Chapter 5

The Seasonal Changes in Reproductive Anatomy of Male *Antechinus stuartii*

5.1 Introduction

The synchronous seasonal endocrine changes of *Antechinus stuartii* have been outlined in Chapter 1. Briefly, in males the plasma testosterone levels begin to rise from basal levels in June and do not reach maximal levels until August, some two months after spermatogenesis has ceased (Bradley *et al.* 1980, Kerr and Hedger 1983). The peak plasma testosterone concentrations are prior to the "die-off" (8.9 $ng{\cdot}mL^{-1}$ or 28.4 $nmol{\cdot}L^{-1}$, Bradley *et al.* 1980). Plasma corticosteroid concentrations in males rise twofold from late July to August. The levels continue to rise until death, a few weeks after the mating period. The free plasma corticosteroid concentrations are 0.38 $\mu g{\cdot}100\ mL^{-1}$ or 13.1 $nmol{\cdot}L^{-1}$ in July, rising to 4.01 $\mu g{\cdot}100\ mL^{-1}$ or 139 $nmol{\cdot}L^{-1}$ in August (Bradley *et al.* 1980). In females the basal corticosteroid concentrations are higher than the males prior to July, but while they increase in the month before mating they return to lower levels after the mating period (Barnett 1973). In females the progesterone levels rise from about 4 $ng{\cdot}mL^{-1}$ (12.7 $nmol{\cdot}L^{-1}$) at ovulation to 11 $ng{\cdot}mL^{-1}$ (35 $nmol{\cdot}L^{-1}$) at days 15-22 gestation (Hinds and Selwood 1990).

These endocrine changes are associated with anatomical changes in the reproductive tract, gastrointestinal tract, and adrenal glands (Barnett 1973, Moore 1974, Woolley 1966). However, the endocrine changes do not appear to be associated with the spermatogenic cycle in *A. stuartii*. The seasonal changes in the testes have been described in earlier studies (Kerr and Hedger 1983, Taggart *et al.* 1993, Woolley 1966). In males, progression of the spermatogenic stages in the seminiferous tubules of the testes is uniform between individuals at the same time of year and commonly only two or three different types of germ cells are found per association, contrasting with the four to six found in other mammalian

species (Kerr and Hedger 1983, Woolley 1966). Spermatogenesis is completed in late June (Kerr and Hedger 1983). Spontaneous spermatogenic failure occurs, during which the spermatogonia and early primary spermatocytes decline while more mature generations of germ cells continue their development (Kerr and Hedger 1983). Thus spermatogenesis is completed prior to the surge of testosterone that begins in late June.

Seasonal alterations in the epididymis are concurrent with the changes in the seminiferous tubules (Taggart and Temple-Smith 1989, 1992). Early epididymal differentiation precedes the completion of spermatogenesis in late June and is completed by mid- July (Taggart and Temple-Smith 1992). The cell structure is similar throughout the epididymis, although the caudal region, which is the site of sperm storage in mammals, increases in epithelial height as well as tubule dimensions from juvenile to adult *A. stuartii* (Taggart and Temple-Smith 1989). However, cell volume was not measured by these authors.

Although the seminiferous tubules decline in activity, the Sertoli and Leydig cells remain numerous and morphologically intact (Kerr and Hedger 1983). The Leydig cells of the testis differentiate and develop early, with the dense interstitial cellular component of the testis present from January/February, when the animals are four or five months old (Taggart *et al.* 1993). This remains unchanged throughout the life of *A. stuartii* (Kerr and Hedger 1983, Taggart *et al.* 1993).

The male accessory reproductive tract does, however, demonstrate some association with the endocrine changes in *A. stuartii*. Woolley (1966) weighed the prostate and the Cowper's glands of *A. stuartii* throughout the year and found that both rapidly increased in weight from June onwards. The increase in prostate weight from this time was confirmed by Bradley *et al.* (1980), who also found that this increase correlated with the increase in plasma testosterone concentrations. In other mammals, the association between plasma androgen concentrations and accessory gland weights has long been recognised (Heller 1930, 1932, Moore *et al.* 1930, Rubin 1944).

However, in *A. stuartii* the microscopic changes of the accessory glands associated with the seasonal cycle have not been

assessed. Rodger and Hughes (1973) described the histology of the prostate and Cowper's glands of three *A. stuartii* that had been deep-frozen after death at an unknown time of year. Their study described general histology and histochemistry of the accessory reproductive tract in *A. stuartii*, but was unable to give any information about the seasonal changes that may be exhibited in the histology of the accessory reproductive tract of male *A. stuartii*.

Thus the present chapter will describe the seasonal changes in the testes, both to confirm the previously reported seasonal changes in the seminiferous tubules, and also to correlate the seasonal changes in the accessory reproductive tract with the changes in the spermatogenic cycle. Moreover, further morphometric assessment of the epididymal changes will be presented. Finally this study will describe the seasonal changes to the accessory reproductive tract, with an aim to determining the changes in structure that may be associated with reproductive function. All these characteristics will be examined to ascertain if there are any parallel changes in the functionally separate systems, the renal and reproductive systems.

5.2 Materials and methods

5.2.1 Animals

Animals were captured, housed and sacrificed as outlined in Chapter 2. Sample sizes are February n = 5, May n = 7, July n = 6, and August n = 11.

5.2.2 Histology

Histological specimens were prepared as outlined in Chapter 2. For the testes, the cytological stains used were Masson's trichrome and Haematoxylin and eosin (see Appendix II). For the accessory reproductive tract, along with the above stains, Periodic acid - Schiff (PAS) with either Alcian blue pH 1.0 or Alcian blue pH 2.5 was used (see Chapter 2, Appendix I).

5.2.3 Morphometry

The scrotal width was measured on capture and used as an external index of male maturity (Woolley 1966). In the present study two measurements were made at a 90° angle to each other using

vernier calipers. The values were then averaged (McAllan *et al.* 1991). This is to eliminate any individual scrotal irregularities.

Caudal and caput epididymis epithelial cell heights were measured and epithelial cell volumes were estimated using the nucleator method and estimating volumes using the formula for a cylinder ($\Pi r^2 h$, Gundersen *et al.* 1988a). Sample sizes were determined by ensuring that the coefficient of error was P<0.05. The accessory reproductive tract was assessed for staining affinity for PAS and Alcian blue pH 1.0 and pH 2.5. Morphometry was not performed on the prostate or Cowper's glands as the morphological distinctions between seasons were so great as to render this unnecessary (see results). Moreover, there was some difficulty in distinguishing the three Cowper's glands in February and May, making separate comparisons impossible.

5.2.4 Statistical analysis

Morphometric data were analysed by performing a one-way ANOVA on each parameter. The mean value of each parameter for each individual was used, and the month in which the animals were collected was used as the grouping variable. If significant, this was followed by Fisher's PLSD pairwise test (Haycock *et al.* 1992, Zar 1984). Significance levels were P<0.05.

5.3 Results

The section will be divided into two sections, i) testes, and ii) accessory reproductive tract.

5.3.1 Testis

5.3.1.1 Scrotal width

The scrotal width increased significantly over the year (P<0.0001, Figure 5.1, Table 5.1). Scrotal width increased from February to May (Fisher's PLSD test P<0.0001), after which the width did not change significantly

5.3.1.2 Testicular morphology

Testis morphology changed over the year. In late February the small seminiferous tubules were surrounded by interstitial cells. The

Table 5.1 Morphometric analyses of testicular structures.

	Testes (scrotal) width (mm)	Caput cell height (μm)	Caput cell volume (μm^3)	Caudal cell height (μm)	Caudal cell volume (μm^3)
February	5.86 ± 0.37 **a**	4.8 ± 0.6 **a**	57.4 ± 8.4 **a**	5.4 ± 0.6 **a**	85.5 ± 20.1 **a**
May	11.21 ± 0.32 **b**	9.2 ± 0.9 **b**	212.5 ± 45.5 **a**	18.8 ± 1.5 **b**	538.6 ± 142.9 **b**
July	11.70 ± 0.35 **b**	13.1 ± 0.7 **c**	878.5 ± 120.1 **b**	32.7 ± 0.8 **c**	1085.3 ± 118.5 **c**
August	11.49 ± 0.23 **b**	10.3 ± 0.2 **b**	877.9 ± 51.7 **b**	34.6 ± 1.6 **c**	1526.1 ± 176.9 **d**
Months	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001

Table 5.1 Morphometric analysis of testes (scrotal) width (mm), caput cell height (μm), caput cell volume (μm^3), caudal cell height (μm), and caudal cell volume (μm^3). Values are means ± standard errors of the means. Below each column are the significance levels following one-way ANOVA. Within each column values with different superscripts differ significantly ($P<0.05$).

