface area (Cruz-Orive and Weibel 1990). Thus the surface areas of structures of interest cannot be assessed in the present study.

I.4 Point estimation

The estimation of the areas of cellular profiles of interest is best done by randomly superimposing a test grid of points over the section (Mayhew 1991). This point estimation method was best described by Weibel (1979) and this "older" morphometric method remains as one of the pivotal bases of modern stereological methods (Bertram and Nurcombe 1992, Cruz-Orive and Weibel 1990, Gundersen *et al.* 1988b, Mayhew 1991). The numbers of points that fall on the item of interest are counted (p) and become an estimator of their area (a). The area that the point represents (usually they are the centre of a square) is known and needs to be calibrated for the image magnification. Thus the point area is a(p) and the total area is $A = P \times a(p)$. This method was used for determining glomerular number per unit area.

I. 5 Volume estimation

Volumes of organelles, cellular complexes (such as renal glomeruli) and whole or part organs (e.g. total renal volume, cortex or medullary volumes) can be measured simply.

Whole organ, or part organ volumes are estimated using the Cavalieri principle (Bertram and Nurcombe 1992, Cruz-Orive and Weibel 1990). The

object is cut in parallel sections of a known distance apart (T). The area of each organ section (A) is calculated using the point counting method. The sum of the section areas (ΣA) times T is ar unbiased estimator of volume (V) (Cruz-Orive and Weibel 1990).

Thus $V = \Sigma A \times T$.

When organs are small (as in this study), serial sections of embedded material are obtained. A uniform systematic sample of sections is selected, and the area (A) estimated for this fraction (f) of sections. For example if one in every 20 sections is selected, f becomes 1/20. The section thickness (t) is known and from this volume can be calculated (Bertram and Nurcombe 1992).

Thus $V = \Sigma A(1/f)t$.

This method can be used to estimate volume of whole organs, part organs and single cells (Mayhew 1991) and an example for the kidney is given by Bertram *et al.* (1992). However, in the present study, more widespread methods of estimating gross morphology were used, RMT, PMT, and renal mass.

I.6 The disector method for counting particle number

This is an extension of the point estimation method of counting but eliminates the problem of height bias, i.e. if a cellular component is very long (e.g. renal proximal tubule) it may be sampled twice (Cruz-Orive and Weibel 1990, Gundersen *et al.* 1988a). Parallel sections are observed through a grid, with two adjacent perpendicular sides of the grid used as the sampling side, and the other two used as the non-sampling sides. This is considered an unbiased sampling grid. The frame is superimposed over the sample section and the cellular component of interest is counted. Any parts that touch the two sampling lines of the grid are counted, any parts that touch the non-sampling lines are not counted. However if any of the cellular components are in the adjacent, or "look-up", section, they are not included. Therefore the only components counted are those present only in the sample section, and only within the sampling grid, reducing height and overcounting biases (Bertram *et al.* 1992, Cruz-Orive and Weibel 1990, Gundersen *et al.* 1988a).

The optical disector method is a modification of this, where a thick (20-25 μm) section is focused through counting components as they come in focus, but meet the grid requirements and omit the first and last few μm of each section, removing section irregularities (Bertram *et al.* 1992, Gundersen *et al.* 1988a).

To calculate the total number of cells in the total volume the section thickness as well as the area of the counting grid (A_g) must be known. Thus number per unit volume (N_{Vcell}) s

$$N_{Vcell} = A_g \times t \times n.$$

If total organ volume is known ($V = A(1/f)t$, see above), then the total number (N_{tot}) can be calculated from

$N_{tot} = N_{Vcell} \times V$ (Bertram and Nurcombe 1992).

I.7 The fractionator

The fractionator method of sampling was devised to eliminate the problems of shrinkage in tissues and also where it is not possible to determine the volume using the Cavalieri method (Bertram and Nurcombe 1992, Gundersen *et al.* 1988a). The principle involves sampling particles uniformly at random (i.e. sections are observed a systematic difference apart (d) after a randomly selected start of a distance between 0 and d. Selection of blocks in large organs is very similar to the vertical section method for unbiased estimation of surface areas. A combination of the disector-fractionator methods will give an unbiased number of cells for the whole volume (Bertram and Nurcombe 1992, Gundersen *et al.* 1988a).

I.8 The nucleator

The area of a circle, **a**, can be defined as $a = \pi r^2$, where r is radius. It is also true that any series of isotropic lines through the object from a fixed point can be used to estimate the area The unbiased estimator of area uses the mean of these lines, L, instead of r, and because it is an estimate of the coefficient of error (CEM) the more lines measured, the better it is an estimate of r. This enables the area of irregular shapes to be calculated (Gundersen *et al.* 1988a). Volume can also be calculated ($V = 4\pi/3 \times (L_n)^3$, from nucleated cells to ovarian follicles (Gundersen *et al.* 1988a)

I.9 Stereology for *A. stuartii*

Several renal parameters were measured for *A. stuartii* using the above methods. The kidney was embedded and serially sectioned longitudinally which enabled the calculation of RMT and PMT. Glomerular number per unit area was calculated using the disector-Cavalieri combination. Glomerular volume was also calculated using the disector-nucleator method. This direct method is better than the estimation described by Bertram *et al.* (1992) as preliminary observations

suggested that there may be differences between cortical and juxtamedullary glomeruli and that populations within individuals may be quite variable.

Surface areas cannot be measured because isotropy cannot be obtained, therefore simple measures were made of epithelial heights and cellular volumes (using the nucleator method) in tubules. Sample sizes for animals were determined by availability from the wild, and were variable, with sample sizes for the groups ranging from 2 to 12. Clearly stereological error must be kept to a minimum by ensuring low CEM. It was hoped with these methods to be able to quantify morphological changes coincident with the physiological changes in *A. stuartii*.

The reproductive parameters in male *A. stuartii* were not extensive as other studies have focused on reproductive morphology in the male, including morphometric analyses (Taggart and Temple-Smith 1992, 1993, Taggart *et al.* 1993). Epididymal cell heights and volumes were calculated using the methods outlined above. Moreover, many of the changes in the male accessory reproductive tract were very distinct and detailed morphometric analyses were not necessary (Chapter 5, 8).

Appendix II

Field Studies

II.1 Introduction and methods

The field work can be divided into two sections, animals collected from nest boxes and animals collected from trapping efforts.

II.1.1 Live trapping

Trapping methods are described in Chapter 2.2. Most *Antechinus stuartii* were collected from traps, so they will be dealt with first. As was discussed in Chapter 2, some animals were released. Female numbers were frequently small during the first half of the animal collection from the wild and consequently not all were kept for experiments as it was thought that this might unnecessarily jeopardise population stability in the wild. Later in the animal collection for experimental work, excess males, not necessary for group numbers, were released. However only total numbers of animals caught are presented below. All other species captured in Elliott traps were released immediately at the trap site.

II.1.2 Nest boxes

The nest boxes were wooden boxes and measured 15 x 15 x 25 cm. The front of the boxes was about 3 cm lower than the other three sides and a lid on a hinge was attached to the back and sat on the other three sides. This meant there was an enclosed nesting box that could be examined easily by lifting the lid upwards.

The boxes had a hook on the back that could be hooked onto a nail in a tree. Each box had plastic fibre bedding placed inside, and the boxes were placed on trees about 2-3 metres above the ground. There were 35 nest boxes in total. Nest boxes were checked by removing them from the tree, opening the lid and looking for signs of disturbance. If this was observed, the contents of the box were carefully emptied into a clear plastic bag and any animals were removed from the bag. Other species were returned to the nest box with the plastic fibre bedding. Any signs of habitation, such as faecal deposits on or in the nest box, or nest reconstruction with the addition of leaf litter were removed when the nest boxes were checked. The first year is a combination of two years

of incomplete seasonal data collection, due to a cessation of the project over this time. Data collected were small for both years.

The data are summarised as follows

i) Total animals captured in traps as a percentage of traps placed in the field for each year.

ii) Total number of *A. stuartii* presented as a percentage of all animals trapped in each year.

iii) Sex ratios for *A. stuartii* for each year. These data include animals caught from nest boxes.

Data were analysed by Chi-square test using $P<0.05$ (Zar 1984).

II.2 Results

II.2.1 Live trapping

In May of the first year of trapping two females and three males were captured in 150 trap nights. Fifteen rats (*Rattus fuscipes*) were caught. In July of the first year of trapping, one female and five males were trapped in 200 trap nights. Twelve *R. fuscipes* were caught. In August of the first year of trapping four females and nine male *A. stuartii* were captured in 245 trap nights. Sixteen *R. fuscipes* were captured.

In February of the second year of trapping one female and three males were captured in 48 trap nights. One Cunningham's skink (*Egernia cunninghami*) was also captured. In May of the second year of trapping two females and ten males were captured in 245 trap nights. Twenty *R. fuscipes* were also caught.

In July of the second year of trapping six female and seven male *A. stuartii* were captured in 280 trap nights. Fifteen *R. fuscipes* and one *Mus musculus* were also caught. In August of the second year of trapping two females and three males were captured in 193 trap nights. No other species were captured.

In the last year of trapping, when males were collected for the testosterone and cortisol administration experiments, trapping was undertaken in the first week of May. Fifty-nine female and 27 male *A. stuartii*, 81 *R. fuscipes* and one *M. musculus* were captured in 1032 trap nights.

II.2.2 Nest boxes

In February in the first year of checking there were two female and three male *A. stuartii* captured. Two of the males and one female were together in the same nest box. In May of the first year of the checking of nest boxes two

females and four males were retrieved from nest boxes. Again some animals were found cohabiting. Nest boxes were checked at other times of year, but no *A. stuartii* were found.

Often evidence of larger species was found and occasionally sugar gliders (*Petaurus breviceps*) were found in the boxes. Faecal remains from *A. stuartii* and *P. breviceps* were observed on nest boxes and it became apparent that these boxes were often used by *A. stuartii* and *P. breviceps*. Frequently ants of several species were observed in the nest boxes, and on one occasion a male *A. stuartii* was observed in a nest box with an ants nest.

In February of the second year of checking, two males were found together in one nest box. In July of the second year one male and one female were found together in a nest box. *A. stuartii* were not found at other collection times of this year. Again evidence of other species were found in the boxes and occasionally sugar gliders were found in the boxes.

The final year of the checking of nest boxes was for collection of males for the testosterone and cortisol administration experiment. Here one male was collected from the nest boxes. There were faecal remains from *A. stuartii* and *P. breviceps* in and on some nest boxes and several ant nests in other boxes.

II.3 Discussion

The trapping data show some trends over the three years of collection for *A. stuartii*. The total number of all species caught as a percentage of trap nights was relatively stable for the first two years and increased slightly, but not significantly in the final year (Figure II.1). However the percentage of all species caught which were *A. stuartii* tended to increase over the course of the study ($P<0.07$, Figure II.2). The final year of the study was drier than the other years (F. Geiser, personal communication), and was the culmination of a dry period throughout the New England district. The drought did not break until several months after the final collection of *A. stuartii*.

The increase in *A. stuartii* over the course of the study may be related to the drying of the trapping sites and the consequent reduction in numbers of *R. fuscipes*. These rodents prefer wet habitats and a dense cover of undergrowth (Braithwaite *et al.* 1978). The drying out of some sites in particular led to fewer *R. fuscipes* being caught. Their place had been taken by *A. stuartii*, as the total numbers of animals caught did not decrease. However, coincident with the drier climate was the recovery of many sites from severe beetle (Scarabaeidae: *Anoplognathus* spp., Heatwole and Lowman 1986) attack of previous years. Cockburn and Lazenby-Cohen (1992) found that *A. stuartii* will shift its habitat

Figure II.1

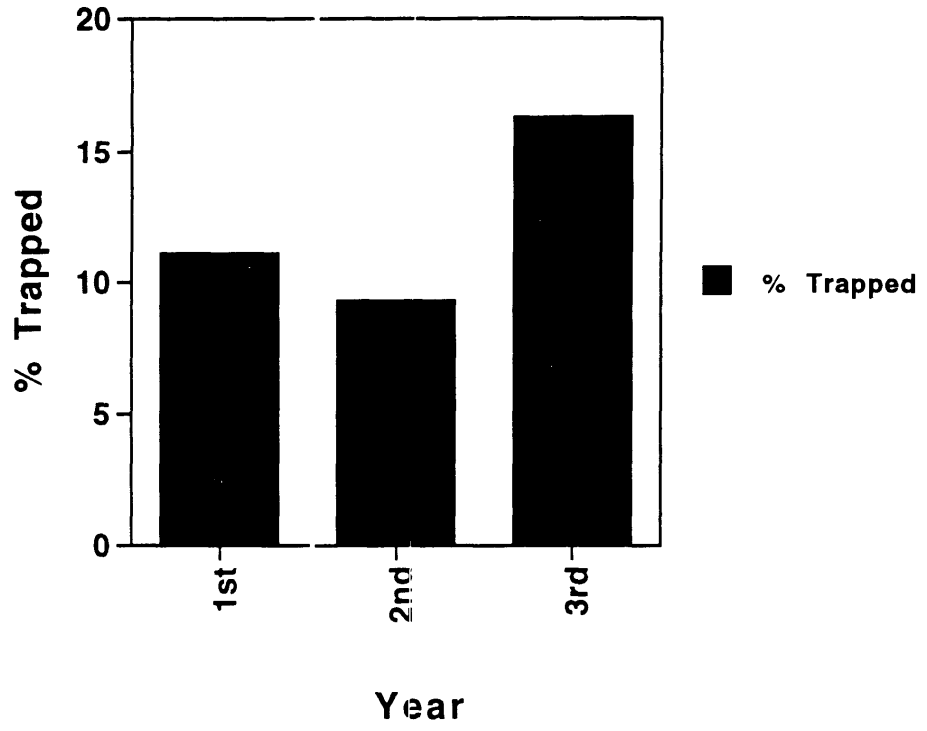

Figure II.1 The total number of vertebrates captured expressed as a percentage of trap nights over the course of the PhD study.