Figure 5.1

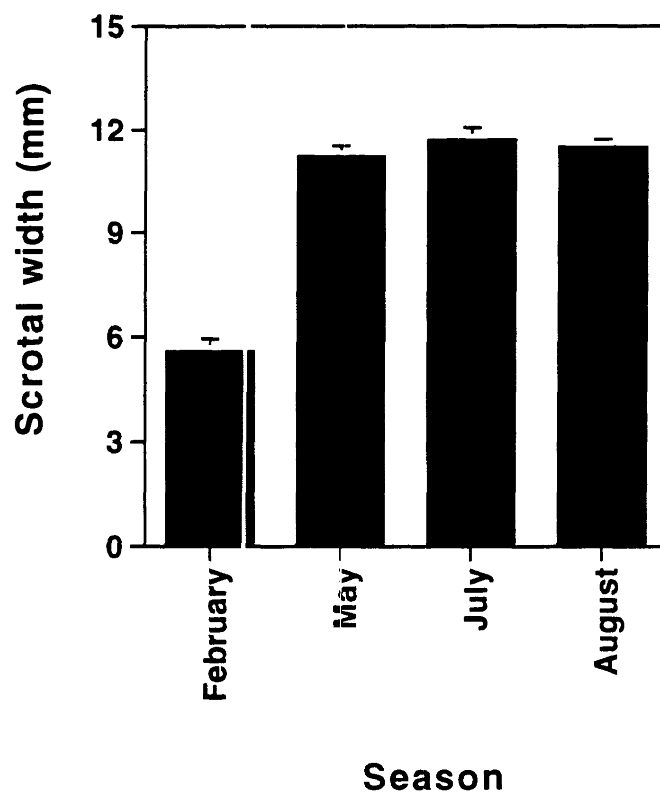


Figure 5.1 Seasonal changes in the scrotal width (mm) in *A. stuartii*. Values are means ± standard errors of the mean.

cells within the seminiferous tubules were of several types. There were more darkly staining cells on the basement of the tubule, presumably Sertoli cells. Adjacent to them were undifferentiated cells, presumably spermatogonia Further into the tubule in some individuals were primary spermatocytes showing conspicuous darkly staining chromatids within the nucleus.

By May the seminiferous tubules had enlarged and less interstitial tissue surrounded the tubules. Both Leydig cells and Sertoli cells had attained a mature appearance, with the Leydig cells appearing with a larger, more three dimensional cytoplasm that stained intensely with Masson's trichrome. The nuclei of the Sertoli cells also stained more darkly, and the cells had an enlarged cytoplasm compared to those present in late February. Large darkly-nucleated spermatocytes were present, mainly leptotene and pachytene spermatocytes, and also some spermatids that were in the process of being transformed into spermatozoa (spermiogenesis). In some individuals there were clear inclusions in the cytoplasm of the spermatids.

In July spermatogenesis was complete with mature sperm observed in the lumen of the seminiferous tubules. Mature spermatids were surrounded by cytoplasmic material. Within the seminiferous tubules, besides the Sertoli cells, there were few other cell types. Occasional primary spermatogonia were seen and the depth of the seminiferous epithelia was reduced. The interstitial material remained plentiful, consisting mostly of darkly-staining polyhedral Leydig cells, surrounding the tubules.

In August the seminiferous tubules had reduced in size and appearance. The plentiful, developed interstitium remained, with intensely staining large Leydig cells, however, few cell types remained within the seminiferous tubules. Some sperm remained in the lumina of the seminiferous tubules of most individuals, although many lumina were devoid of sperm. Much cellular debris was present in the seminiferous epithelia and residual bodies were seen in the tubules of many individuals. Sertoli cells were the only epithelial cells seen in most individuals, although occasionally spermatogonia were seen in one or two individuals.

5.3.1.3 Epididymal morphology

The epididymal epithelia of *A. stuartii* consisted of a simple cuboidal epithelium in the caudal region and a simple columnar epithelium in the caput region. The epithelium was surrounded by a basement that consisted of smooth muscle cells. Connective tissue surrounded the epididymal tubule and this was surrounded by the tunica vaginalis.

There were distinct seasonal changes in the epididymides of male *A. stuartii*. In late February the epididymis consisted of simple cuboidal cells, with little evidence of differentiation along the epididymis. Cilia were not evident nor were there any secretory products within the lumen of the epididymal tubule. The epididymis was loosely bound by connective tissue.

In May the epididymis of most individuals had become differentiated from the caput to the caudal regions. Cilia were more obvious and the epithelium in the caudal region had become more columnar in appearance. The epithelium in the caput region remained simple cuboidal in appearance. The connective tissue surrounding the epididymis had become more densely arranged and the blood supply for the epididymis was more pronounced. Little secretory material was observed in the lumen of the epididymis.

In July all regions of the epididymis showed extensive hypertrophy. The lumina were full of mature sperm and there was also intensely staining material within the lumina. The dense connective tissue was well vascularised. The simple columnar cells of the caudal epithelia were ciliated and large. Hypertrophy of the epithelial cells of the caput region was also observed.

In August the epididymis appeared similar to that seen in July, although the epididymal epithelium appeared more hypertrophied.

5.3.1.4 Epididymal morphometry

The caput epithelial cell height differed significantly across the year ($P<0.0001$, Figure 5.2a, Table 5.1). Epithelial cell heights in February were significantly smaller than those for all other seasons (Fisher's PLSD test $P<0.0005$). Epithelial cell heights in May were significantly smaller than those in July (Fisher's PLSD test $P=0.0005$), but not significantly different from those in August. Epithelial cell

heights in July were significantly larger than those from August (Fisher's PLSD test $P<0.005$).

The caput epithelial cell volume differed significantly across the year ($P<0.0001$, Figure 5.2b, Table 5.1). Values in February and May were significantly smaller than those found in July and August (Fisher's PLSD test $P<0.0001$).

The caudal epithelial cell height differed significantly across the year ($P<0.0001$, Figure 5.3a, Table 5.1). Epithelial cell heights in February were significantly smaller than those for all other seasons (Fisher's PLSD test $P<0.0001$). Epithelial cell heights in May were significantly smaller than those for July and August (Fisher's PLSD test $P<0.0001$, Figure 5.3a).

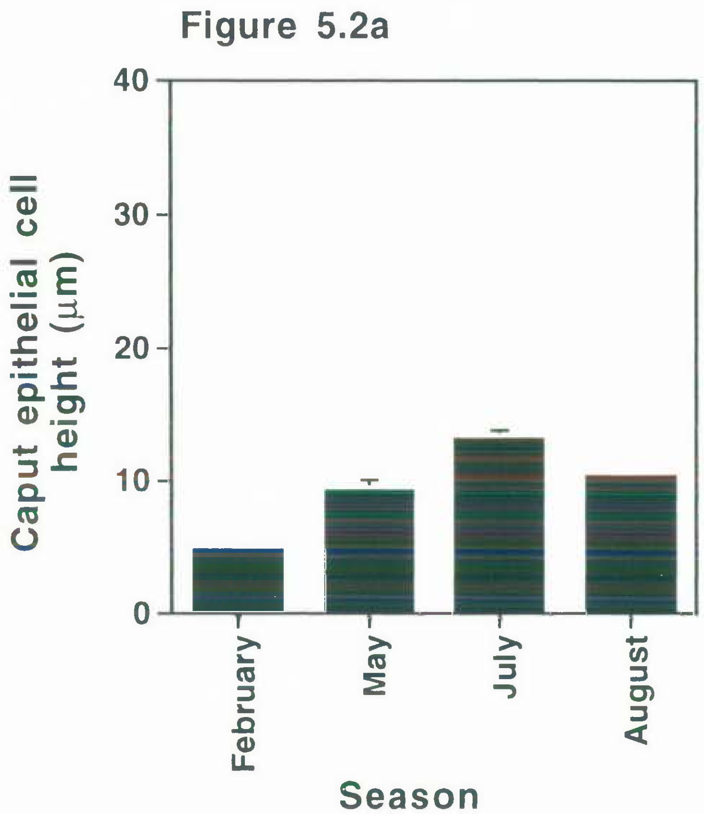
The caudal epithelial cell volume differed significantly across the year ($P<0.0001$, Figure 5.3b, Table 5.1). Epithelial cell volumes in February were significantly smaller than those for all other seasons (Fisher's PLSD test $P<0.05$). Epithelial cell volumes in May were significantly smaller than those for July and August (Fisher's PLSD test $P<0.05$) and epithelial cell volumes in July were significantly smaller than those for August (Fisher's PLSD test $P<0.05$).

5.3.2 Accessory reproductive tract

5.3.2.1 Prostate

The prostate of *A. stuartii* surrounded the urethra from the bladder to the penis. It was carrot-shaped and was attached at the anterior end to the bladder Posteriorly the prostate was continuous with the penile urethra. Structurally the prostate was disseminate and consisted of simple tubules that were lined by a simple columnar epithelium. These tubules were oriented towards the lumen of the prostate. The lumen was also continuous with the bladder and the cellular structure of the urethral lining of the prostate was continuous with that of the bladder. Exteriorly, the prostate was surrounded by a smooth muscle coat. Differentiation will be discussed with the seasonal changes of the prostate.

The prostate changed significantly over the course of the year. In February it was small and macroscopically somewhat transparent in nature, and the prostatic epithelium was undifferentiated along its length. The epithelium did not appear to be secretory at this stage as



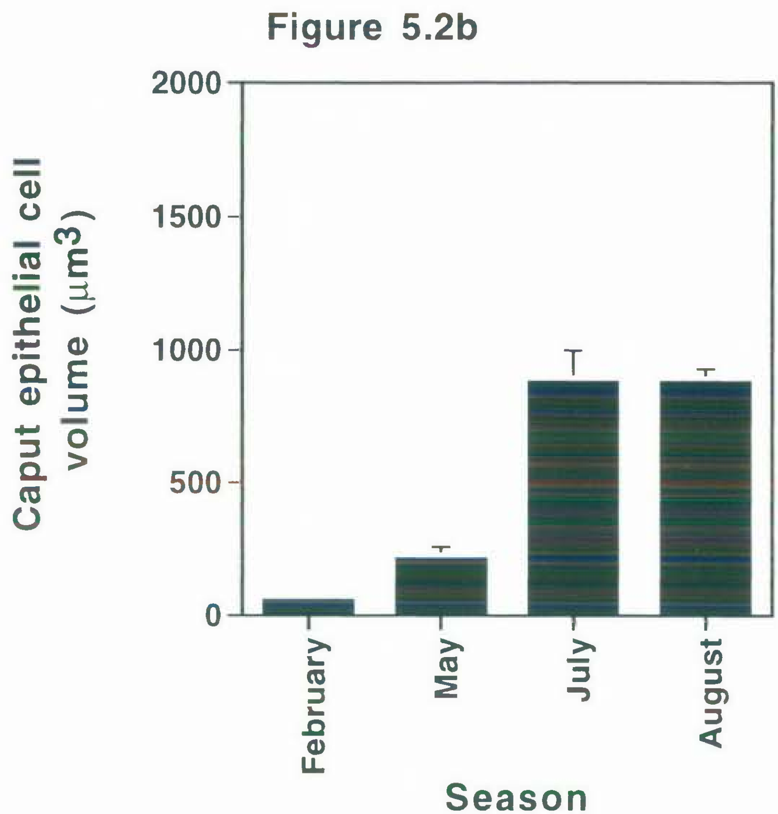
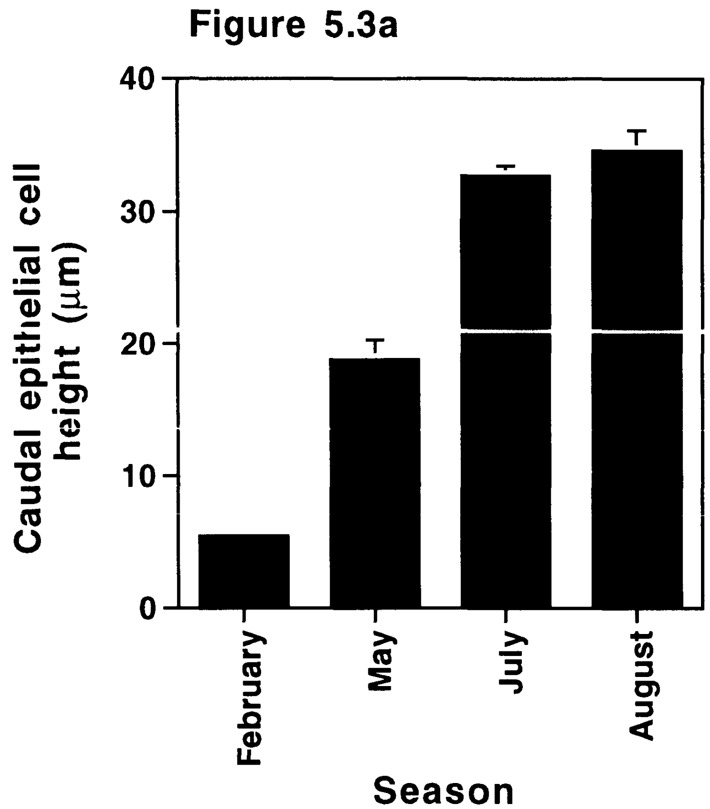


Figure 5.2 Seasonal changes in epididymal cell volume of *A. stuartii.* a) caput epithelial cell height (μm) and b) caput epithelial cell volume (μm^3). Values are means ± standard errors of the mean.



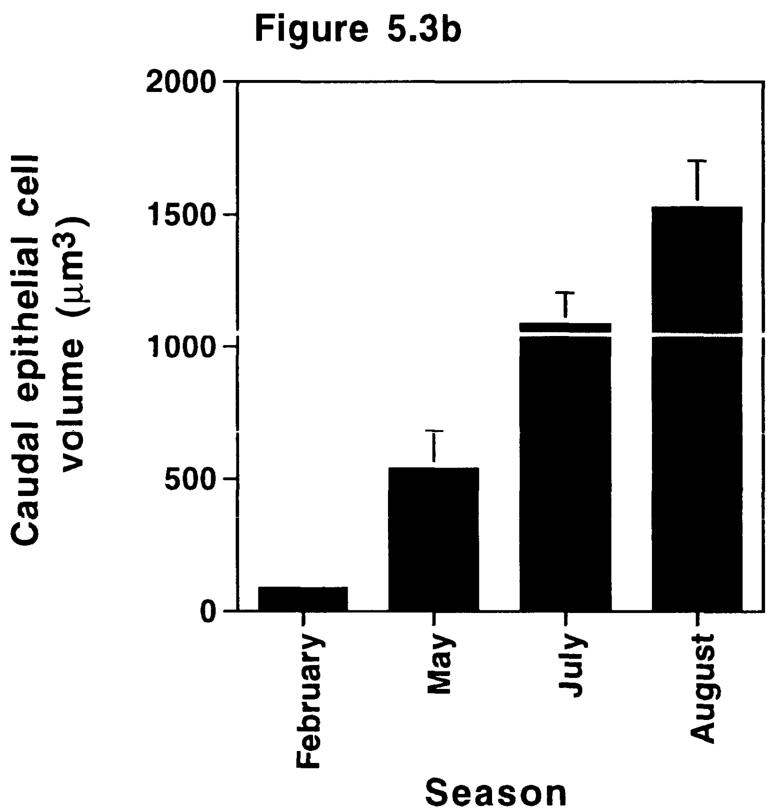


Figure 5.3 Seasonal changes in epididymal cell volume of *A. stuartii.* a) caudal epithelial cell height (μm) and b) caudal epithelial cell volume (μm^3). Values are means ± standard errors of the mean.

the cytoplasm of the cells was small and no secretory material was present in the lumina of the tubules themselves nor in the urethral lumen (Figure 5.4a). The connective tissue stained with PAS, but the tubular epithelium did not stain with PAS and stained weakly with Alcian blue pH 2.5 and pH 1.0 (acid mucopolysaccharides). The urethral epithelium, like that of the bladder, was PAS positive.

In May the prostate was still undifferentiated along its length although it had increased in size and was macroscopically more opaque in the posterior portion of the gland. The staining for PAS- Alcian blue was the same as for February (Figure 5.4b).

In July, however, the prostate had become more segmented. The anterior portion was macroscopically translucent and the posterior portion remained white and opaque. The anterior quarter of the prostate had simple cuboidal epithelium with an enlarged lumen. The epithelium was positive for neither PAS nor Alcian blue although the secretory material found in the lumen was positive for PAS. The posterior three-quarters was composed of tubules that were of a more columnar nature and secreted material into small lumina (Figure 5.5a). The tubules were arranged perpendicular to the length of the prostate and joined into simple ducts at the urethral epithelium. The tubular epithelium stained differentially with both Masson's trichrome and PAS-Alcian blue. When stained with Masson's trichrome the cytoplasm of some groups of cells stained blue and next to them in the same tubule were groups of cells whose cytoplasm stained mauve or red. Changes in the cellular staining were observed along the length of individual tubules. The epithelial cells which stained blue were PAS positive, while the regions of epithelia that stained mauve were PAS negative. Neither stained with Alcian blue. The prostate of males from August was similar, although the cells were more hypertrophied and there was more secretory material present than for males from July (Figure 5.5b and 5.5c)

5.3.2.2 Cowper's or Bulbourethral glands

The bulbourethral glands of *A. stuartii* lay dorsally to the penis (Figure 5.6) and were observed to enter the urethra by separate ducts at the base of the muscular urethral bulb (Figure 5.7). In animals from July and August there were three distinct secretory glands and a