Figure II.2

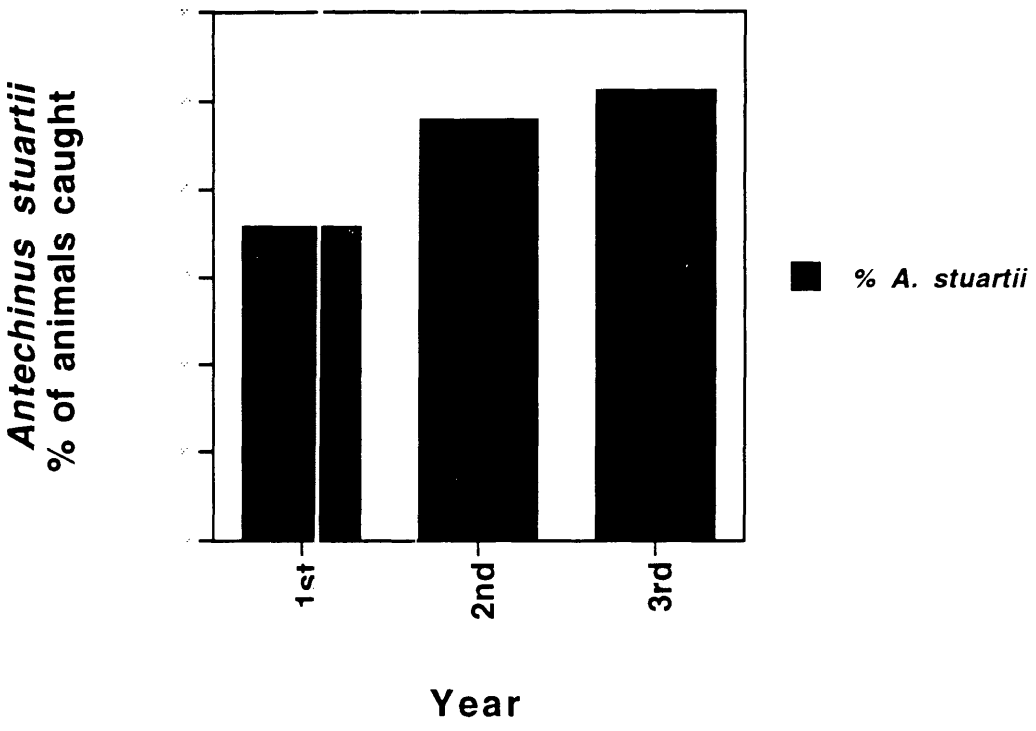

Figure II.2: Numbers of *A. stuartii* caught expressed as a percentage of the total number of vertebrates captured each year.

preference as the forests change and also as a consequence of competition with other species. This may have been occurring at these sites for *A. stuartii*.

However, one of the largest changes over the course of the study was the change in sex ratio over time. The first two years showed a distinct bias towards males (Figure II.3) and in the final year there was a bias towards females (Figure II.3). This contrasts with other studies that found that the sex ratio does not change within populations, but can be quite different between populations (Cockburn *et al.* 1985a, Cockburn 1990). In part this may be because of the removal of animals for the studies that may have disrupted the population structure for each site. However, the main impact on the population by removal of animals was in the first year of trapping, and the reversal of the sex ratio was in the third year of trapping.

Male *A. stuartii* disperse after weaning (Cockburn *et al.* 1985b), and Cockburn and Lazenby-Cohen (1992) suggest that because female young have more potential to compete with their mother, the mothers will invest in dispersing male young. The implication is that when competition for resources is high, such as under conditions of marginal habitat, drought or habitat destruction, females will produce more males for dispersal to colonise more favourable areas. Under less competitive conditions females will produce more daughters, which are more costly to rear (Cockburn and Lazenby-Cohen 1992). In this study, only two second year females were captured in the whole of the first two years, but in the last year four were captured, perhaps indicating that the sites had improved in suitability for longer survival of the females. Perhaps the regrowth of eucalyptus forest damaged by beetles altered the prey availability and thus the competitive structure of the areas trapped.

While the results of present study are an interesting comparison to other studies the main difference is the methodology and larger number of observations in other studies (Cockburn *et al.* 1985a, Cockburn 1990). The field work in this study was not aimed at systematic sampling of the area and marking of animals to obtain population information and structure, merely to obtain *A. stuartii* for the physiological studies. The change in sex ratios may be the result of other ecological parameters, such as logging, clearing or fires nearby, not accounted for by the present study.

Another contrast to other studies was the use of nest boxes by *A. stuartii*. Cockburn and Lazenby-Cohen (1992) found that males apparently always nest communally, and always with females. This contrasts with the present study where about half the animals were found nesting alone. Both males and females were found alone and male only groups were also found.

Figure II.3

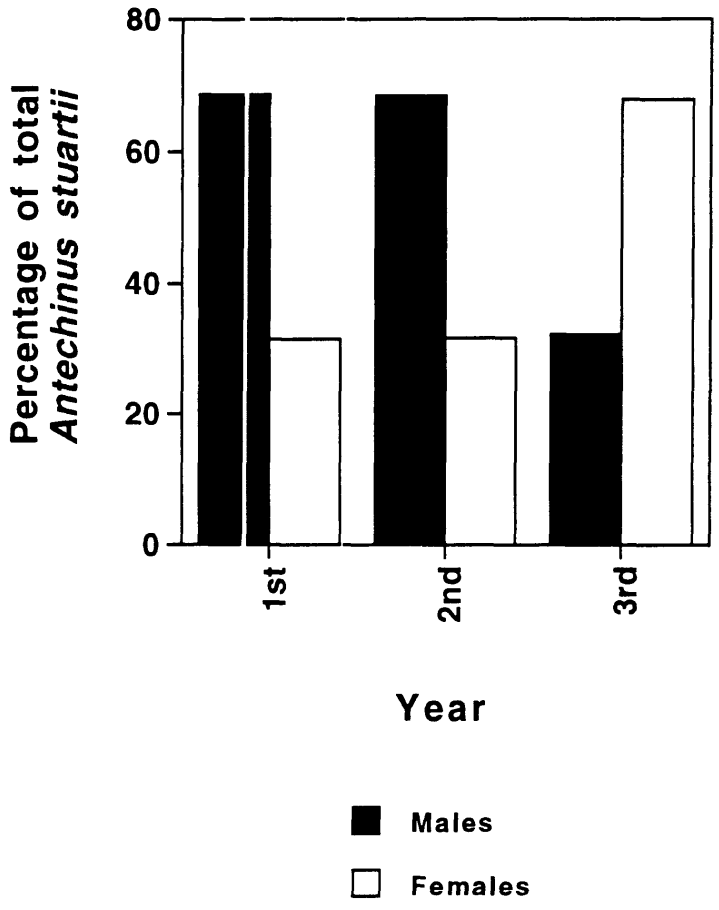

Figure II.3 The sex ratio of *Antechinus stuartii* over the course of the PhD study. The ratio is expressed as percentage of the total numbers of *A. stuartii* captured.

While numbers are small, the unequivocal finding of males nesting alone is different from the other study.

Although the field data are not a complete population study of the areas around the New England tablelands, they show some interesting parallels and contrasts with other studies that have concentrated on population dynamics in *A. stuartii*.

Appendix III

Histological Methods

III.1 Fixatives for light microscopy

Neutral Buffered 10% Formalin (Kiernan 1990)

Formalin	100 mL
Distilled water	900 mL
Sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$)	4.0 g
Disodium phosphate (Na_2HPO_4)	6.5 g

Dissolve sodium phosphates in water, add formalin.
Optimum fixation is 7-10 days, suitable for paraffin or frozen sections.

Bouin's fixative (Kiernan 1990)

Saturated Picric acid	750 mL
Formalin	250 mL
Acetic acid	50 mL

Optimum fixation is 6-24 hours, and then transferred to 70% ethanol. Remove yellow of picric acid before staining with basic aniline dyes with 2.5% sodium thiosulphate. This fixative was used for some reproductive tracts.

III.2 Tissue processing

Tissues were first placed in 30% ethanol for 4-8 hours and then processed using a histokinette. The processing protocol used was:-

Solution	Time
30%	2 hours
50%	2 hours
70%	2 hours
80%	2 hours
95%	1 hour
100%	1 hour
100%	1 hour
1:1 100% ethanol: xylene	1 hour
Xylene	1 hour
Xylene	1 hour
Paraplast	2 hours
Paraplast	2 hours

Followed by vacuum embedding and solidification on ice.

III.3 Histological Stains

III.3.1 Masson's trichrome (Drury and Wallington 1980)

1. Take sections to water.
2. If fixed in neutral buffered formalin, place in dichromate mordant for 15 minutes. Wash three times in distilled water.
3. Stain with Celestine blue haemalum for 5 minutes.
4. Stain with Weigert's haematoxylin for 5 minutes.
5. Differentiate nuclear stain with 0.5% hydrochloric acid in 70% ethanol, until background is a greyish colour.
6. Wash well in tap water, rinse in distilled water.
7. Stain in red cytoplasmic stain for 3-5 minutes.
8. Rinse in water, then in distilled water.
9. Differentiate in 1% phosphomolybdic acid until collagen is decolourised, usually 5-10 minutes.
10. Rinse in water, then distilled water.
11. Counterstain in Aniline blue for 2-5 minutes.
12. Wash well in 1% acetic acid for 1-2 minutes.
13. Blot off excess fluid, dehydrate in two changes of absolute ethanol, then clear in xylene, mount in DPX or Eukitt mounting medium.

Results

Black - Nuclei

Red - muscle, red blood cells, some cytoplasmic granules

Blue - collagen, some reticulin, amyloid and mucin

Weigert's haematoxylin	
Solution A (stain)	
Haematoxylin (Gurr, BDH)	10 g
100% ethanol	1000 mL
Solution B (mordant)	
30% aqueous ferric chloride (anhydrous) (Merck, Germany)	40 mL
Concentrated hydrochloric acid	10 mL
Distilled water	950 mL

Store separately and mix together immediately before use. If the solutions have been made up fresh, use equal proportions of each, if solution A is aged, use less

of it. The mixed solutions last only a few hours and if the stain is purplish black it is usable, if it is muddy brown it should be discarded.

Celestine blue - haemalum	
Celestine blue B (Gurr, BDH)	2 g
Ferric ammonium sulphate (BDH)	20 g
Glycerol	56 mL
Distilled water	400 mL

Dissolve iron alum without heat, add celestine blue B and boil for three minutes. Cool, filter, and add glycerol. Keeps for 6 months.

Cytoplasmic stain

A. 1% Ponceau de xylidine (Ponceau 2R, Gurr, BDH) in 1 % acetic acid

B. 1% Acid fuchsin (Gurr, BDH) in 1% acetic acid

Mix 2 parts A to 1 part B.

Fibre stain

2% Methyl (Aniline) blue (Gurr, BDH) in 2% acetic acid.

Dichromate mordant

This is to "brighten" the staining of tissues that have not been fixed in a picric acid fixative. Use 3:1 10% potassium dichromate: 90% ethanol solution mixed immediately before use.

III.3 2 Periodic acid - Schiff's - Alcian blue (Drury and Wallington 1980)

1. Take sections to water.
2. Stain with Alcian blue for 30 minutes.

3a. For Alcian blue pH 2.5 wash with water for 5 minutes.

3b. For Alcian blue pH 1.0 briefly wash with 0.1 N hydrochloric acid.

4. Treat control slides with 0.1% malt diastase in distilled water for 30 minutes at 37° C. Leave other slides in distilled water for 30 minutes.
5. Oxidize for 5 minutes in 1% aqueous periodic acid (Sigma, U. S. A.), leaving a few slides in distilled water as a control.

6. Wash in running water for 5 minutes, then rinse in distilled water.
7. Place in Schiff's reagent for 20 minutes.
8. Wash for 10 minutes in running water, then rinse in distilled water.
9. Stain with Weigert's haematoxylin or Celestine blue for 5 minutes, avoid differentiation and overstaining as this will mask some of the results.
10. Dehydrate, clear in xylene and mount in DPX or Eukitt mounting medium.

Results

The oxidation by periodic acid allows hydroxyl groups on adjacent carbons to be oxidized to aldehyde groups. This reaction breaks the pyranose ring of many sugars producing a dialdehyde and, when followed by staining with Schiff's reagent, gives a pink reaction when positive. Substances that stain positive include polysaccharides, glycoproteins, and glycolipids. Acid mucopolysaccharides (glycosaminoglycans) are PAS negative but Alcian blue pH 2.5 positive. Sulphated mucopolysaccharides are stained by Alcian blue pH 1.0 (Kiernan 1990). Table III.1 has been modified from Drury and Wallington (1980).

Schiff's reagent

Schiff's reagent (Feulgen stain) was purchased from BDH Laboratories, Poole, England

Alcian blue pH 2.5 (Drury and Wallington 1980)

Alcian blue 8GX (Gurr, BDH)	2.5 g
Glacial acetic acid	15 mL
Distilled water to	500 mL

Mix together.

Alcian blue pH 1.0 (Drury and Wallington 1980)

Alcian blue 8GX (Gurr, BDH)	5.0 g
0.1 N hydrochloric acid	500 mL

Mix together.

III.3.3 Haematoxylin and eosin (Drury and Wallington 1980)

1. De-wax sections and take to 70% ethanol.
2. Stain in Harris' or Ehrlich's haematoxylin for 20 minutes.

Table III.1 Results of treatment with periodic acid-Schiff's reagent, Alcian blue pH 2.5, and Alcian blue pH 1.0 (from Drury and Wallington 1980).