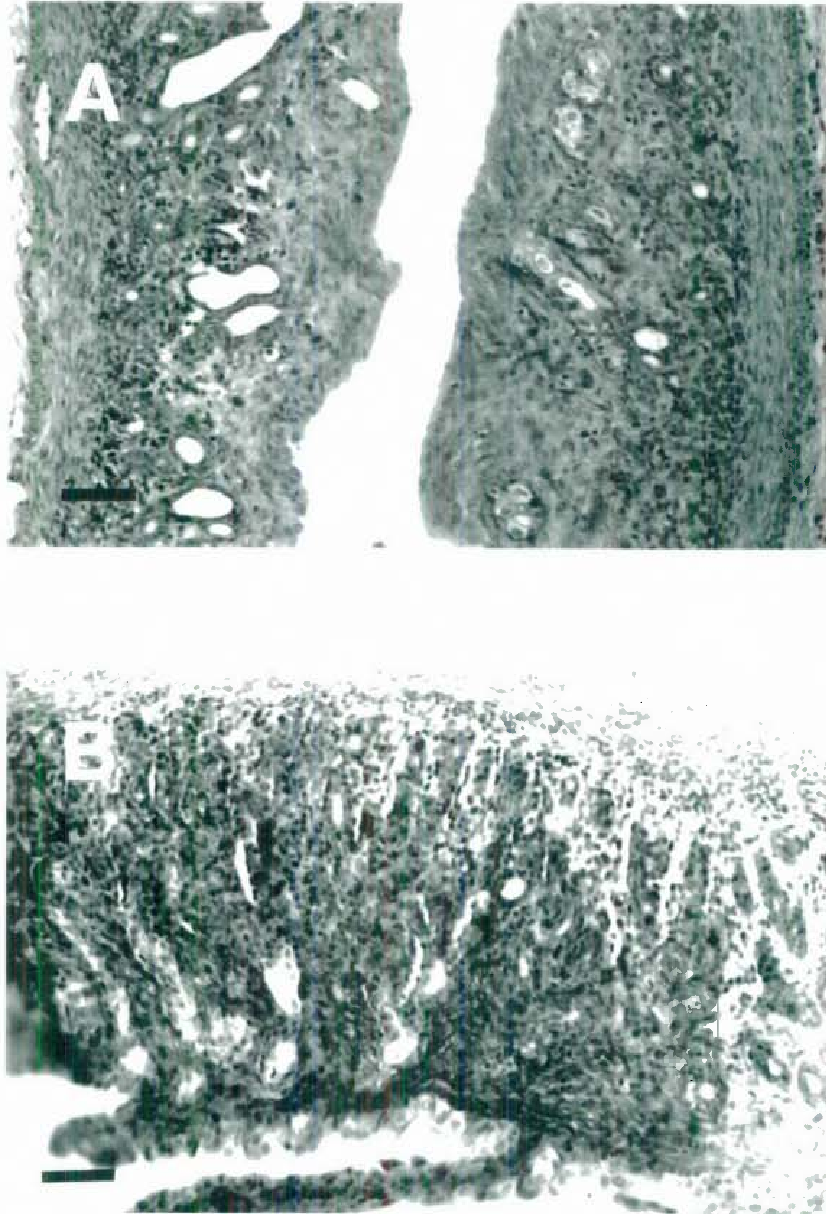


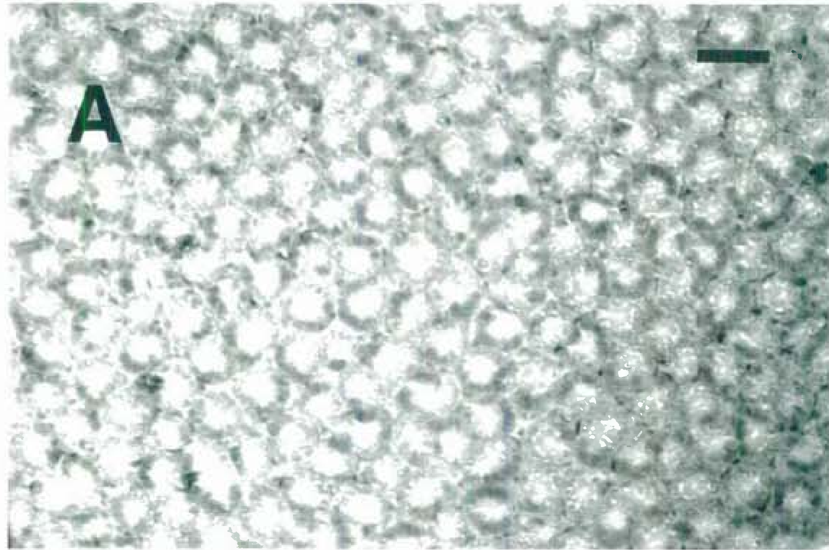
Figure 5.4 Prostate from A) Febuary. B) May. The prostates from both February and May are from the anterior end, into the posterior portion, and demonstrate the lack of differentiation between the anterior and posterior ends. Bars are 50 μm.

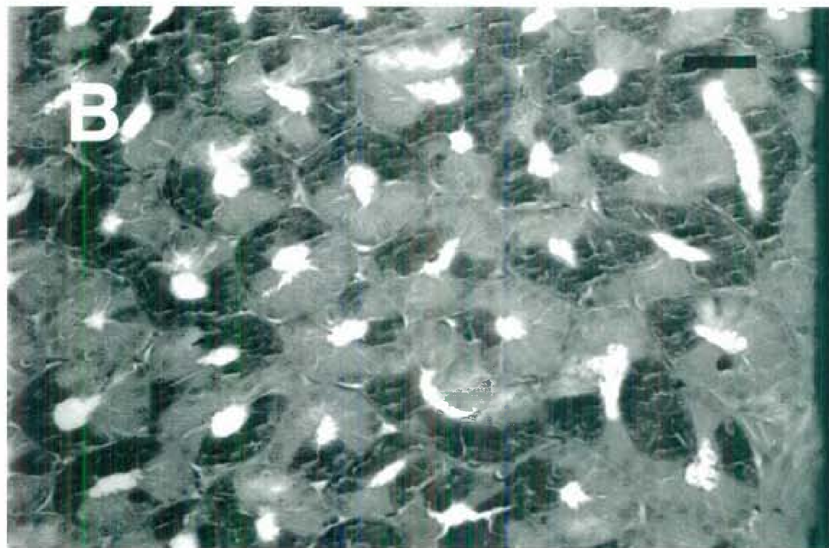
Figure 5.5 Prostate glands of males from A) July and B), C) August. Scale bars are 50μm.

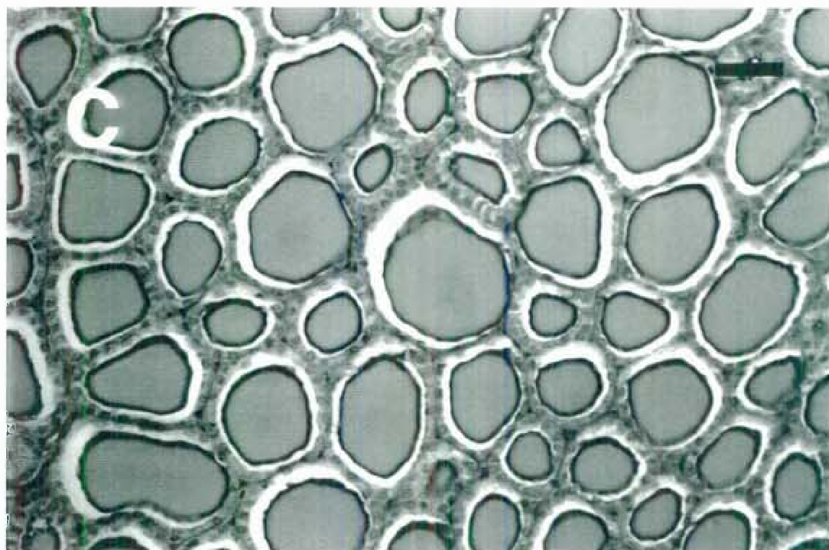
A) The posterior prostate of a male from July.

B) The posterior prostate of a male from August. The tubules of the prostate of the male from August are considerably more hypertrophied and show the differential staining more strongly than in the prostate of the male from July

C) The anterior prostate of a male from August. Note the open lumen full of colloidal-like secretory material.


A


B


C

fourth gland whose duct was not actually observed to enter the urethra in any histological preparation that included the penis. This gland would usually dissect away with the bulbourethral glands proper. All glands showed distinct seasonal changes in their morphology and the glands are defined in figure 5.7.

In February and May gland IV was the only bulbular structure that could be easily identified (Figure 5.8). The other three bulbourethral glands (I, II, and III) were made up of small loosely associated tubules connected by connective tissue. It was not always possible to distinguish the three glands from one another in February and May and macroscopically they were often not visible and were missed in dissection (Figure 5.8a, 5.8c). Their tubular epithelium was simple columnar and stained weakly with PAS and the luminal surface stained with Alcian blue pH 1.0. Gland IV had a muscular coat and an open lumen, which in some individuals was observed to contain parasites and their eggs (Figure 5.8b). The stratified cuboidal glandular epithelium was loosely folded and stained weakly with PAS, and the luminal material stained with Alcian blue pH 1.0.

In July the bulbourethral glands had increased considerably in size and differentiation. The muscular layers of the urethral bulb had increased in size (Figure 5 9a). Glands I and II were large white and sacular and the branching tubules were lined by simple columnar epithelium secreting material into an open irregularly shaped lumen (Figure 5.10a, 5.10b). The tubules were attached by connective tissue and the whole glands were surrounded by a muscular coat. The colloidal-like secretory material of gland I stained red with Masson's trichrome and was PAS positive (Figure 5.10a). The secretory material in gland II was crystalline in appearance and stained blue and red with Masson's trichrome (Figure 5.10b). This material, too, was PAS positive and Alcian blue negative (polysaccharides).

Externally, gland III was large and creamy coloured and was considerably enlarged compared to those found in February and May. The secretory cuboidal epithelium was branched and the ducts drained into a few common ducts that traveled towards the urethra (Figure 5.10c). The epithelium stained weakly with PAS and the luminal surface stained with Alcian blue pH 1.0 and pH 2.5 (acid

Figure 5.6 Dissection of an August male *A. stuartii*. The penis is seen to extend out of the urogenital sinus or cloaca, and to be folded anteriorly. The crura of the penis are seen lateral to the anterior fold. To the left of the penis the bulbourethral glands have been dissected away. On the right side of the penis lies a small superficial paracloacal gland that is loosely connected to the urogenital sinus by connective tissue. The large whitish mass that lies on the left in the image and in situ lies dorsolaterally to the tail is the largest bulbourethral gland, gland III (see text). The other three glands, and the urethral bulb lie ventrally to this and are hidden in this dissection by the penis, crura, tail and upper leg muscles.

Figure 5.6 Dissection of ar August male *A. stuartii*.