Substance	Specific substance	Periodic acid Schiff	Diastase control	Alcian blue pH 2.5	Alcian blue pH 1.0
Polysaccharide	Glycogen	+ve	-ve	-ve	-ve
Acid mucopolysaccharide	Carboxylated (COOH), hyaluronic acid	-ve	-ve	-ve	+ve
Acid mucopolysaccharide (Connective tissue "mucins")	Sulphated, (COOH and SO_4), chondroitin sulphate A, B & C, heparin, keratosulphate	-ve	-ve	+ve	+ve
Mucoproteins and glycoproteins (Epithelial "mucins")	Neutral (1:2 glycol), gastric mucin. Carboxylated (1:2 glycol & COOH), sialomucin.	+ve	+ve	+ve	-ve
Mucoproteins and glycoproteins (Epithelial "mucins")	Sulphated (1:2 glycol, COOH & SO_4), sulphated sialomucins	+ve	+ve	+ve	+ve
Glycolipids	Lipids (1:2 glycol and lipid), cerebroside	+ve	+ve	-ve	-ve

3. Wash well in running tap water for 2-3 minutes. Check that the nuclei are sufficiently stained.
4. Differentiate in 1% Hydrochloric acid in 70% ethanol for a few seconds. Remove stain until nuclei are reddish and the background is a pale yellowish colour.
5. Wash in running tap water for 15 minutes. The nuclei should be an intense dark blue colour.
6. Stain in 1% aqueous eosin for 1-3 minutes. Blot off excess stain.
7. Dehydrate in two to three changes of absolute ethanol, this removes excess stain.
8. Pass through two changes of xylene and mount in DPX or Eukitt mounting mediums.

Results

Blue to blue black - nuclei

Purplish blue to blue - nucleoli, cartilage, cement line of bone, calcium or calcified bone, basophil cytoplasm

Bright orange to red - red blood cells, plasmasomes, eosinophil granules, Paneth cell granules, zymogen granules, keratin

Shades of pink - cytoplasm, muscle fibres, colloid, thick elastic fibres, decalcified bone matrix, collagen

Many structures are, however, not easily differentiated or even stained by this method. The best example of this is the kidney, where all tubules stain the same pink colour. With a trichrome stain the medullary region stains mauve and the cortex stains reddish purple, with the brush borders of the proximal tubules staining mauve and the glomeruli red, due to the red blood cells in the glomeruli. Drury and Wallington (1980) also maintain that neuroglia fibres, axons, nerve endings, reticulin, golgi bodies and mitochondria are not stained, even when preserved by the fixative.

III.3.4 Ehrlich's (1886) haematoxylin (Drury and Wallington 1980)

Haematoxylin (Gurr, BDH)	6 g
Absolute ethanol	300 mL
Glycerol	300 mL
Distilled water	300 mL
Glacial acetic acid	30 mL
Aluminium potassium sulphate (potassium alum) in excess	10-14 g

Dissolve the haematoxylin in the ethanol before adding the other ingredients. The stain must be aged, and this is achieved by leaving in a large flask, loosely stoppered with cotton wool, for several weeks in the sunlight. The colour should go from purplish to deep red, and the odour should change from acetic acid to a vinous one.

III.3.5 Harris' (1890) haematoxylin (Drury and Wallington 1980)

Haematoxylin (Gurr, BDH)	5 g
Absolute ethanol	50 mL
Aluminium potassium sulphate (potassium alum)	100 g
Distilled water	1000 mL
Mercuric oxide	2.5 g

Dissolve the haematoxylin in the ethanol before adding to the potassium alum, which has been dissolved in the warmed distilled water in a large flask. Bring to the boil and add the mercuric oxide, upon which the solution will turn dark purple. The flask needs to be quite large as the solution froths considerably on boiling. Cool rapidly under the tap and filter before use.

III.4 Histological methods for electron microscopy

III.4.1 Introduction and general methods

Tissues were immersed in Karnovsky's fixative and while immersion is not an optimum method for fixation of samples for electron microscopy, because the plasma was to be used for hormone analysis and the frozen sections for *in vitro* autoradiography or enzyme analysis, the preservation of tissues for electron microscopy was necessarily compromised.

Small, firm tissues, such as kidney, ovary, adrenals and prostate were cut into pieces 1-2 mm^3 prior to immersion. Tissue was fixed for 2 hours at room temperature with rotation. Larger pieces which did not hold their structure on dissection, such as the bulbourethral glands and the testes were immersed in fixative overnight and then cut into pieces 1-2 mm^3 the following morning and placed into fresh fixative for 1 hour. After fixation tissues were washed 3x in 0.1 M phosphate buffer pH 7.3, while on a rotator. They were then post-fixed for 1.5 hours in 1.0% Osmium tetroxide in 0.1 M phosphate buffer, pH 7.3, at room temperature. The tissue was then washed once in 0.1 M phosphate buffer pH 7.3, and then dehydrated and embedded in Spurr's resin "A" (Probing and Structure, Queensland). The resin: ethanol concentration was increased gradually over 48 hours before embedding.

While tissues were collected for electron microscopic analysis, because of limitations of time only semi-thin sections of kidney tissues were assessed. The thesis concentrated on the morphometric analysis of the light microscopy samples. These complemented the physiological data.

III.4.2 Fixative

Initially fixatives were tested on *Mus musculus* and *Rattus norvegicus* kidneys, as these tissues are the most difficult to fix for electron microscopy. The osmolality of kidney tissue has at least a fourfold range between tissues and optimal fixation often needs to be obtained by trial and error (Hayat 1990). The most complex part is the medulla, where osmolal changes can range from about 300 mosm to 1200 mosm in humans (Guyton and Hall 1996). The osmolality of several buffers with glutaraldehyde percentages increasing from 1% to 4% was given by Hayat (1990). While buffer only osmolality was 212-345 mosm, with 4% glutaraldehyde it was 710-895 mosm. Fixation for most mammalian tissues requires a slightly hypertonic vehicle (400-450 mosm) because the glutaraldehyde is diluted while it penetrates the tissue block (Hayat 1990).

Phosphate buffers were used in the present study. Cacodylate buffers are preferred by many electron microscope histologists because they are easy to

prepare, stable for long periods of time, do not form precipitates, and do not support the growth of micro-organisms (Glauert 1975, Hayat 1990). However, the arsenic content of cacodylate always requires careful handling. Phosphate buffers have the advantage in that they are not toxic, mimic certain components of extracellular fluids, and pH does not change with temperature (Glauert 1975). However, they are stable for only about a month, support micro-organismal growth, decrease nuclear mass, and can cause preciptiates with fixatives (Glauert 1975, Hayat 1990).

Three fixatives were tried on mouse (*Mus musculus*) and rat (*Rattus norvegicus*) tissues. These included 3% Glutaraldehyde in 0.1 M Phosphate buffer pH 7.4, 505 mosm (Glauert 1975, Hayat 1990), Karnovsky's fixative (2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M Phosphate buffer, pH 7.4, 555 mosm, in Glauert 1975) and half strength Karnovsky's (diluted with buffer, pH 7.4, 316 mosm). While the glutaraldehyde only fixative is used routinely by many histologists (Glauert 1975), and was recommended by the Electron Microscope Unit at the University of New England, a combination of a paraformaldehyde - glutaraldehyde fixative also has advantages.

Paraformaldehyde penetrates tissue more quickly than glutaraldehyde and it is believed that the paraformaldehyde temporarily stabilises structures that are then fixed more permanently by the glutaraldehyde (Glauert 1975). The adverse effect of hypoxia is reduced and the combination appears to catalyse protein cross-linking (Hayat 1990). This combination also reduces the "myelinic figures" artifact seen with the use of glutaraldehyde alone (Glauert 1975). Phospholipid dissolution is also reduced. Glutaraldehyde alone also does not destroy the osmotic properties of the cells and thus the fixative buffer is extremely important. This is less important if paraformaldehyde is used as well (Glauert 1975).

The examination of the rat and mouse semi-thin sections did show some differences. When examined, the kidney tissue (cortex and medulla) was different between the fixatives More cytoplasmic detail was evident in the tissue preserved with Karnovsky's fixative. Futhermore there was some hyperosmotic distortion in the medullary cells preserved by 3% glutaraldehyde fixative. However, the dilute fixative showed less cytoplasmic detail than the full strength fixative. Thus Karnovsky's fixative was used for the preservation of *A. stuartii* tissues for electron microscopy.

The osmolality of the fixative was 555 mosm/kg and the osmolality of the 0.1 M phosphate buffer pH 7.3 was 230 mosm/kg. The lower pH of 7.3 was used because my prior experience with marsupial tissue culture indicated that a

slightly lower pH (7.2-7.3) than the usual pH of 7.4 for eutherian cell cultures, was optimum for marsupial fibroblastic cell growth. Although Glauert (1975) states that the pH is not critical, as long as it is in the range of 6.5-8.0, it was thought better to limit some of the problems that immersion fixation produces (see Chapter 2) by keeping other factors as controlled as possible.

Karnovsky's (1965) Fixative (Glauert 1975)

0.2 M Phosphate buffer pH 7.3	100 mL
10% paraformaldehyde in distilled water	40 mL
25% glutaraldehyde in distilled water	20 mL
distilled water to make	200 mL

pH was always determined after aldehydes were added, and adjusted if necessary.

Phosphate buffer (Sörensen, in Glauert 1975)

Solution A

0.2 M dibasic sodium phosphate $Na_2HPO_4.2H_2O$	35.61 g
Distilled water	1000 mL

Solution B

0.2 M monobasic sodium phosphate $NaH_2PO_4.H_2O$	27.6 g
Distilled water	1000 mL

The 0.1 M phosphate buffer is made by mixing x mL of "A" with y mL of "B" and diluting to 1 litre with distilled water.

pH	x mL	y mL
7.2	360	140
7.3	385	115
7.4	405	95

The osmolality of the phosphate buffer was 228 $mosm \cdot kg^{-1}$.

10% Paraformaldehyde (Glauert 1975)

Dissolve 3.0 g of paraformaldehyde powder in about 25 mL of distilled water and heat to between 60-65°C in a fume cupboard. Add a few drops of freshly made

1.0N sodium hydroxide until the solution becomes clear. Make up to 30 ml and filter when cool. It is best to make the paraformaldehyde solution a day before its addition to Karnovsky's fixative.

III.4.3 Embedding procedure

All procedures, with the exception of the Osmium post fixation are performed with rotation.

Immersed in fixative	2 hours
3x washes in	
0.1 M phosphate buffer pH 7.3	20 minutes each
Post fixed in 1% Osmium tetroxide in	
0.1 M Phosphate buffer pH 7.3	1.5 hours
Wash in	
0.1 M phosphate buffer pH 7.3	20 minutes
Dehydration in ethanol	
30% ethanol	10 minutes
50% ethanol	10 minutes
70% ethanol	10 minutes
80% ethanol	10 minutes
90% ethanol	10 minutes
95% ethanol	10 minutes
100% ethanol (desiccated)	10 minutes
100% ethanol (desiccated)	10 minutes
100% ethanol (desiccated): Spurr's "A" Resin	
2:1	4 hours
1:1	overnight
1:2	8 hours
Full strength	overnight

Embedded in fresh resin and polymerised at 65^{o} C for 24 hours.

Semithins were stained with Toluidine Blue (from Hayat 1993).

1. Warm sections on slide and then add several drops of the stain. Replace onto hot plate.
2. When the stain is drying around the edge rinse with distilled water.
3. Rinse with ethanol, and dry on hotplate.
4. Mount with DPX or Eukitt

Toluidine blue

Toluidine blue	1.0 g
(National Aniline Division)	
30% Ethanol	100 mL

Borate buffer (pH 7.8)

Boric acid	2 g
Sodium tetraborate	1 g
distilled water	100 mL

Mix Toluidine blue: Borate buffer 3:2 before use.

Results

All cellular components are shades of blue.

Appendix IV

In vitro Autoradiography

IV 1 Introduction

IV.1.1 Autoradiography

Autoradiographic localisation of hormone receptor sites has long been used in endocrinology (Bitensky and Poulter 1969, Morrell and Pfaff 1981). The principles of autoradiography are simple, the electromagnetic radiation emitted by radioactive isotopes affects photographic emulsions in the same way as light. When the gelatin emulsion is developed, the silver bromide crystals are induced to form silver grain formation at the sites of radiation. Very small amounts of radiation can be detected because emulsions can be exposed over long periods of time (Møller and Krogh 1991). Thus receptor sites for radioactive hormones can be identified in their target tissues.

The radioactive molecule is absorbed and metabolised in the same way as the non-radioactive molecule and therefore the fate of the molecule can be traced (Møller and Krogh 1991). Many studies have mapped the sites of uptake of hormones, both steroid and peptide, by the *in vivo* autoradiographic method (Bidmon *et al.* 1990, Callard *et al.* 1986, Ghar *et al.* 1992, Joss 1981, Watson *et al.* 1989). The radioactive molecule of interest is usually injected intraperitoneally into the animal and then the animal is sacrificed up to 24 hours later (Morrell and Pfaff 1981). By this time the level of isotope circulating in the blood has decreased from its peak value and is low (Morrell and Pfaff 1981).

However, the *in vivo* method, while delivering information about the target sites of labeled hormones, has several disadvantages. Large quantities of isotope are required (25-200 μCi or 0.9-7.4 MBq per 100 g body weight, Morrell and Pfaff 1981), the isotope may not pass the blood brain barrier (for brain autoradiography), or it may be a substrate of uptake processes and become concentrated within specific cell types (Stewart and Bourne 1992). The *in vitro* autoradiographic method, first described by Young and Kuhar (1979) eliminates some of these problems.

Two new techniques were discussed by Young and Kuhar (1979). Firstly, the *in vitro* incubation of frozen sections with the ligand of interest, and secondly the use of emulsion covered coverslips rather than emulsion covered slides for the autoradiography. Both these techniques significantly changed the flexibility

of autoradiography. The use of emulsion covered coverslips rather than emulsion covered slides meant that all cryostat sectioning did not need to be in a special darkroom.