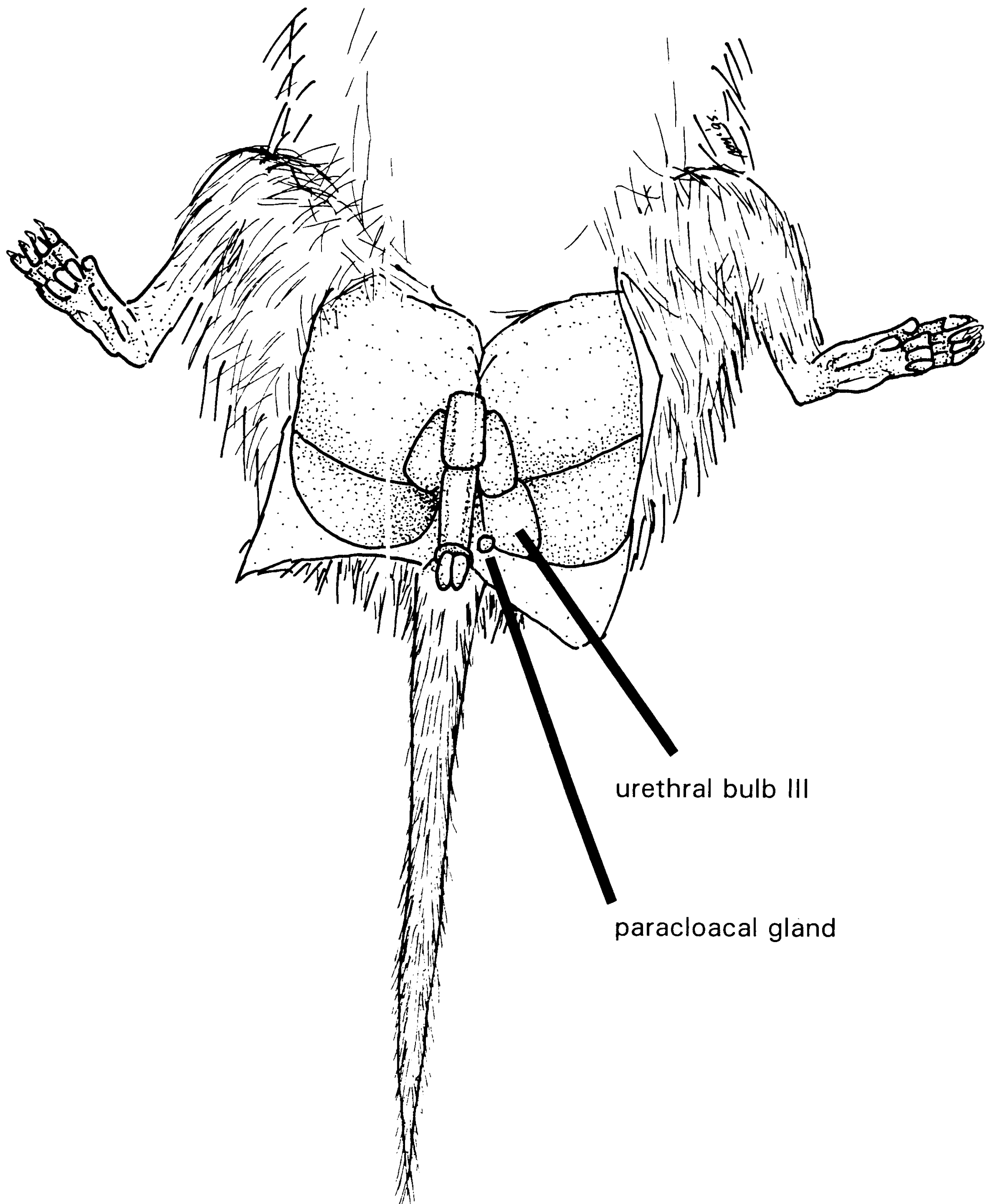


Figure 5.7 Cross-section of the male reproductive tract of *A. stuartii*.

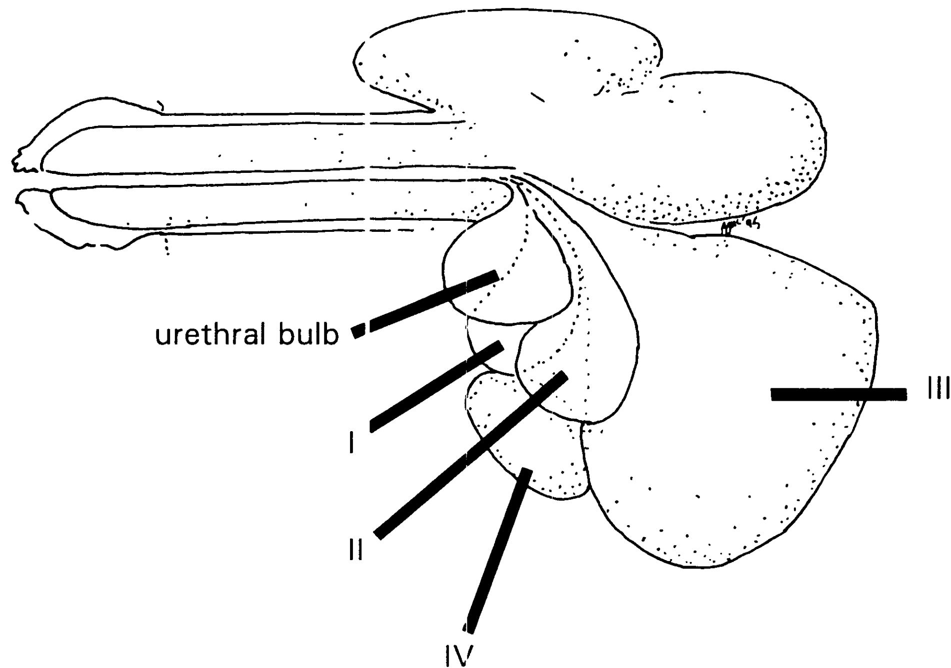


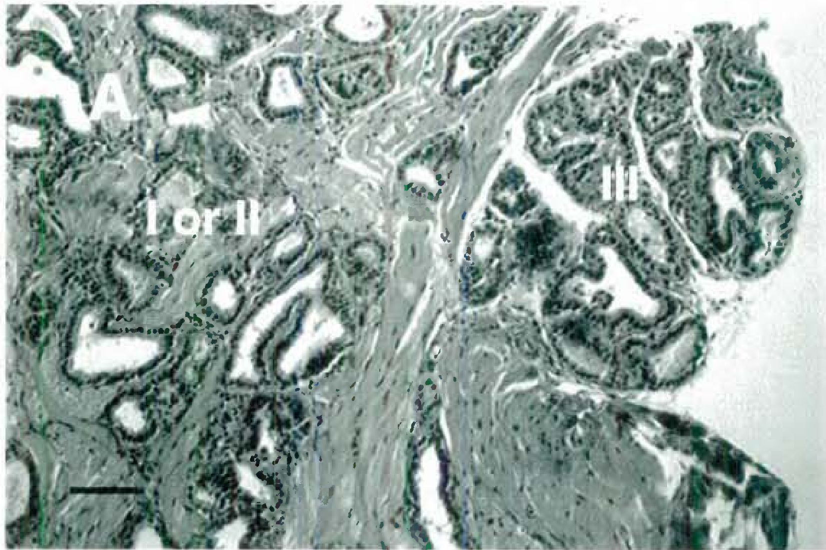
Figure 5.7 The lateral v ew of the bulbourethral glands and their relationship to the penile urethra. The drawing was taken from a series of histological sections from an August male which included the musculature of the penis. While the glands depicted here are taken from a histological preparation it was noted that, in other individuals, glands I and II and the urethral bulb were often situated more anteriorly. Glands I, II and IV and the urethral bulb were often seen to be closer to the base of the crura of the penis. The glands referred to in the text and in histological figures are identified by the arrows.

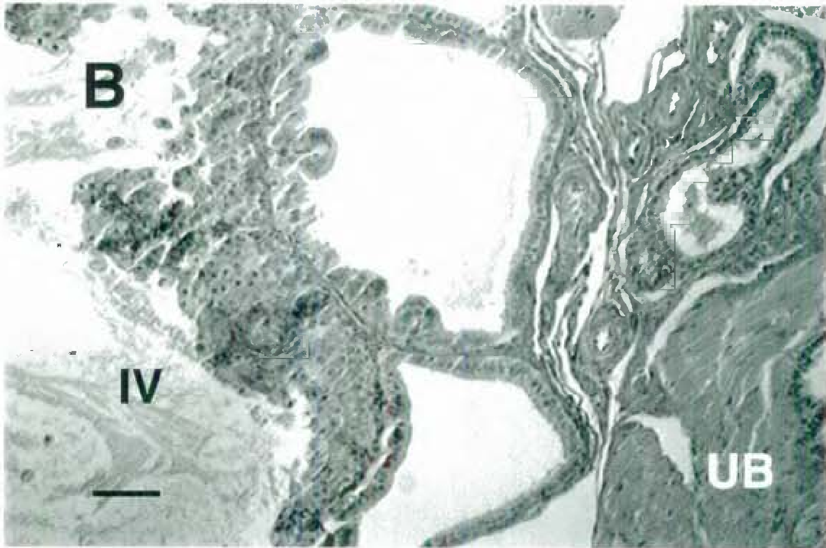
Figure 5.8 The bulbourethral glands of males from A), B) February, and C) May. Scale bars are 50μm.

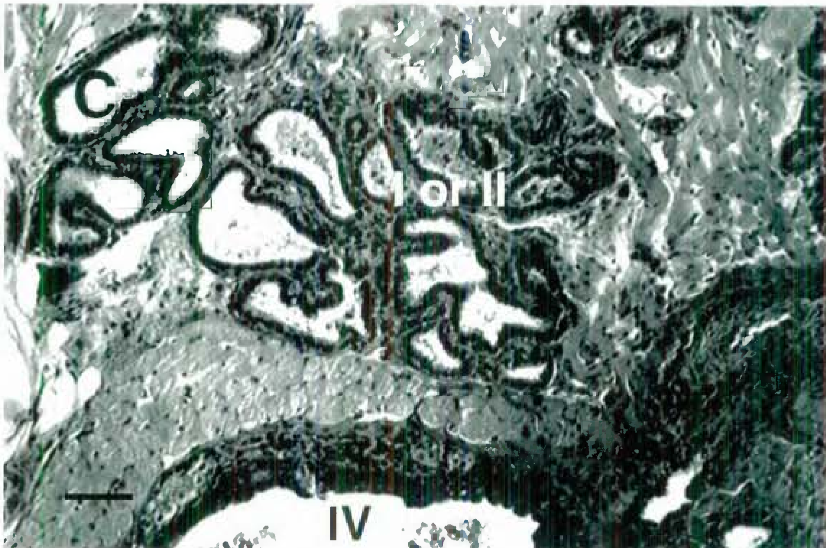
A) Demonstrates the loose configuration of the bulbourethral glands and the lack of differentiation between them. I, II and III indicate the glands.

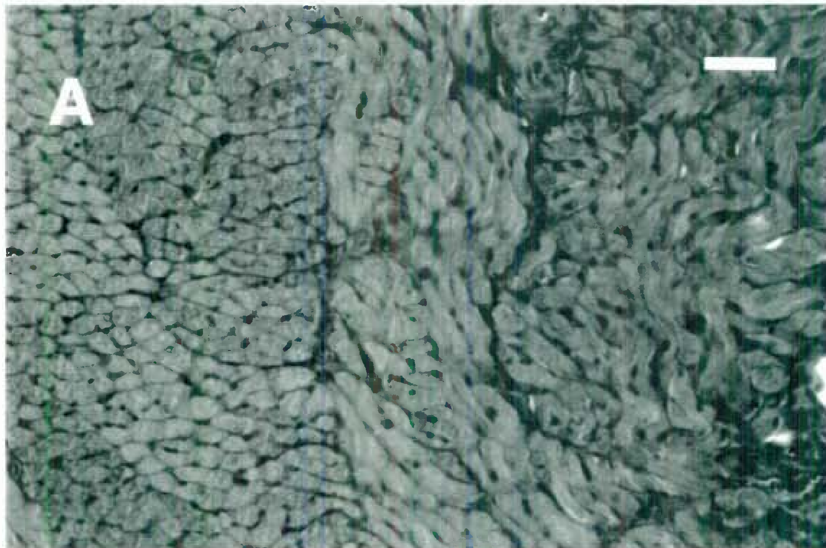
B) Gland IV (IV) and part of the urethral bulb (UB) in February. The open structures in the centre of the image are the ducts from gland IV.

C) Demonstrates the continuation of the undifferentiated appearance of the bulbourethral glands in May. I, II and IV indicate the relevant glands.


A
I or II
III


B
IV
UB


C
I or II
IV



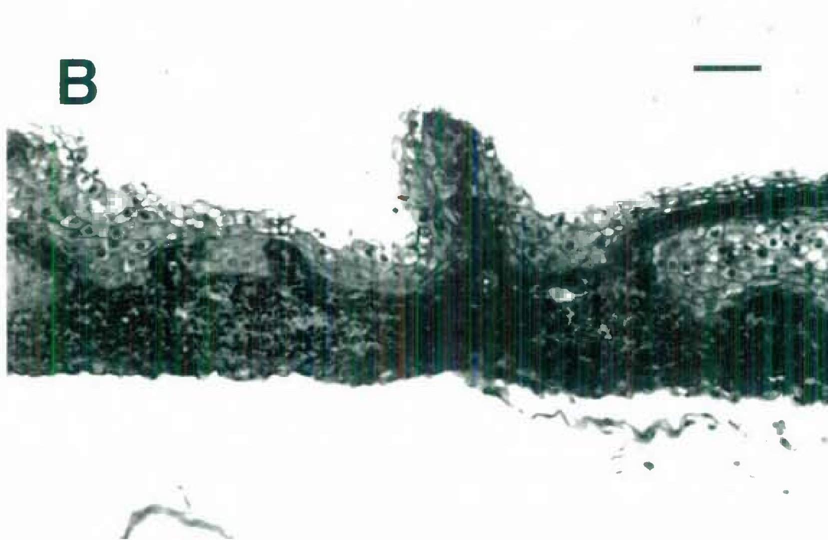


Figure 5.9 Urethral bulb and Gland IV. A) Urethral bulb from July, and B) Gland IV from August. The urethral bulb and Gland IV were similar in July and August. Bars are 50 μm.

mucopolysccharides). The secretory material in the lumen was weakly PAS positive. The tubules were attached by connective tissue and the gland was surrounded by a muscular coat. Gland IV was larger than in February and May, although the cellular structure was very similar. The epithelial cells appeared larger and the lining thicker. Parasites were observed in the lumen of the gland.

In August the bulbourethral glands were more hypertrophied than in July (Figure 11). The muscular urethral bulb was further enlarged and the lumina of glands I, II, and III were full of secretory material (Figures 5.11a, 5.11b, 5.11c). Gland III became so enlarged that the bulk was comparable to that of the testes. Gland IV was similar in appearance to those seen in July (Figure 5.9b). Staining affinities of all glands were the same as for July.

5.4 Discussion

This study demonstrates that the reproductive tract exhibits seasonal changes in *A. stuartii*. The seasonal changes in the epididymis, prostate and bulbourethral glands have not previously been described in detail, nor in comparison to the spermatogenetic cycle. All three associated tissues show parallel changes throughout the year. In February and May there is little specialisation within the structures and all show considerable development in July. The morphology of the structures continues to hypertrophy throughout August. These changes parallel the glandular weight changes observed in other studies (Bradley *et al.* 1980, Woolley 1966).

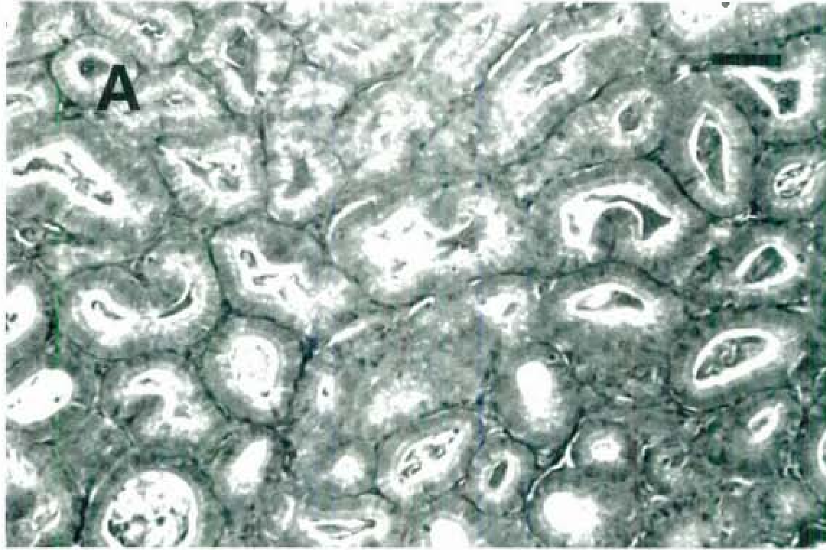
The epididymis shows some specialization along its length in May, confirming findings by Taggart and Temple-Smith (1992). This precedes the completion of spermatogenesis that occurs in late June (Kerr and Hedger 1983 Taggart and Temple-Smith 1992) and corresponds to the changes in the scrotal width in the present study. The increase in differentiation is reflected in the change in epithelial cell height and volume. Values were similar in caput and caudal ends of the epididymis in February and, while they increased in both areas, by May values in the caudal end were more than twice as high as those from the caput end. The increase in cell height and volume continued through to July, when all values remained similar into

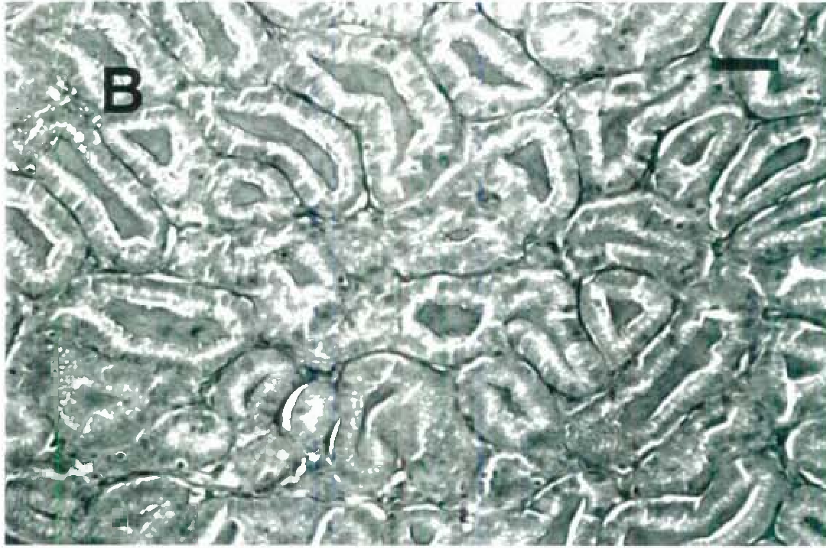
Figure 5.10 The bulbourethral glands in males from July. Note the considerable hypertrophy and differentiation compared to males from May. Scale bars are 50μm