The *in vitro* incubation method meant that adjacent sections can be assessed for different hormone receptors, only small quantities of isotope are needed, and tissue can be stored at -70° C for long periods of time before use, as the receptor binding characteristics remain stable (Dashwood 1992, Young and Kuhar 1979).

Since then some authors have used tritium sensitive film, [^{3}H]hyperfilm (Amersham) or Ultrofilm (LKE) instead of emulsion covered coverslips (for example Kloas and Hanke 1992a,b, Panzica *et al.* 1994, Walters *et al.* 1993). This allowed the densitometric evaluation of autoradiographic images to be performed (Dashwood 1992). Previously the "grain counting" of autoradiographic images provided a simple means of evaluating receptor densities (Dashwood 1992). Grain counting autoradiography, while simple, has some resolution problems. The source of the radiation, thickness and uniform coating of the emulsion, and the length of development time will all affect the resolution of the grain (Bitensky and Poulter 1969, Csillag 1992).

The use of image analysis in evaluating autoradiographic material has led to more sensitive receptor measurement, with some authors presenting their data in fmol per mg protein (Brown and Zuo 1993, Dashwood 1992, Walters *et al.* 1993). This is the traditional unit of measurement for homogenate binding studies, where the protein content can be accurately measured (Casolini *et al.* 1993, Geelen *et al.* 1981, Meaney *et al.* 1993).

There are many problems with the use of this as an accurate measurement of radioactivity from an autoradioactive image. These include tritium quenching within the tissue, non-linearity of the image with time, differing protein content across the tissue of interest, and the problems associated with the use of carbon and iodine labeled compounds (Dashwood 1992, Stewart and Bourne 1992). One solution to this is to use d.p.m. per mm^2 (disintegrations per minute) (Dashwood 1992). Another method that some authors have chosen to use is fmol per mm^2 (Kloas and Hanke 1992a,b, Stewart and Bourne 1992) where a compromise between knowledge of receptor concentration per unit tissue is reached without the corresponding information of protein estimation. Consensus about preferential use of receptor units has yet to be reached.

The concern over units of measurement has arisen due to some of the recent advances in the use of standards. Standard curves are made by homogenising the tissue of interest in a small amount (50μL) of low viscosity

silicone oil at 4° C. Then aliquots of a series of at least six dilutions of the isotope (40 μL each) are used with 500 mg of homogenised tissue in 1.5 mL Eppendorf tubes and centrifuged to remove air bubbles. It takes about 0.5-50 nCi/mg (0.02-2 kBq/mg) tissue (Stewart and Bourne 1992). The tubes are then frozen in an isopentane/CO_2 mixture and frozen sections can be cut by cutting the base, removing the paste onto a cryostat and proceeding to section and thaw mount in an identical manner to the tissue sections (Stewart and Bourne 1992). Parts of the frozen blocks can also be collected and put into scintillation vials to estimate isotope level. The homogenate sections are treated in the same manner as the tissue of interest and the optical density of each standard can be measured with an image analyser and then converted to d.p.m. per unit area of the standard by referring to the value estimated from the scintillation counting of the sections (Stewart and Bourne 1992). If the specific activity of the experimental radioligand is known, this can be incorporated into the calculation and the absolute concentrations of the isotope can be expressed as fmol per mg or fmol per mm^2 (Stewart and Bourne 1992).

However, standard polymer strips can now be purchased from suppliers. These have the advantage that known quantites of isotope can be distinguished as grades along the strip, and the densitometric analysis of the tissue can be made by referring to these strips. They are as accurate as tissue homogenate paste standards (Stewart and Bourne 1992).

IV.1.2 Autoradiography and *Antechinus stuartii*

In vitro autoradiography was attempted on tissues of *A. stuartii* to determine the receptor sites for cortisol. This technique has many obvious advantages over other methods for determining cortisol binding sites. *In vivo* autoradiography would have been extremely expensive, because of the large amounts of isotope needed. The use of competitive binding assays for cortisol requires whole tissue homogenates (Casolini *et al.* 1993, Meaney *et al.* 1992), which leaves none left for other analyses. *A. stuartii* is a native marsupial which must be captured from the wild, therefore as much information as possible must be obtained from each individual. Clearly methods that enable tissue to be conserved for other purposes must be employed. The *in vitro* autoradiography technique enables adjacent sections to be assessed for binding with several hormones, such as androgens and corticosteroids, and for immunocytochemical techniques for the presence of enzymes.

To the best of my knowledge this method of ascertaining cortisol receptor sites on frozen sections had not been previously attempted. Other authors had

reported glucocorticoid receptor binding in the mesolimbic system of rats using homogenate incubation with [^{3}H] corticosterone (Casolini *et al.* 1993, Meaney *et al.* 1992). In the kidney both mineralocorticoid and glucocorticoid receptors have been demonstrated using homogenate incubation with tritiated hormones (Funder *et al.* 1973b, Marver *et al.* 1975 . *In vivo* autoradiography has been successful for glucocorticoid receptors of the kidney, where *in vitro* incubation with excess aldosterone or cortisone determined specificity (Strum *et al.* 1975). This study of glucocorticoid receptors by Strum *et al.* (1975) included *in vitro* incubation using fresh tissues that were dry-mounted and freeze dried and sectioned at -65^o C. However in the present study fresh tissue from *A. stuartii* was collected over many months, and therefore freezing before analysis was the only practical method of collecting tissue to analyse in one or two batches (to reduce within-batch error). Kidney tissue has also been successfully targeted for other substances using *in vitro* autoradiography (Calianos and Muntz 1990, Kloas and Hanke 1992a).

The endocrine changes in male *A. stuartii* have been well documented (Bradley *et al.* 1980, Lee *et al.* 1977, McDonald *et al.* 1981). The role of cortisol in the death of the males and the failure of the glucocorticoid feedback mechanism in this genus opens up several avenues of experimentation. If the feedback of cortisol fails to restrict ACTH stimulation of the adrenal cortex, then it would be expected that mesolimbic receptor sites may be impaired. An alteration of receptor sites should be detected by *in vitro* autoradiography.

Furthermore, if cortisol is saturating not only glucocorticoid receptors, but also mineralocorticoid receptors in the brain and peripheral tissues, then receptor cross-reactivity should be demonstrated by using either *in vitro* autradiography to demonstrate the presence of aldosterone receptors on an adjacent slide, or by immunocytochemically demonstrating the presence of 11ß-hydroxysteroid dehydrogenase (11ß-OHSD) on the same, or adjacent slide (Mercer and Krozowski 1992). 11ß-hydroxysteroid dehydrogenase is an $NADP^+$ dependent microsomal enzyme which catalyses cortisol to cortisone, which is then unable to bind to mineralocorticoid receptors (Moisan *et al.* 1990, Stewart and Whorwood 1994). Mineralocorticoid receptors exhibit an identical affinity for aldosterone and cortisol *in vitro*, but their specificity *in vivo* is maintained by the inactivation of cortisol by 11ß-OHSD, thus enabling aldosterone to bind with the mineralocorticoid (Funder *et al.* 1988, Stewart and Whorwood 1994).

In the kidney there is normally a much higher affinity for aldosterone by the mineralocorticoid receptors, with only 2-4% of mineralocorticoid receptor sites showing affinity for glucocorticoids (Funder 1993, Funder *et al.* 1973b,

Mercer and Krozowski 1992). However in some instances the glucocorticoid receptors can mediate mineralocorticoid like effects on the cortical collecting ducts (Lee *et al.* 1983, Marver 1984, Náray-Fejes-Tóth *et al.* 1994b). The result of this has led Funder (1993) to suggest that the transepithelial sodium flux can be indistinguishably produced by glucocorticoid activation, providing the steroid can reach the receptor.

If the failure of the glucocorticoid feedback mechanism in *A. stuartii* is mediated by changes in the mineralocorticoid and glucocorticoid receptors, this should be demonstrated by *in vitro* autoradiography. Furthermore, because there is an eightfold increase in plasma levels of testosterone over the life of male *A. stuartii*, it was also thought that determining the receptor sites, and any changes in them throughout the year, would help determine the interplay between these hormone systems in *A. stuartii*. Methods for brain, kidney, and reproductive tract were examined for similarities in an effort to ascertain the most universal method for these tissues.

However, before trialling these methods on *A. stuartii*, *in vitro* autoradiography was attempted on tissues from *Mus musculus*. Many methods for *in vitro* autoradiography have been determined for the sex steroids (Callard *et al.* 1986, Peters and Barrack 1987, Walters *et al.* 1993), and thus it was thought that establishment of published methods would be the best to start with on mice using sex steroids. Moreover, because these methods could be used in determining sex steroid receptors in tissues from *A. stuartii*, this seemed a useful experimental approach.

To ensure that there would be differences between groups, old and young, castrate and intact mice were used. This would ensure pronounced differences between individuals for receptor numbers, so that when quantitative assessment was attempted the sensitivity of the grain counting method could be determined. The trials of *in vitro* radiography will be treated separately below, with a short discussion of each attempt following the method.

IV.2 Methods and results

IV.2.1 Animals

Mus musculus were obtained from the Animal House, University of New England, and were used for mating studies before the collection of tissues for *in vitro* autoradiography. Permission to perform the castrations was obtained from the Animal Ethics Committee, UNE. Twenty four males were used for the castration study. Twelve young males (two months old) and twelve old males (over four months old) were used with six from each group castrated.

Animals were castrated under ether anaesthesia by making an inguinal incision and ligating the testicular arteries and then excising the testes. The wounds were then stitched and Teramyacin antiseptic spray applied to the wound. Two old males died under ether anaesthesia, and one young male died post-operatively from chewing at the mouse cage.

Glomerular filtration rate (GFR) experiments were performed on the young intact males, and all other males were injected with approximately 1MBq of ^{51}Chromium-EDTA so as to expose all animals to the same procedures. The GFRs of eight females were also measured. Animals were killed by an overdose of pentobarbitone (see Chapter 2 between four and nine days after exposure to ^{51}Chromium-EDTA.

IV.2.2 Tissue preparation

Tissue was quickly immersed in Isopentane, Analytical grade, cooled to -80° C by placing solid carbon dioxide in and around the vessel containing the isopentane. Tissues were quickly transformed to chilled storage vessels and stored at -80° C.

IV.2.3 Radioligands

Purchase of radioligands is costly, therefore the first three trials of *in vitro* autoradiography were performed using substances remaining from previous experiments. For all but one radioligand the specific activity and the radioactive concentrations were known and are indicated in the respective trial. The final trial, using [^{3}H]-cortisol, was undertaken with new isotope and unlabelled hormone (for non-specific binding). The radioligands were dried down with nitrogen and reconstituted in 100% ethanol.

IV.2.4 Microscope Slides

This procedure was the same for all trials. Microscope slides were washed well in hot running tap water followed by three rinses in distilled water. They were then coated in a gelatin/chrome alum solution (2.5g gelatin and 0.25g chrome alum in 500 mL distilled water). The slides were then left to dry for two days and stored at 4° C until needed. The gelatin/chrome alum coating helps to hold the section to the slide (Bitensky and Poulter 1969).

IV.2.5 Preparation of Emulsion covered coverslips

The emulsion used was Amersham emulsion for high resolution microautoradiography, LM-1 (code RPN.40) for light microscopy (Amersham

International, UK). The protocol supplied with the emulsion was strictly followed. Coverslips were stored for less than 24 hours at 4° C in a light proof box before use.

IV.2.6 Development of emulsion covered coverslips

Development in Ilford Phenisol developer was performed following the LM-1 emulsion instructions. Coverslips were separated from the slides by using plastic covered paperclips placed between the coverslip and the slide on the unglued end of the coverslip.

IV.2.7 Trial 1

The method used was an adaptation of Kloas and Hanke (1992b) and Calianos and Muntz (1990). These authors had used the most simple buffers and had the most complete description of their procedures. Seminal vesicles from intact males were used and [1,2,6,7, ^{3}H(N)]-Testosterone was the isotope used.

Method

1) Sections were cut at 10 µm at -20° C and thaw mounted onto gelatin coated slides.
2) The sections were stored desiccated at -80° C until use, within a week of sectioning. Kloas and Hanke (1992b) placed under vacuum overnight, but the sections were not refrozen before incubation with radioligand. Other authors stored desiccated at -20° C until use, for up to three weeks (Calianos and Muntz 1990 Young and Kuhar 1979).
3) Slides were pre-incubated at room temperature for 15 minutes in buffer A, pH 7.4.
4) Slides were incubated with radioligand in buffer A, pH 7.4 at room temperature. These were total binding sections. For kinetic studies the time course of between 1 to 120 minute incubation time should be employed (Kloas and Hanke 1992b). Ideally at least five time periods should be set, and while when setting up a complete trial time periods of 1, 30, 60, 90 and 120 minutes would be used to determine optimum time, for this initial trial the average optimum incubation time of 30 minutes was used (Calianos and Muntz 1990, Kloas and Hanke 1992b). Other authors used single incubation times of up to three hours (Helliwell *et al.* 1994, Latouche *et al.* 1989).

   Non-specific binding was determined by incubating sections with radioligand and 100 x concentration of unlabelled hormone.

Background was determined by leaving some sections in buffer A with no radioligand or unlabelled hormone.

Thus for this trial 0.4 nmol of [1,2,6,7, ^{3}H(N)]-Testosterone (approximately 48 Ci/mmol specific activity) was used and non-specific binding was determined by incubating with 500nmol of testosterone propionate (dissolved in 100μL 100% ethanol in buffer A). Background was also determined.

5) Slides were washed with buffer B two times for five minutes each, followed by a wash in distilled water at 4° C for two minutes.
6) Slides were dried in a cold air stream (about five minutes).
7) Stored at 4° C, desiccated, until coverslipped with emulsion coated coverslips (overnight).
8) Slides were coverslipped in darkroom following the method of Young and Kuhar (1979). Coverslips were allowed equilibrate to room temperature and then attached to the sides by superglue along one edge. The slides were then placed in light proof boxes, desiccated at 4° C and exposed for 2, 4, 6, or 8 weeks.
9) Slides were developed, rinsed in deionised water and then stained in 1% Toluidine blue.