A) Gland I

B) Gland II

C) Gland III


A


B

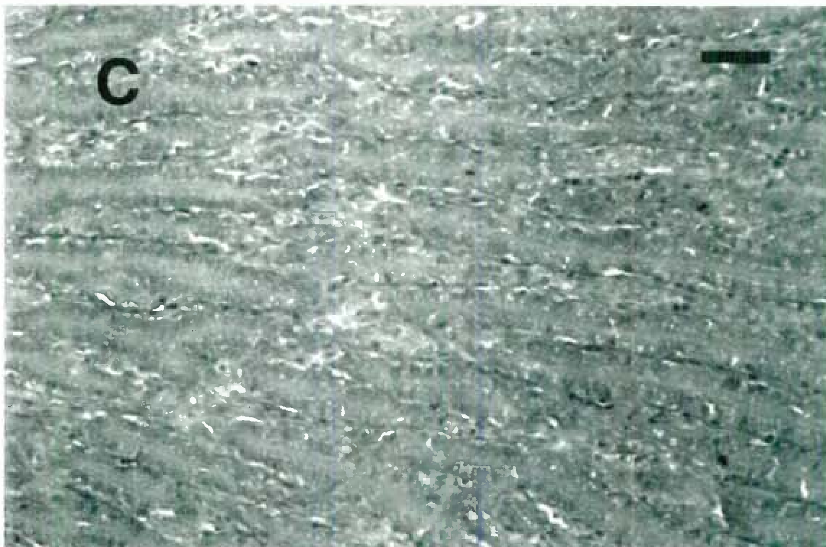
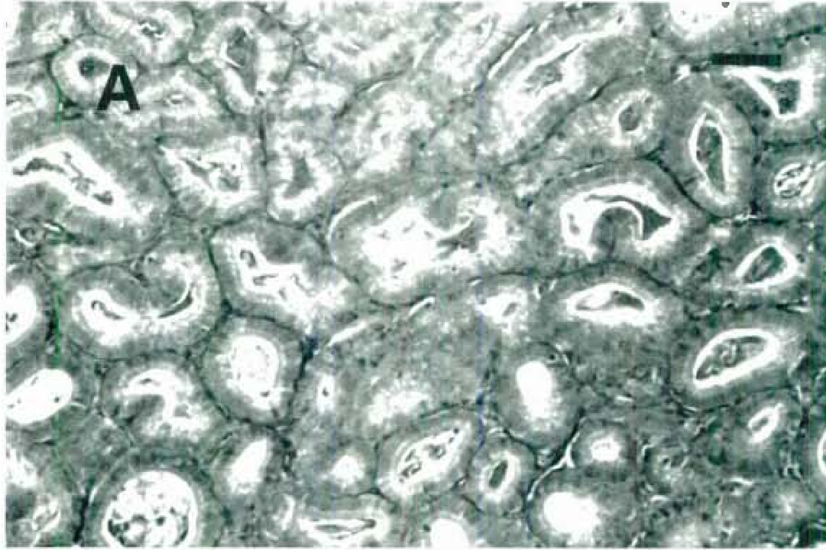

C

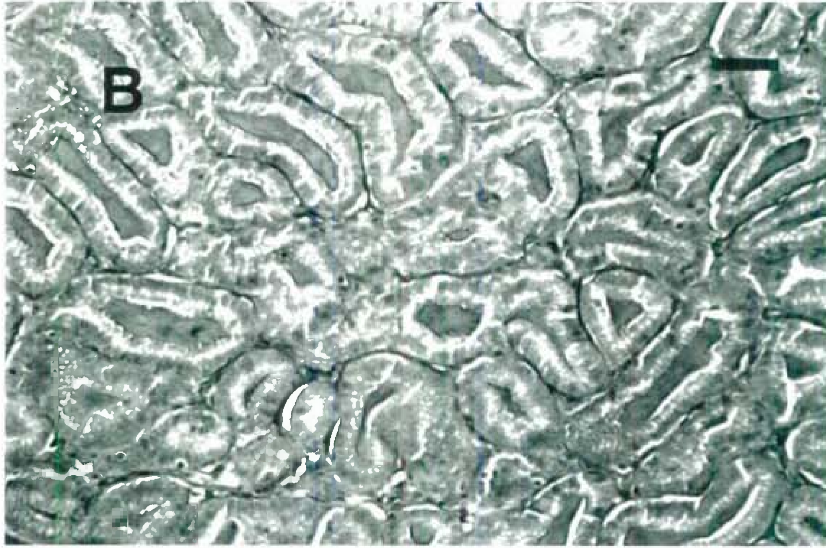
Figure 5.11 The bulbourethral glands in males from August. Note the considerable hypertrophy and lumina full of secretory material compared to males from July. Scale bars are 50μm.

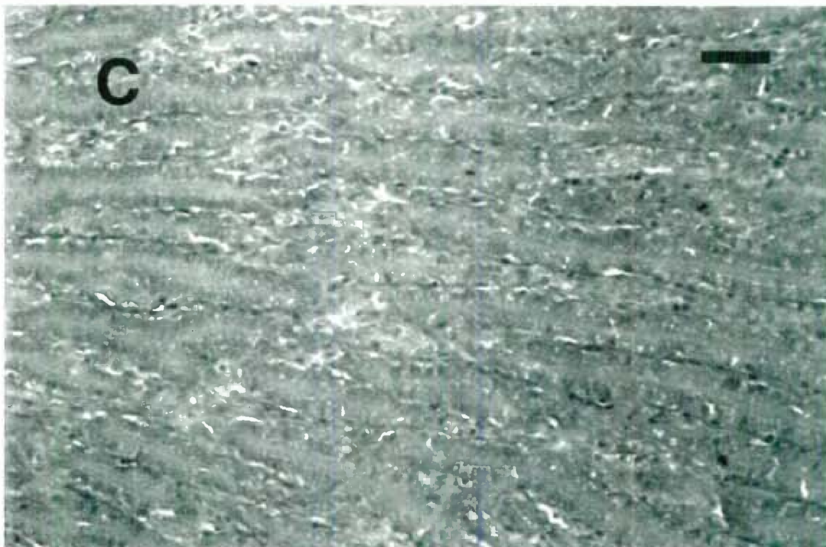
A) Gland I

B) Gland II

C) Gland III


A


B


C

August, with the exception of caput cell height which declined in August. The significance of the decline in epithelial height is not clear, especially as cell volume was not compromised because the cellular hypertrophy was maintained by an increase in cellular radius.

The extensive hypertrophy of the epididymis in July and August was matched by changes in the prostate. Like the epididymis, the prostate was easily identified in February and May and did not show any secretory activity. The morphological hypertrophy and secretory activity present in July and August were similar, but not identical, to those observed by Rodger and Hughes (1973). These authors found that *A. stuartii* had a carrot shaped prostate, which was divided into three histological types with the anterior part made up of simple branched tubules, lined with a single layer of columnar cells and secreting acid mucosubstances (Rodger and Hughes 1973). However, Rodger and Hughes (1973) found an internal posterior segment (which surrounded the urethral epithelium and secreted proteinaceous, neutral and small quantities of acid mucosubstances) and an external posterior segment whose proteinaceous secretions must pass along the tubule to be secreted into the urethral lumen. In the present study the simple branched tubules and the cells surrounding the urethral lining did not demonstrate any differential staining. The differences between the studies may be because of more diverse staining methods used by Rodger and Hughes (1973) or because of post-mortem changes to the tissue structure because they were frozen at an unspecified point after death, but before fixation, in the study of Rodger and Hughes (1973).

The microanatomy of the prostate of other marsupials has been described in varying detail by Chase (1939), Disselhorst (1904), Fordham (1928), Inns (1982), Martan and Allen (1965), Rodger and Hughes (1973), Todhunter and Gemmell (1987) and Woolley and Scarlett (1984). All studies describe a distinct anterior portion with a similar simple structure with open lumina full of prostatic secretions. The differentiation of the posterior portion seems to differ between species, with some, such as the macropods, showing two distinct separations and others, notably the possums and bandicoots, showing no further differentiation (Disselhorst 1904, Inns 1982, Martan and Allen 1965, Rodger and Hughes 1973, Todhunter and Gemmell 1987,

Woolley and Scarlett 1984). The posterior portion of the prostate of *A. stuartii* does not appear to have any further specialization.

The lack of differentiation observed in *A. stuartii* in February and May is most pronounced in the bulbourethral glands. The three secretory glands are often indistinguishable from one another. The dramatic increase in size and secretory activity in July and continuing into August, is even more extreme than that observed in the prostate, because, with the exception of gland IV, all bulbourethral glands were undifferentiated and unrecognizable as part of the accessory reproductive tract. Both gland IV and the urethral bulb increase in size to July and August, but it would appear that while there is an association of gland IV with the bulbourethral glands, it is more likely to be a paracloacal gland. In some other marsupials, paracloacal glands can be quite large and can change seasonally (Bolliger and Whitten 1948, Cowper 1704, Green 1963, Nogueira 1988, Owen 1868, Rotenberg and Glauert 1928, Smith 1984, Sweet 1907). The seasonal changes in the paracloacal glands are associated with high plasma concentrations of androgens in the sugar glider (*Petaurus breviceps*, Bradley and Stoddart 1993). Certainly the microscopic morphology more closely resembles the paracloacal glands described in other species (Bolliger and Whitten 1948, Green 1963, Helder-José and Freymüller 1995, Sweet 1907).