Buffer A

40 mmol	Na_2HPO_4
5 mmol	NaH_2PO_4
120 mmol	NaCl
5 mmol	Na_2EDTA

0.1% Bovine Serum Albumin

pH with NaOH, use within a few days.

Buffer B

50 mmol	Tris-HCl, pH 7.56

Toluidine blue (Disbrey and Rack 1970)

1% Aqueous toluidine blue

1) Rinse in water
2) Toluidine blue two minutes
3) Rinse well in water
4) Differentiate in 70% ethanol
5) Dehydrate, clear and mount

Nuclei - blue, greenish blue; capillary walls - lighter blue; red blood cells - dull green; other structures - pale blue to colourless.

IV.2.7.1 Results

None of the slides incubated with radioligand showed silver grains above the background level at any of the development times.

IV.2.7.2 Discussion

It was unclear whether the failure of this trial was due to insufficient radioligand or insufficient incubation time. Thus, a second trial would try to address these issues.

IV.2.8 Trial 2

The method used was the same as trial 1, however in this trial ^{125}I-Inhibin was used as the radioligand. Inhibin is a gonadally derived hormone which suppresses Follicle stimulating hormone (FSH) release (Woodruff *et al.* 1990). Two tissues were cut, brain and ovary from intact females.

IV.2.8.1 Method

The procedure was identical to trial 1, except for step 4 and 8, which are explained below.

4) Incubate with radioligand in buffer A, pH 7.4 at room temperature. To determine the point at when binding reaches equilibrium, five incubation times were used. They were 1, 30, 60, 90 and 120 minutes. Non-specific binding was not determined, however background was determined by incubating slides in the buffer with no radioligand or unlabelled hormone.

8) Coverslip slides in darkroom following the method of Young and Kuhar (1979). Coverslips are allowed to equilibrate to room temperature and then attached to the slides by superglue along one edge. The slides were then placed in light proof boxes, desiccated at 4° C and exposed for 4 or 6 days. ^{125}I-labels have a half life of 60 days (Møller and Krogh 1991), and most authors using this isotope to label the hormones under study specify an exposure time of between 2 and 7 days (Duncan 1994, Kloas and Hanke 1992a,b, Panzica *et al.* 1994).

IV.2.8.3 Results

Many of the ovarian sections floated off the slides during the incubation with the radio ligand. Others floated off in the subsequent washes. The few remaining sections exhibited no evidence of silver grain precipitation.

IV.2.8.4 Discussion

The main objective in using ^{125}I-inhibin was to determine the efficacy of continuing with the combined method of Kloas and Hanke (1992b) and Calianos and Muntz (1990). The advantage is that any problems with the method are more quickly discovered because the time between incubation with the radioligand and the development of the slides is much shorter.

There are limitations with this isotope, as iodination frequently distorts the shape of the hormone under investigation, and binding capacity of the hormone for its receptor is often reduced (Woodruff *et al.* 1990). However, the disadvantage of the possibility of reduced binding ability of this radioligand was less important than the short completion time of the experiment and possible resolution of the problems confronted in trial 1. Unfortunately the use of this ligand proved not to solve any of these problems. At this point it was decided to abandon the procedures outlined in trials 1 and 2.

IV.2.9 Trial 3.

The method used in this trial was based on that of Peters and Barrack (1987). There were several differences in the protocol for treatment of sections used by these authors. Firstly, all buffers were tissue culture based solutions rather than simple phosphate or Tris based buffers. Before incubation the thaw mounted sections were lightly fixed, a process which other authors consider to interfere with the radioligand binding (Dashwood 1992, Stewart and Bourne 1992). Unlike most other studies which were performed at room temperature (Calianos and Muntz 1990, Heliwell *et al.* 1994, Kloas and Hanke 1992b, Latouche *et al.* 1989), the incubation was performed for 20-24 hours at 4° C. Finally, the sections were rinsed in three washes of a weak Triton-X solution.

The tissues used were prostate glands from intact and castrated males. The radioligand used was [^{3}H]Dehydroepiandrosterone.

IV.2.9.1 Method

1) Sections were cut at 10 µm at -20° C and thaw mounted onto gelatin coated slides.

2) The sections were stored desiccated at -80° C until use, within a week of sectioning.
3) Sections were placed in Zamboni's fixative at 4° C for 10-15 minutes.
4) Sections were rinsed three times in Dulbecco's Phosphate buffered saline with Calcium chloride and Magnesium chloride.
5) Sections were incubated in Phosphate buffered saline with radioligand at 4° C for 20-24 hours. Four to eight replicates of each tissue were incubated. Non-specific binding was determined by incubating with label and 100 x concentration of hormone.
6) Sections were washed three times in PBS and 0.1% Triton-X detergent, for five minutes each wash at 4° C.
7) Sections were rinsed in deionised water at 4° C, then dried in a cool air stream.
8) Sections were processed for autoradiography on the same day as incubation finished, placed in light proof boxes for 5-8 weeks at 4° C.
9) Slides were developed in the darkroom, rinsed in deionised water and stained with 1% Toluidine Blue.

Dulbecco's balanced salt solution (Kiernan 1990)

Sodium chloride	8.0 g
Potassium chloride	0.2 g
Dibasic sodium phosphate (Na_2HPO_4)	1.15g
Monobasic potassium phosphate (KH_2PO_4)	0.2 g
Calcium chloride	0.1 g
Magnesium chloride ($MgCl_2.6H_2O$)	0.1 g
Phenol Red	0.01 g
distilled water	1000 mL

Mix together

Phosphate buffered saline (Kiernan 1990)

0.1M Phosphate buffer, pH 7.4	1000 mL
Sodium chloride	9.0 g

Mix together

Zamboni's fixative (Kiernan 1990)
0.5-1.0% Paraformaldehyde in 0.1M Phosphate buffer pH 7.4. See appendix III for methods for paraformaldehyde and phosphate buffer.

IV.2.9.2 Results

Dense silver grains were found above the prostate tissue on the coverslips of the total binding slides. Not all of this was removed by the treatment with 100 x concentrations of Androstenedione, dihydrotestosterone and testosterone propionate, as would be expected. There was a considerable amount of background labelling in many of the slides. This method seemed to be far superior to the methods used in the first trials.

IV.2.9.2 Discussion

One serious problem with this method was the long incubation time at 4° C. As with the previous trials at room temperature, some sections did not adhere to the slides for the whole incubation time. A compromise between these times seemed the best solution. Furthermore, because background was quite noticeable at the development at 5 weeks, it was decided to develop the slides at this time as a standard procedure. Longer development times will only increase the background labelling. As the *in vitro* autoradiography had worked for androgens in *M. musculus*, it was then decided to attempt the procedure using [^{3}H]-cortisol in *A. stuartii* in the next trial.

IV.2.10 Trial 4.

In this trial a first attempt at determining cortisol receptor sites was made. The target tissues of interest in *A. stuartii* were brain and kidney, so these tissues were used in intact old and young *M. musculus* and in *A. stuartii*. The radioligand was [1,2,6,7-^{3}H]cortisol (Amersham International, England), specific activity 76 Ci/mmol (2.31 TBq/mmol). The [1,2,6,7-^{3}H]cortisol was dried down with nitrogen and reconstituted in 100% ethanol. A concentration of 1.3 nmol was used for the incubation and 100 x concentration of unlabelled cortisol was added for the non specific binding incubation.

IV.2.10.1 Methods

The methods followed were identical to trial 3, with the exception that the incubation time was shorter. Step 5 incubation time (for total and non-specific binding sections) was 6 hours. There were polymer strip standards of 80 Ci and 8.0 Ci (Amersham International, England) attached to a slide, and with

the coated coverslips placed over them in the same manner as the incubated tissue slides.

IV.2.10.2 Results and Discussion

No binding of [1,2,6,7-^{3}H]cortisol was observed. There was also little background labelling. There was some concern that the brain tissues were less well preserved than would be expected. If tissue was damaged, either by the freezing process in the isopentane/CO_2 mixture, or by accidental freeze-thawing during the sectioning of the tissue, then this would compromise the *in vitro* autoradiography attempt. This would be addressed in the next trial.

However, there were few sections removed by the incubation procedure. It appears that the reduction in incubation time was important in minimising the loss of sections. Whether this reduction in incubation time coupled with 4° C incubation temperature lowers the binding capacity of the radioligand remains to be determined by a successful *in vitro* radiography trial.

IV.2.11 Trial 5.

In this trial a second attempt at determining cortisol receptor sites was made. Again brain and kidney tissues were used in intact, old and young *M. musculus* and *A. stuartii.* The radioligand was [1,2,6,7-^{3}H]cortisol (Amersham International, England), specific activity 76 Ci/mmol (2.81 TBq/mmol). A concentration of 1.3 nmol was used for the incubation and 100 x concentration of unlabelled cortisol was added for the non specific binding incubation.

IV.2.11.1 Methods

The methods were essentially the same as for trial 4. The differences were that at the end of each step a series of sections were stained with Toluidine blue, and sections were incubated for 2, 4 or 6 hours with [1,2,6,7-^{3}H]cortisol alone or with 100 x concentrated unlabelled hormone.

IV.2.11.2 Results

Again no binding of [1,2,6,7-^{3}H]cortisol was observed. There was also little background labelling. This was independent of the incubation time. The brain and kidney sections that were stained after each step did not appear to deteriorate over the time. However, the sections were not well preserved, this was especially true of the brain sections, where the cytoarchitecture of the brain was poorly discerned.

IV.2.11.3 Discussion

The poor tissue structure may be the reason for the failure of the *in vitro* autoradiography in this trial. The brain and, to a lesser extent, the kidney tissue, were not well preserved. This contrasts with the prostate, seminal vesicles and the brain in the previous trials, where the cytoarchitecture was clearly discernible. However, at this point in the overall study the limitations of time meant that the *in vitro* autoradiography of cortisol was abandoned.

IV.3 General discussion

The failure of the final trial of *in vitro* autoradiography was a disappointing end to the series of trials endeavouring to find a reliable method of identifying cortisol receptors in brain, kidney and reproductive tract. While cortisol receptors have been detected by homogenate or tissue slice incubation (Casolini *et al.* 1993, Funder *et al.* 1973b, Lee *et al.* 1983, Meaney *et al.* 1992) and *in vivo* autoradiography (Strum *et al.* 1975), the transposition of these methods to an *in vitro* autoradiographic method of identifying cortisol receptors was not forthcoming. While corticosterone is the main glucocorticoid for *M. musculus*, it was thought that because the trial for sex steroids had worked for mice that inclusion in the cortisol trial should at least indicate non-specific binding and perhaps a small amount of specific binding. Unfortunately no binding was observed for *A. stuartii* either, even though cortisol is the main glucocorticoid for this species.

The success with the method using sex steroids implies that the general procedures followed during the trials were adequate. However the failure of the method using [1,2,6,7-^{3}H]cortisol suggests that the receptors are less robust than those for androgens. This indicates that the analysis of tissue for cortisol receptors should be done on fresh tissue.

Many studies determining corticoid receptors used fresh tissue homogenates (Beaumont and Fanestil 1983, Funder *et al.* 1973a,b, Lee *et al.* 1983, Náray-Fejes-Toth *et al.* 1994b) and *in vivo* studies have the advantage in that the radioligand can attach to the receptor before death. If *in vitro* labelling requires fresh tissue sections for adequate receptor detection, then the method is limited to studies where planning for suitable sample sizes can limit the number of experimental sessions and hence reduce between group error. For a study such as the present one on *A stuartii,* where the animals are collected at discrete times of the year and where sample sizes are not easily controlled, this would appear to have some limitations.

The importance of fresh tissue may explain the problems seen with the poorer quality tissues observed in the later trials. It is unclear whether the poorer quality of the sections was due to a deterioration of the tissue at -80° C or due to a failure of these tissues to be frozen adequately in the initial isopentane/CO_2 mixture. If the isopentane does not replace the water quickly and thoroughly the tissue can fracture and lose its structure (Kiernan 1990). This may explain some of the problems encountered with the brain tissue, but fails to explain the poorer profile of the kidney tissue. While cortex and medullary regions were well defined, the finer cytoarchitectural structures were less well identified.

However the reproductive structures used in the other trials remained well preserved so it is unclear whether the preservation method, the inherent structural fragility, or the thaw mount method of attaching sections to slides, was the main problem with the poorer definition in the later trials. The receptors for the androgens on the prostate glands may be robust enough to withstand the procedural buffeting that occurred.

Although the *in vitro* autoradiography trials for the detection of cortisol receptors failed, the trial determining androgen receptors in *M. musculus* prostate was successful. Unfortunately the limitations of time for the PhD study meant that the pursuance of the androgen receptors in *A. stuartii* was not practicable. The concentration of the study on the renal physiology of *A. stuartii* and the effects of testosterone and cortisol on renal structure and function made this a less important aspect of the total study.

If the study was to continue, it is unclear which direction would be the best to follow. It is not practical to obtain fresh tissues from *A. stuartii* and obtain batch homogeneity and so standardisation of trials would be impossible. However, one approach would be to run a trial using fresh tissue. This would also be true for *M. musculus*. Trials using the appropriate glucocorticoid for *M. musculus*, cortisone, and fresh tissues may eliminate many of the problems observed in the present study. Certainly the problems of sampling and sample times would be eliminated. If the above methods were able to work on *M. musculus* tissues using cortisone, one could then move to modify the approach for marsupials, perhaps eventually enabling *in vitro* autoradiography to be used for *A. stuartii* as well.

Appendix V

Carnivore and Non-carnivore Data Used in Regression Analyses, and Analyses of Variance.

The data used for all calculations are presented in this appendix. The orders are listed first (Bold, capitals), followed by the family (bold) and then the information is listed for each species (italics) in the family. Body mass (kg) is always present, followed from left to right with kidney mass (g), kidney size (mm), RMT, PMT, and MT (mm).

The environmental categories are listed to the right of the data, with mesic environments including wet and forest environments, and xeric environments including arid and grassland savannah environments. Data were also sorted by taxonomic order and habitat type. Monotreme and Marsupial orders were based on Strahan's (1983) classification, and Placental orders were based on Nowak and Paradiso (1981). Taxonomic revisions of scientific names were based on Chiarelli (1972) Corbet (1978), Corbet and Hill (1992) and Strahan (1983). Habitat types were as follows:-

Wet - only found in or beside streams, lakes or swamps.

Forest - living in wetter wooded areas, dense tree cover.

Grassland savanna - found in open grazing areas, drier lightly treed areas.

Arid - living in cold or warm desert areas.

Habitat descriptions were based on the original published sources or on Corbett (1978), Nowak and Paradiso (1981) or Strahan (1983). Many sources did not define habitat by rainfall or temperature but the above classifications would best allow analysis of broad habitat niches.

Data were log transformed and least square regression analyses were performed (Pagel and Harvey 1988). Multiple regression comparisons were performed for slope or Y-intercept, followed by the modified Tukey test (Zar 1984). Body mass equations presented in the results are in the following format, $Y = aM^b$ where Y is the variable under consideration, a is the Y-intercept, M is mass in kilograms and b is the slope of the equation. The right column lists the source of the data. When separate body masses are available for different data points, the different sources are indicated for each body mass and relevant data.

ORDER **Family** *Species*	**Body mass (kg)**	**Kidney mass (g)**	**Kidney size (mm)**	**RMT**	**PMT**	**Environ-ment**	**Author(s)**
MONOTREMATA **Ornithorhynchidae**							
Ornithorhynchus anatinus	1.25		18.64	2.41	52.94	Wet	Sperber (1944)
POLYPROTODONTA **Dasyuridae**							
Antechinomys laniger	0.020		5.5	8.0	81.5	Arid	Sperber (1944))
Antechinus melanurus	0.048		5.5	6.0	84.4	Wet	Brooker and Withers (1994)
Antechinus stuartii stuartii	0.038	0.40	6.56	6.31	74.5	Forest	This study (May males)
Antechinus stuartii agilis	0.035	0.26	6.79			Forest	Unpublished data
Antechinus swainsonii	0.041	0.59	7.92	3.7	75.2	Wet	Unpublished data, Brooker and Withers (1994)
Dasycercus cristicauda	0.078			8.4	86.4	Arid	Brooker and Withers (1994)
Dasykaluta rosamundae	0.020			7.2	84.9	Arid	Brooker and Withers (1994)
Dasyuroides byrnei	0.120	1.21	10.44			Arid	Unpublished data
Dasyurus viverrinus	1.0		15.0			Forest	Sperber (1944)
Ningaui ridei	0.006	0.13	4.76	11.1	89.0	Arid	Brooker and Withers (1994) Unpublished data
Ningaui timealeyi	0.0045			10.8	87.7	Arid	Brooker and Withers (1994)
Phascogale calura	0.042			5.8	84.3	Grassland Savannah	Brooker and Withers (1994)
Planigale maculata	0.010			7.9	85.6	Grassland Savannah	Brooker and Withers (1994)
Pseudoantechinus macdonellensis	0.030			11.5	89.5	Arid	Brooker and Withers (1994)
Pseudoantechinus woolleyii	0.041			7.7	82.6	Grassland Savannah	Brooker and Withers (1994)
Sarcophilus harrisii	7.0		31	5.0	75.8	Forest	Sperber (1944)

Sminthopsis crassicaudata	0.015	0.27	6.39	8.9	87.7	Arid	Brooker and Withers (1994) Unpublished data
Sminthopsis dolichura	0.0165			5.6	81.6	Grassland Savannah	Brooker and Withers (1994)
Sminthopsis granulipes	0.018			6.6	83.1	Grassland Savannah	Brooker and Withers (1994)
Sminthopsis hirtipes	0.011			8.0	86.0	Arid	Brooker and Withers (1994)
Sminthopsis macroura	0.023	0.36	6.44	10.1	88.9	Grassland Savannah	Brooker and Withers (1994), Unpublished data
Sminthopsis youngsonii	0.010			9.0	87.8	Arid	Brooker and Withers (1994)
Peramelidae							
Echymipera kalabu	0.695			4.7		Forest	Hulbert and Dawson (1974a)
Echymipera rufescens rufescens	1.276			4.5		Forest	Hulbert and Dawson (1974a)
Echymipera rufescens australis	0.616			5.5		Forest	Hulbert and Dawson (1974a,b)
Thylacomyidae							
Macrotis lagotis	0.943			5.9		Arid	Hulbert and Dawson (1974a,b)
Didelphidae							
Caluromys philander	0.222	4.0				Forest	Crile and Quiring (1940)
Marmosa robinsoni	0.080	1.064			80.0	Forest	Barnes (1977)
DIPROTODONTA Petauridae							
Acrobates pygmaeus	0.012		5.1	6.7	70.83	Forest	Sperber (1944)
Petaurus breviceps	0.120		8.2			Forest	Sperber (1944)
Phascolarctidae							
Phascolarctos cinereus	6.91*		29.6	3.0*	63.64	Forest	*Degabrielle *et al.* (1980) Sperber (1944)
Vombatidae							
Lasiorhinus latifrons	24.63	64.12	36.59	5.71	74.03	Arid	McAllan *et al.* (1995)

Macropodidae							
Bettongia lesueur	1.74		17.4	8.4	94.67	Grassland Savannah	Yadav (1979)
Bettongia pencillata	1.3		14.5	5.9	71.90	Forest	Yadav (1979)
Dendrolagus matscheii	6.5		22.5	3.9	60.0	Forest	Yadav (1979)
Lagorchestes conspicillatus	1.7		20.1	8.7	80.73	Arid	Yadav (1979)
Lagorchestes hirsutus	1.74		17.4	6.6	69.70	Arid	Yadav (1979)
Lagostrophus fasciatus	1.5		12.0	7.5	75.0	Arid	Yadav (1979)
Macropus eugenii	2.93	17.78	26.0	6.82	77.43	Grassland Savannah	Yadav (1979) Purohit (1974)
Macropus robustus erebuscens	17.63	57.69	35.2	6.48	76.08	Grassland Savannah	Yadav (1979)
Macropus robustus robustus	20.88	84.3		5.67	77.68	Grassland Savannah	Freudenberger (1991)
Macropus rufus	14.4 *22.8		36.6	5.0 *5.8	70.82	Arid	Yadav (1979) * Denny & Dawson (1977)
Peradorcas concinna	1.4		15.0	7.3	73.33	Arid	Yadav (1979)
Potorous tridactylus	0.86	2.90				Forest	J. R. Roberts (Unpub.)
Setonix brachyurus	3.5		21.3	4.9	68.18	Grassland Savannah	Yadav (1979)
Thylogale thetis	5.3			5.7		Forest	Hume & Dunning (1979)
INSECTIVORA **Erinacoidae**							
Hemiechinus auritus	0.301	3.196	12.82	6.96	81.83	Arid	Yaakobi & Shkolnik (1974)
Parachinus aethiopicus	0.352	3.864	14.74	7.55	80.12	Arid	Yaakobi & Shkolnik (1974)
Soricidae							
Neomys fodiens	0.0088	0.19	5.0	5.0	78.13	Wet	Sperber (1944)
Notiosorex crawfordi	0.004			8.6		Arid	Lindstedt (1980)
Talpidae							
Desmana moschata	0.060		10.97	3.92	66.15	Wet	Sperber (1944)

Macroscelididae							
Macroscelides proboscideus	0.040		7.0	9.40	82.5	Arid	Sperber (1944)
DERMOPTERA **Cynocephalididae**							
Cynocephalus volans	1.40		21.0			Forest	Sperber (1944)
CHIROPTERA **Pteropodidae**							
Pteropus giganteus	1.45		14.0	3.79	66.25	Forest	Sperber (1944)
Pteropus vampyrus	1.60		13.0	3.46	60.0	Forest	Sperber (1944)
Rhinopomatidae							
Rhinopoma hardwickeri	0.0115				90.27	Arid	Vogel & Vogel (1972)
Rhinopoma kinneari	0.031		5.7	15.26	89.69	Arid	Purohit *et al.* (1973)
Emballonuridae							
Taphozous perforatus	0.028		5.5	9.64	84.13	Arid	Purohit *et al.* (1973)
Rhinolophidae							
Rhinolophus ferrumequinum	0.016				82.76	Forest	Vogel & Vogel (1972)
Phyllostomidae							
Leptonycteris sanborni	0.024		5.7	4.3	61.45	Arid	Carpenter (1969)
Macrotis californicus	0.016		4.9	8.53	82.9	Arid	Carpenter (1969)
Vespertilionidae							
Antrozous pallidus	0.016				88.2	Arid	Geluso (1978)
Eptisicus fuscus	0.013		4.59	9.33	83.01	Forest	Carpenter (1969)
Eptisicus nilssoni	0.010	0.12	5.0	8.2	82.83	Forest	Sperber (1944)
Lasionycteris noctivagus	0.010				86.2	Forest	Geluso (1978)
Lasiurus cinereus	0.025				87.1	Forest	Geluso (1978)
Myotis auriculus	0.007				83.1	Arid	Geluso (1978)
Myotis lucifugus	0.0074				86.8	Forest	Geluso (1975)
Myotis subulatus	0.0043				86.0	Forest	Geluso (1978)
Myotis velifer	0.0095				80.9	Forest	Geluso (1978)
Myotis volans	0.0069				85.4	Forest	Geluso (1978)

Myotis vivesi	0.0025		7.29	6.12	83.26	Forest	Carpenter (1969)
Myotis yumanensis	0.0049				82.8	Forest	Geluso (1978)
Pipistrellus hesperus	0.0039				90.7	Forest	Geluso (1978)
Vespertilio murinus	0.014	0.11	4.8	7.7	82.35	Forest	Sperber (1944)
PRIMATES **Tupaiidae**							
Tupaia glis	0.165				77.01	Forest	Munkasci & Palkovits (1977)
Tupaia javanica	0.120		8.3	6.0	76.92	Forest	Sperber (1944)
Lorisidae							
Galago crassicaudata	1.2				71.05	Forest	Munkasci & Palkovits (1977)
Nycticebus coucang	1.38			5.8	73.92	Forest	Muller (1979)
Perodicticus potto	1.225				65.26	Forest	Munkasci & Palkovits (1977)
Lemuridae							
Lemur catta	2.1		21.0	3.6	60.0	Forest	Sperber (1944)
Daubentoniidae							
Daubentonia madagascariensis	2.0		12.93	6.19	76.19	Forest	Sperber (1944)
Tarsiidae							
Tarsius spectrum	0.122		9.0	6.7	75.0	Forest	Sperber (1944)
Callitrichidae							
Callithrix jacchus	0.250		10.0	5.0	71.43	Forest	Sperber (1944)
Saguinus geoffroyi	0.504	3.362				Forest	Hrdlicka (1925)
Saguinus oedipus	0.510				62.5	Forest	Munkasci & Palkovits (1977)
Cebidae							
Alouatta caraja	6.588	32.94				Forest	Hill (1962)
Alouatta palliata	7.257	38.72				Forest	Hrdlicka (1925)
Ateles geoffroyi	7.63	31.2				Forest	Crile & Quiring (1940)
Brachyteles arachnoides	9.5		22.45			Forest	Hill (1962)
Cacajao melanocephalus	3.4		22.51			Forest	Hill (1960)
Cebus apella	2.2				65.81	Forest	Munkasci & Palkovits (1977)

Cebus capucinus	2.2 *3.101	*14.3			60.0	Forest	Munkasci & Palkovits (1977) *Crile & Quiring (1940)
Lagothrix lagotricha	5.0		38.85			Forest	Hill (1962)
Cercopithecidae							
Cercocebus atys	7.5	36.76	38.94			Forest	Hill (1974)
Cercopithicus galeritus	6.0	20.34				Forest	Hill (1974)
Cercopithicus cephus	4.5	19.05				Forest	Hill (1966)
Cercopithicus nigroviridis	5.0		28.3			Forest	Hill (1966)
Cercopithicus talapoin	1.225		14.57			Forest	Hill (1966)
Macaca sinica	2.181	6.2	17.37			Forest	Hill (1974)
Macaca sylvanus	11.145		34.0	3.24	57.89	Forest	Sperber (1944)
Papio leucophaeus	19.0		26.21			Forest	Hill (1970)
Papio papio	13.6		27.97			Forest	Hill (1970)
Theropithecus gelada	20.0		37.24			Grassland Savannah	Hill (1970)
Pongidae							
Pan troglodytes	56.69	210				Forest	Crile & Quiring (1940)
EDENTATA **Bradypodidae**							
Bradypus griseus	3.121	16.4				Forest	Crile & Quiring (1940)
Bradypus sp.	4.0		23.0	4.35	71.43	Forest	Sperber (1944)
Myrmecophagidae							
Cyclopes didactylus	0.250		8.3	7.2	75.0	Forest	Sperber (1944)
Dasypodidae							
Chaetophractus vellerosus	0.850			7.5		Grassland Savannah	Greegor (1975)
PHOLIDOTA **Manidae**							
Manis javanica	10.0		23.0	5.65	74.29	Forest	Sperber (1944)

LAGOMORPHA **Leporidae**							
Lepus arcticus	2.271	22.365				Arid	Crile & Quiring (1940)
Ochotona alpina	0.250		8.2			Arid	Sperber (1944)
Oryctolagus cuniculus	1.8	29.92	28.0	5.36	75.0	Grassland Savannah	Sperber (1944)
Sylvilagus aquaticus	1.8			6.18	75.18	Wet	Heisinger & Breitenbach (1969)
Sylvilagus audubonii	0.9			7.17	84.47	Arid	Heisinger & Breitenbach (1969)
Sylvilagus floridanus alacer	0.9			6.35	78.59	Grassland Savannah	Heisinger & Breitenbach (1969)
Sylvilagus floridanus similis	0.9			6.83		Grassland Savannah	Heisinger & Breitenbach (1969)
Sylvilagus palustris	0.9			6.05	76.12	Wet	Heisinger & Breitenbach (1969)
Sylvilagus transitionalis	0.9				76.35	Forest	Heisinger & Breitenbach (1969)
RODENTIA **Aplodontidae**							
Aplodontidia rufa	0.364		17.0	2.94	54.35	Wet	Sperber (1944)
Sciuridae							
Cynomys inauris	0.809			5.85		Arid	Bakko (1977)
Cynomys ludovicianus	1.5			6.32		Arid	Bakko (1977)
Eutamias alpinus	0.039			11.15		Arid	Heller & Poulson (1972)
Eutamias amoenus	0.0424			10.39		Arid	Heller & Poulson (1972)
Eutamias minimus	0.035			12.08		Arid	Heller & Poulson (1972)
Eutamias quadrivittatus	0.068			7.66		Grassland Savannah	Blake (1977)
Eutamias speciosus	0.071			9.32		Forest	Heller & Poulson (1972)
Funambulus pennantii	0.200		6.8	6.91	73.44	Grassland Savannah	Purohit *et al.* (1973)
Glaucomys volans	0.080				71.43	Forest	Munkasci & Palkovits (1977)

Sciurus carolinensis	0.196 *0.673	1.349		*5.15		Forest	Crile & Quiring (1940) *Bakko (1975)
Sciurus vulgaris	0.550		12.0	5.33	78.05	Forest	Sperber (1944)
Spermophilus beechyi	0.318	2.6		7.07		Grassland Savannah	Baudinette (1974)
Spermophilus lateralis	0.212			5.44	73.33	Grassland Savannah	Blake (1977) Munkacsi & Palkovits (1966)
Spermophilus parryi	0.918	7.14				Arid	Crile & Quiring (1940)
Tamias striatus	0.109			6.21		Forest	Blake (1977)
Tamiasciurus hudsonicus	0.207			5.79		Forest	Bakko (1975)
Heteromyidae							
Dipodomys deserti	0.105			7.2	85.0	Arid	Lawler & Geluso (1986)
Dipodomys merriami	0.037	0.227	5.87	8.52	83.33	Arid	Sperber (1944) Carpenter (1966) MacMillen (1972)
Dipodomys ordii	0.044			8.20	83.4	Arid	Lawler & Geluso (1986)
Dipodomys spectabilis	0.100				81.05	Arid	Munkasci & Palkovits (1977)
Liomys adspersus	0.045		6.5			Arid	Sperber (1944)
Microdipodops pallidus	0.0125			9.52	86.9	Arid	Lawler & Geluso (1986)
Perognathus ampulus	0.0058	0.1338			88.31	Arid	Altschuler *et al.* (1979)
Perognathus baileyi	0.0257	0.2352			89.30	Arid	Altschuler *et al.* (1979)
Perognathus formosus	0.018			9.68	86.8	Arid	Lawler & Geluso (1986)
Perognathus longimembris	0.0082			11.55	88.2	Arid	Lawler &Geluso (1986)
Perognathus pencillatus	0.016	0.1886			92.65	Arid	Altschuler *et al.* (1979)
Castoridae							
Castor canadensis	19.0 *5.83	*72.60	36	1.3	61.03	Wet	Schmidt-Nielsen & O'Dell (1961) Munkasci & Palkovits (1977) *Crile & Quiring (1940)
Castor fiber	18.0		36	3.89	66.67	Wet	Sperber (1944)
Pedetidae							
Pedetes capensis	3.0		22	5.91	72.22	Arid	Sperber (1944)

Muridae							
Arvicola terrestris	0.200		9.5			Wet	Sperber (1944)
Deomys ferrugineus	0.060		7.5			Forest	Sperber (1944)
Dicrostonyx tornuatus	0.0537	0.8075				Arid	Crile & Quiring (1940)
Gerbillus gerbillus	0.044	0.396	6.28	10.51	82.5	Arid	Khalil & Tawfic (1963)
Gerbillus pyramidum	0.030		6.2			Arid	Sperber (1944)
Gerbillurus paeba	0.028			5.97	85.27	Arid	Buffenstein (1985) Downs & Perrin (1991)
Gerbillurus setzeri	0.035			8.35	89.09	Arid	Downs & Perrin (1991)
Gerbillurus tytonis	0.035			6.26	83.41	Arid	Downs &Perrin (1991)
Gerbillurus vallinus	0.045			6.14	87.53	Arid	Downs & Perrin (1991)
Hydromys chrysogaster	0.680		15.0	3.87	68.24	Wet	Sperber (1944)
Lemmus sibiricus	0.032	0.4404				Arid	Crile & Quiring (1940)
Meriones hurrianae	0.072			12.5		Arid	Goyal *et al.* (1988)
Meriones unguicaudatus	0.068			7.5	81.3	Arid	Edwards *et al.* (1983)
Mesocricetus auratus	0.105			8.01	80.56	Arid	Munkasci & Palkovits (1977) Trojan (1977)
Microtus orchogaster	0.047				81.54	Grassland Savannah	Heisinger *et al.* (1973)
Microtus pennsylvanicus	0.038				77.21	Wet	Heisinger *et al.* (1973)
Neofiber alleni	0.272		8.4	3.57	60.00	Wet	Sperber (1944)
Notomys alexis	0.030		6.5	8.0	85.25	Arid	Purohit (1974)
Onchyomys torridus	0.022	0.3316			82.90	Arid	Altschuler *et al.* (1979)
Ondatra zibethicus	0.900	7.45				Wet	Crile & Quiring (1940)
Peromyscus eremicus	0.0228	0.4454			84.73	Arid	Altschuler *et al.* (1979)
Phodopus sungorus	0.030			8.29		Arid	Trojan (1977)
Psammomys obesus	0.212		13	10.7	86.88	Arid	Sperber (1944)
Pseudomys delicatula	0.0105		4.3	7.67	84.61	Grassland Savannah	Purohit (1974)

Pseudomys hermansbergensis	0.012		5.1	7.45	77.55	Arid	Purohit (1974)
Reithrodontomys megalotis	0.012				88.46	Grassland Savannah	Reaka & Armitage (1976)
Reithrodontomys montanus	0.012				89.93	Grassland Savannah	Heisinger *et al.* (1973)
Tatera indica	0.093			9.35		Arid	Goyal *et al.* (1988)
Dipodidae							
Jaculus jaculus	0.042	0.410	7.54	9.66	84.88	Arid	Khalil & Tawfic (1963)
Hystricidae							
Artherurus africanus	2.75		28.0			Forest	Sperber (1944)
Erethrozontidae							
Erethizon dorsatum	3.105	32.485	33.0			Forest	Crile & Quiring (1940) Sperber (1944)
Cavidae							
Dolichotis patagonium	12.0		35.0	5.71	80.00	Arid	Sperber (1944)
Hydrochaeridae							
Hydrochaeris hydrochaeris	27.67 *40.0	69.75	*53.0	*2.08	*55.0	Wet	Crile & Quiring (1940) *Sperber (1944)
Dasyproctidae							
Agouti paca	3.627	22.7				Forest	Crile & Quiring (1940)
Dasyprocta punctata	3.172	15.39				Forest	Crile & Quiring (1940)
Chinchillidae							
Chinchilla laniger	0.55		13.0	6.7	77.68	Arid	Sperber (1944)
Capromyidae							
Myogaster coypus	6.0		24.0	3.54	60.71	Wet	Sperber (1944)
Ctenodactylidae							
Ctenodactylus gundi	0.180		6.1			Arid	Sperber (1944)
Ctenodactylus vali	0.180	0.664			87.5	Arid	de Rouffignac *et al.* (1981)

CARNIVORA **Canidae**							
Canis lupus pallipes	20.0		62.1	5.36		Arid	Afik & Pinshow (1993)
Canis mesomelas	2.85	23.03				Grassland Savannah	Crile & Quiring (1940)
Fennecus zerda	1.277		18.4	5.35		Arid	Noll-Banholzer (1979)
Otocyon megalotis	3.335	31.91				Grassland Savannah	Crile & Quiring (1940)
Urocyon cinoreoargentus	3.749	17.43				Grassland Savannah	Crile & Quiring (1940)
Ursidae							
Ailuropoda melanoleuca	115.0		57.0			Forest	Sperber (1944)
Ursus maritimus	258.29	1011.0				Arid	Crile & Quiring (1940)
Procyonidae							
Ailurus fulgens	3.75		29.0			Forest	Sperber (1944)
Nasuas nasua	5.0		26.0	3.85	64.52	Forest	Sperber (1944)
Potos flavus	2.0		17.0			Forest	Sperber (1944)
Procyon lotor	3.381 *5.25	36.425	*28.0	*3.75	*65.63	Forest	Crile & Quiring (1940) *Sperber (1944)
Mustelidae							
Lutra lutra	7.4	52.0	38.3	3.0	76.67	Wet	Sperber (1944)
Martes martes	0.94		18.0	3.61	72.22	Forest	Sperber (1944)
Meles meles	5.0		33.0	4.55	76.92	Forest	Sperber (1944)
Musteles vison	1.654	13.1	22.93	4.57	75.0	Wet	Sperber (1944)
Viverridae							
Arctictus binturong	11.5		21.0	3.33	58.33	Forest	Sperber (1944)
Ichneumia albicauda	4.40	34.96				Grassland Savannah	Crile & Quiring (1940)
Prionodon linsang	0.698		14.0	6.43	76.92	Forest	Sperber (1944)
Hyaenidae							

Crocuta crocuta	62.37	400				Grassland Savannah	Crile & Quiring (1940)
Hyaena sp.	55.5		54.0	4.81	78.79	Grassland Savannah	Sperber (1944)
Felidae							
Acinonyx jubatus	40.82	290				Grassland Savannah	Crile & Quiring (1940)
Felis lynx	18.0		45.0	4.56	75.93	Forest	Sperber (1944)
Felis serval	7.887	70.17				Grassland Savannah	Crile & Quiring (1940)
Panthera leo	190.8 *151.0	1810.0	*89.0	*4.72	*85.71	Grassland Savannah	Crile & Quiring (1940) *Sperber (1944)
Panthera tigris	134.0		78.0	4.23	80.49	Forest	Sperber (1944)
HYRACOIDEA **Procaviidae**							
Procavia capensis	3.8		16.64	5.42	81.82	Grassland Savannah	Sperber (1944)
PERISSODACTYLA **Equidae**							
Equus caballus	412.28 *260.0	1310	*115.70	*3.20	*72.55	Grassland Savannah	Crile & Quiring (1940) *Sperber (1944)
Equus hemionus	188.0		75.0	5.07	64.41	Arid	Purohit (1969) in Purohit (1974)
Tapiridae							
Tapirus bairdii	14.26	167.0				Forest	Crile & Quiring (1940)
Tapirus indicus	258.5		84.0			Forest	Sperber (1944)
Rhinocerotidae							
Diceros bicornis	763.0	3000.0				Grassland Savannah	Crile & Quiring (1940)

ARTIODACTYLA **Suiidae**							
Phacochoerus aethiopicus	65.32	300.0				Grassland Savannah	Crile & Quiring (1940)
Sus scrofa	150.0 *182.06	*276.5	85.57	1.64	41.18	Forest	Sperber (1944) *Crile & Quiring (1940)
Hippopotamidae							
Hippopotamus amphibius	1351.0 *3500.0	3160.0	*155.87	*1.03	*57.14	Wet	Crile & Quiring (1940) *Sperber (1944)
Camelidae							
Camelus bactrianus	450.0		109.86	3.64	72.73	Arid	Sperber (1944)
Camelus dromedarius	570.0			7.89	83.33	Arid	Abdalla & Abdalla (1979)
Tragulidae							
Tragulus javanica	1.5		21.0	2.62	55.0	Forest	Sperber (1944)
Cervidae							
Alces alces	500.0	1080	93.36	2.68	52.63	Wet	Sperber (1944)
Axis axis	88.45	247.0				Grassland Savannah	Crile & Quiring (1940)
Giraffidae							
Giraffa camelopardis	1220.0 *800.0	2268.0	*110.0			Grassland Savannah	Crile & Quiring (1940) *Sperber (1944)
Bovidae							
Aepyceros melampus	42.5	153.5	47.0	4.33	71.56	Grassland Savannah	Stafford & Stafford (1992)
Antidorcas marsupialis	34.0			5.3		Arid	Hofmeyr & Louw (1987)
Antilope cervicarpa	37.0		38.0	4.74	73.47	Grassland Savannah	Sperber (1944)
Bos taurus taurus	506.0 *225.0	1207.0	*98.13	*1.93	*67.86	Grassland Savannah	Crile & Quiring (1940) *Sperber (1944)
Bubalus depressicornis	225.0		54.0	2.41	65.0	Forest	Sperber (1944)

Capra hircus mambricus	47.0			5.83		Arid	Dunson (1974)
Connochaetes gnou	200.0		50.0	4.2	75.0	Grassland Savannah	Sperber (1944)
Connochaetes taurinus	200.0	315.0	58.67	4.23	71.37	Grassland Savannah	Stafford & Stafford (1992)
Damaliscus dorcas	100.0			4.9		Grassland Savannah	in Hofmeyr & Louw (1987)
Gazella thomsonii	24.37	105.0				Grassland Savannah	Crile & Quiring (1940)
Kobus kob	175.0				38.0	Wet	Schoen (1969)
Kobus leche	175.0	160.72	12.5	3.13	56.69	Wet	Stafford & Stafford (1992)
Madoqua kirkii	4.57 *5.3	21.4			*47.0	Grassland Savannah	Crile & Quiring (1940) Schoen (1969)
Ovibos moschatus	310.0		79.0	3.04	57.14	Wet	Sperber (1944)
Ovis aries	40.0	114.0	50.0	3.4	70.83	Grassland Savannah	Sperber (1944))
Redunca redunca	31.7	102.0				Grassland Savannah	Crile & Quiring (1940)
Raphicerus campestris	8.62	38.51				Grassland Savannah	Crile & Quiring (1940)
Syncerus caffer	447.0	1725.0				Wet	Crile & Quiring (1940)
Tragelaphus scriptus	44.23 *50.0	178.0			*31.0	Wet	Crile & Quiring (1940) *Schoen (1969)

Appendix VI

Publications arising from the thesis

Agar, N. and B. M. McAllan. 1995. Red cell metabolism in a small dasyurid marsupial, the brown antechinus (*Antechinus stuartii*). Comparative Haematology International 5: 201-205

McAllan, B. M., J. R. Roberts, and T. O'Shea. 1996. Seasonal changes in the renal morphometry of *Antechinus stuartii* (Marsupialia: Dasyuridae). Australian Journal of Zoology 44: 337-354

McAllan, B. M., J. R. Roberts, and T. O'Shea. Seasonal changes in the reproductive morphology of *Antechinus stuartii* (Marsupialia: Dasyuridae). Journal of Morphology: in press

McAllan, B. M., J. R. Roberts, and T. O'Shea. Seasonal changes in the renal function of *Antechinus stuartii* (Marsupialia: Dasyuridae). Journal of Comparative Physiology B (submitted)

Published Conference Abstracts

McAllan, B. M., J. R. Roberts, and T. O'Shea. 1993. Glomerular filtration rate in *Antechinus stuartii*. The Sixth International Theriological Congress, University of New South Wales.

McAllan, B. M., J. R. Roberts, and T. O'Shea. 1993. Glomerular filtration rate in *Antechinus stuartii* (Marsupialia). Tenth Meeting of Australian Comparative Physiologists, University of Tasmania

McAllan, B. M., J. R. Roberts, and T. O'Shea. 1994. Effects of cortisol and testosterone administration on renal structure and function in *Antechinus stuartii.* Annual meeting of Australian and New Zealand Comparative Physiologists, University of Queensland

McAllan, B. M., J. R. Roberts, and T. O'Shea. 1995. Renal function in *Antechinus stuartii*. Proceedings of the 1995 Scientific meeting and Annual General Meeting of the Australian Mammal Society, James Cook University.

McAllan, B. M., J. R. Roberts, and T. O'Shea. 1995. Effects of testosterone and cortisol on the accessory reproductive tracts of *Antechinus stuartii*. Proceedings of the 1995 Scientific meeting and Annual General Meeting of the Australian Mammal Society, James Cook University.

McAllan, B. M., J. R. Roberts, and T. O'Shea. 1996. Testosterone and cortisol administration alter the renal morphology of the marsupial *Antechinus stuartii*. Environmental and Conservation Endocrinology, Proceedings of the third congress of the Asia and Oceania Society for Comparative Endocrinology pp 253-254

Appendix VII

Single injection method of GFR estimation

Determination of GFR in small mammals is difficult. There are few studies that have focussed on renal function in small (< 100 g) mammals. Most studies are content to describe urine osmolalities and electrolyte and urea concentrations. The present study sought to explore more than just these parameters. The least complicated and least invasive method for estimating GFR in small mammals is the single injection method. Many published accounts of GFR measurement include both components of the exponential decay curve (Hall *et al.* 1977, Schuster and Seldin 1992). However, the measurement of the fast component of the curve involves venipuncture at 1, 5, 10, 15 and 20 minutes before bleeding every 20 minutes. As in *A. stuartii* there is a limit to the number of blood collections that can be made, estimating the fast component of the curve jeopardises the completion of the GFR measurement by the lack of venipuncture sites to estimate the slow component of the decay curve (at 40, 60, 80, 100 and 120 minutes).

Because of the limitations of venipuncture in a small mammal, the single injection method, using the slow component of the exponential decay curve, was used. However, the exact limitations of this method are unclear. Schuster and Seldin (1992) have summarised the recent available information on methods for renal clearance and indicated that, using the second compartment method only, there has been found to be good agreement with inulin clearance methods in many studies, but closer values may be found with the two compartment method. It is apparently more problematic to measure the GFR using either the one or two compartment method when there is extracellular volume expansion (Schuster and Seldin 1992).

In the present study GFRs were performed on *Mus musculus* as well as *A. stuartii* using the single injection method. Calculated GFR values (mL·hr) were compared to the allometric equation of Yokota *et al.* (1985). GFR values (mL·hr) for *M. musculus* and for *A. stuartii* for February, May and female values for July and post-mating August of the

seasonal study were found to lie within 95% confidence limits of the equation:

GFR (mL·hr) = $1.24M^{0.765}$, where M is body mass in grams (Yokota *et al*. 1985).

GFR (mL·hr) values for female *A. stuartii* in pre-mating August and for male *A. stuartii* in July and both August sampling times were outside the 95% confidence limits.

Moreover, an unpublished study by Bayley (1994) found that when estimating GFR for *M. musculus* measurements using the one compartment single injection method did not differ significantly from measurements using Alzet minipumps. Thus the one compartment single injection method for estimating GFR in small mammals does appear to be robust under normal circumstances.

An example of the method is illustrated below. Values are plotted using a semilog scale and then the GFR is calculated using the slope and the Y-intercept of the regression equation. The range of r^2 for the regression equations was 0.703 to 0.999. The statistical analysis and the GFR calculation are also illustrated.

Figure VII.1

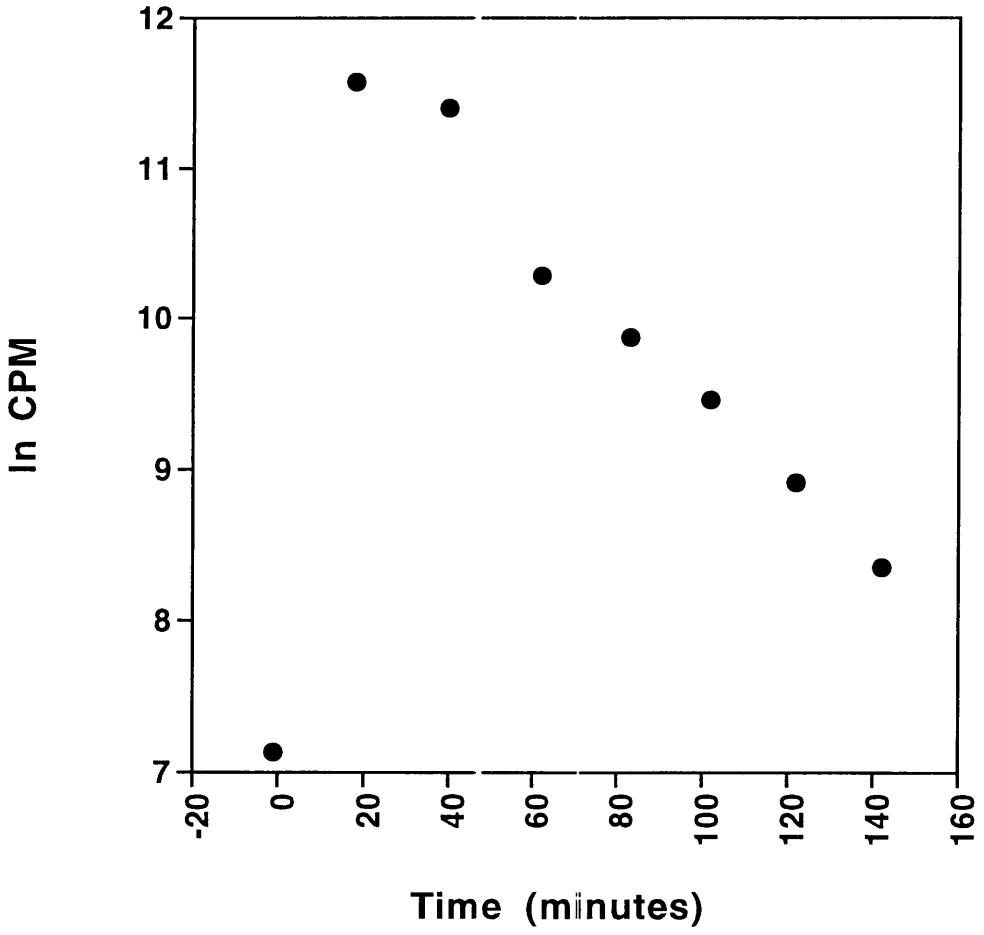

Figure VII.1 Plot of n CPM for a male Antechinus stuartii from post-breeding August. The plot includes a value from a sample taken prior to injection of ^{51}Cr-EDTA.

Regression Summary
UNEAs25 male #2 vs. As25m #2 time (mins)

Count	7
Num. Missing	0
R	.99089
R Squared	.98186
Adjusted R Squared	.97823
RMS Residual	.17766

ANOVA Table
UNEAs25 male #2 vs. As25m #2 time (mins)

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Regression	1	8.54221	8.54221	270.63155	<.0001
Residual	5	.15782	.03156		
Total	6	8.70003			

Regression Coefficients
UNEAs25 male #2 vs. As25m #2 time (mins)

	Coefficient	Std. Error	Std. Coeff.	t-Value	P-Value
Intercept	12.1616	.14863	12.1616	81.8121	<.0001
As25m #2 time (mins)	-.02684	.00163	-.99089	-16.45088	<.0001

Confidence Intervals
UNEAs25 male #2 vs. As25m #2 time (mins)

	Coefficient	95% Lower	95% Upper
Intercept	12.1616	11.77948	12.54373
As25m #2 time (mins)	-.02684	-.03103	-.02265

Residual Statistics
UNEAs25 male #2 vs. As25m #2 time (mins)

# >= 0	4
# < 0	3
SS[e(i) - e(i-1)]	.47608
Durbin-Watson	3.01663
Serial Autocorrelation	-.54363

Figure VII.2

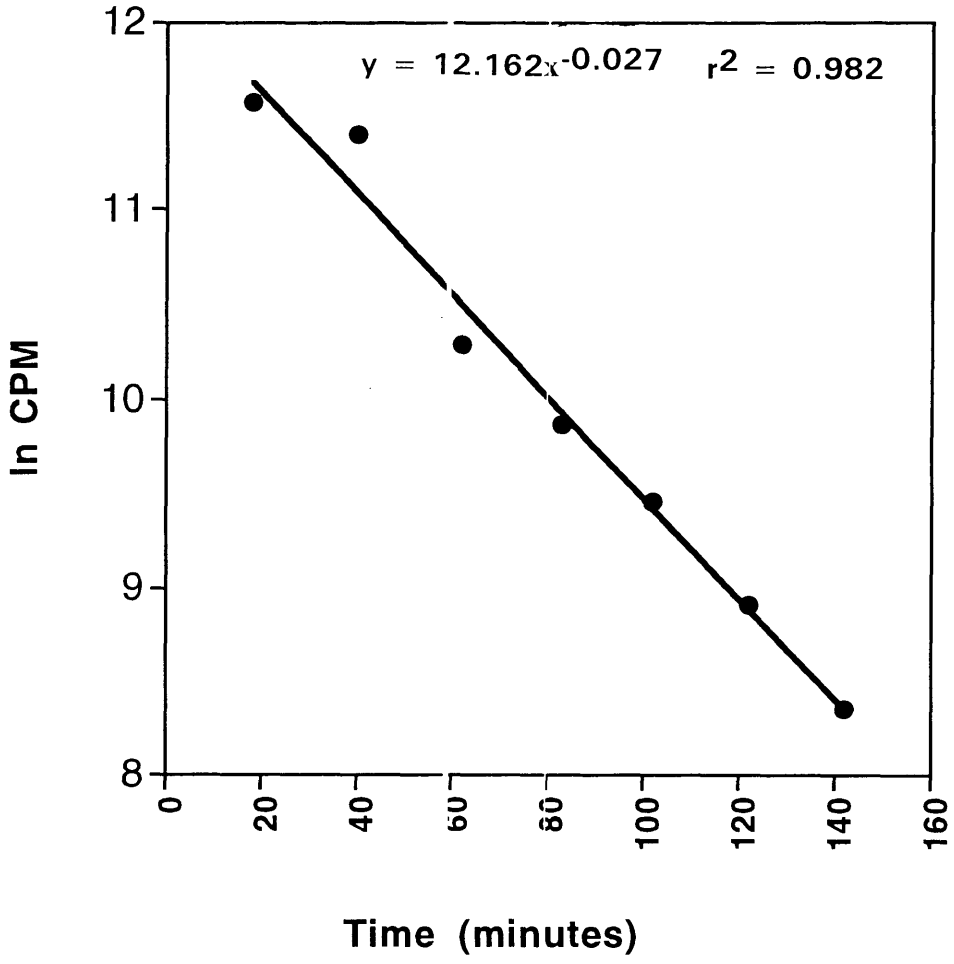

Figure VII.2 Regression line for the plot from figure VII.1, excluding the pre-injection value. The equation and the r^2 value are on the graph.

GFR calculation

Animal number: UNEAs25 male (*Antechinus stuartii*), post mating

Body weight = 44.64 g
Total counts, Q = 2,768,263
Slope = -0.027
Y intercept = 12.162

Haematocrit = 62.05 %
Plasma = 37.95 %

GFR (mL·mir) $= \frac{Q \times |\text{slope}|}{\text{Y intercept}}$

$= \frac{2,768,263 \times 0.027}{191377}$

$= 0.3906$

GFR (mL·mir) plasma $= 0.3906 \times 0.3795$
$= 0.1482$

$\frac{\text{GFR (mL.min) plasma} \times 1000}{\text{body weight (g)}} = 0.1482 \times 1000$

$= 3.320$ mL·min·kg