The general microscopic morphology of glands I, II, and III was similar to the findings of Rodger and Hughes (1973), who further found that the largest secreted proteinaceous material and neutral mucosubstances, the second secreted a small amount of protein or mucosubstances and the smallest secreted neutral and acidic mucosubstances (Rodger and Hughes 1973). The results of the PAS-Alcian blue staining confirm some of these results. The main difference between the two studies is the presence of crystalloid material in gland II, which may have been observed in the present study on *A. stuartii* because the tissues were preserved soon after death. Like the present study on *A. stuartii*, colloidal-like material was observed in one bulbourethral gland of the wombat (*Vombatus ursinus*, Brooks *et al.* 1978).

The main contribution of the present study is the description of the seasonal changes in all of the non-testicular sections of the

reproductive tract. Most of the changes occur after spermatogenesis has been completed. The accessory reproductive tract is important as it produces the supporting material for the sperm released on ejaculation. An increase in size leading up to the breeding season is of obvious functional significance for reproduction. The increase in cellular differentiation and secretory activity correlates with the proliferation of the Leydig cells in the interstitium. In mammals the Leydig cells are the main source of testicular testosterone (Weinbauer and Nieschlag 1990) and in *A. stuartii* the Leydig cells of the testis differentiate and develop early, remaining plentiful throughout the life of *A. stuartii* (Kerr and Hedger 1983, Taggart *et al.* 1993).

Previous studies on the *Antechinus* genus suggest that spermatogenesis is maintained by small amounts of locally acting hormones interacting between the Leydig and Sertoli cells in the *Antechinus* genus (Kerr and Hedger 1983, Wilson and Bourne 1984). The 8-fold increase in plasma testosterone levels is coincident with the cessation of spermatogenesis in this genus (Kerr and Hedger 1983, Wilson and Bourne 1984). Testosterone is known to act on the accessory reproductive tract in a stimulatory manner. Moreover, the bulbourethral glands and prostate were used previously as testes hormone "indicators" (Chase 1939, Heller 1930, 1932, Rubin 1944).

These early reports have since been expanded to show that the accessory reproductive glands in marsupials are sensitive to the effects of testosterone. The increases in weights of the prostate and Cowper's glands are directly related to the plasma testosterone concentrations (Bradley *et al.* 1980, Curlewis 1991, Curlewis and Stone 1985, Todhunter and Gemmell 1987). Administration of testosterone to castrate animals results in an increase in weight and secretory properties of these glands, especially those of the prostate (Cook *et al.* 1978, Curlewis and Stone 1985, Jones *et al.* 1988).

Studies on other marsupials have shown that there are seasonal changes in the accessory reproductive tracts of marsupials (Curlewis 1991, Curlewis and Stone 1985, Fletcher 1985, Inns 1982, Nogueira 1988, Todhunter and Gemmell 1987, Woolley 1966). The seasonal secretory activity of the accessory reproductive tract often is correlated with high plasma testosterone concentrations, although in

macropods prostatic activity may continue throughout the year, even when testosterone levels are lower (Inns 1982, Jones 1989).

These findings suggest that the marsupial reproductive tract may be sensitive to local actions of androgens. In *A. stuartii* there appear to be local interactions between the Sertoli cells and Leydig cells that occur before the eightfold increase in plasma testosterone levels from the end of June (Bradley *et al.* 1980, Kerr and Hedger 1983, Taggart *et al.* 1992). Epididymal differentiation may be stimulated by small amounts of locally acting hormones before this surge of testosterone (Bradley *et al.* 1980, McDonald *et al.* 1981). The cellular hypertrophy accelerates with the further stimulation by testosterone. The prostate and bulbourethral glands would appear to be less sensitive to low plasma concentrations of testosterone, and appear to rely on the large concentrations produced later in the year to fully differentiate and reach full secretory potential.

An alternative explanation is that the epididymis and all parts of the testes are both relatively insensitive to the impact of testosterone and development is under endogenous control, where few external factors influence the initiation of local development but external factors, such as photoperiod, stimulate the later stages of the reproductive cycle. Underlying endogenous rhythms in the reproduction of *A. stuartii* have been implicated previously (Dickman 1985, McAllan *et al.* 1991, Scott 1986).

This may explain why the reproductive tissues contained within the scrotum begin to develop when plasma testosterone concentrations are low. In *A. stuartii,* when spermatogenetic activity is at its peak, the plasma testosterone levels are low. In mice and humans these concentrations would be associated with hypogonadism (Crawford *et al.* 1993, Handelsman 1990), and are associated with low testicular size and activity in other mammals (Curlewis 1991, Fuentes *et al.* 1993, Inns 1982, Kaplan and Mead 1993, Weinbauer and Nieschlag 1990). The development of the mature Leydig cell occurs early in life, although the proliferation of Leydig cells occurs before the cessation of spermatogenesis and before the plasma testosterone levels rise. Hypertrophy does occur in the epididymis following the cessation of spermatogenesis in the seminiferous tubules, but this is not as dramatic as in the bulbourethral glands or

the prostate. This finding suggests that the non-scrotal reproductive tissue is directly responsive to the actions of testosterone, and is similar to the results of studies on other marsupials where maximum plasma testosterone concentrations correspond with maximum accessory gland activity. This suggestion also correlates with the concept of endogenous, or spontaneous testicular development in *A. stuartii*. as the later surge of testosterone does not induce such an extreme hypertrophic response as occurs in the non-scrotal reproductive organs (Curlewis 1991, Curlewis and Stone 1985, Inns 1982, Todhunter and Gemmell 1987). The precise mechanism initiating testicular development in *A. stuartii* is yet to be determined. There are, however, clear seasonal changes in the epididymis, prostate, and bulbourethral glands that are associated with the breeding cycle and which correlate with the seasonal changes in plasma concentrations of testosterone reported by other studies.

One final observation, concerning the hypertrophy of the reproductive tract of *A. stuartii*, is that the changes in July and August correspond to the renal morphometric changes observed in the previous chapter. This would suggest that the physiological response to testosterone is systemic in male *A. stuartii*, with the sensitivity to androgens not just restricted to the "traditional" target organs, the reproductive tract, but extending to other organ systems. The next three chapters will deal with the elucidation of the pervasive effects of testosterone, and any interaction with cortisol.

Chapter 6

The Effects of Cortisol and Testosterone on Renal Function in Male *Antechinus stuartii*

6.1 Introduction

As discussed previously, *Antechinus stuartii* is a small carnivorous marsupial that undergoes a highly synchronised life history, which culminates in a brief mating period, of one to two weeks' duration, which is followed by complete male mortality before the young are born (Wood 1970, Woolley 1966). The male mortality is characterised by a failure of the glucocorticoid feedback mechanism, and the resulting excess of corticosteroids produces a cascade of stress related diseases. These include immunosuppression, gastric bleeding, parasitic invasion negative nitrogen balance, and a drop in plasma glucose levels (Barker *et al.* 1978, Barnett 1973, Bradley *et al.* 1980, Lee *et al.* 1977, McDonald *et al.* 1981, Woollard 1971).

Coincident with the high plasma cortisol concentrations are high plasma concentrations of testosterone (Bradley *et al.* 1980). The excessive amounts of circulating plasma cortisol clearly have a dramatic effect on the physiology of male *A. stuartii*, and the high steroid hormone concentrations may well contribute to alterations in other physiological systems including renal function.

The seasonal changes and sex differences in renal function were examined in Chapter 3. Glomerular filtration rate (GFR) in males was found to differ seasonally with a significant reduction in July and August, a time that other studies have found to be when plasma free testosterone and corticosteroid concentrations are high in the males (Bradley *et al.* 1980, McDonald *et al.* 1981). The GFR was also significantly different between sexes, with that of the females lowering only during the premating period in August. At other times when male GFR was low, the GFR of the females remained significantly higher. The urine electrolytes sodium, potassium and chloride did not change markedly, although urea concentration was higher in females than males and urine osmolality increased in July and

August. Plasma sodium and chloride concentrations increased in July and August and plasma potassium concentration decreased over the same period.

The changes in renal function were associated with alterations in kidney structure throughout the year (Chapter 4). While renal structure remained essentially unchanged in female *A. stuartii* across the yearly cycle, in males, significant morphometric changes occurred in July and August. Glomerular numbers decreased and the glomeruli themselves enlarged, the Bowman's capsule was lined by proximal tubule-like cells at the tubular pole, the proximal tubule hypertrophied and the distal tubule showed some tubular disturbance. In general, all tubular epithelial cells enlarged in males in July and August.

Cortisol and testosterone can cause changes in renal structure and function (Bardin and Catterall 1981, Baylis and Brenner 1978, Koibuchi *et al.* 1993, Oudar *et al.* 1991, Selye 1939). The changes induced by testosterone include higher proteinuria in intact male rats (Remuzzi *et al.* 1988, Sellers *et al.* 1950), more glomerular injury in intact males than in both castrates and in females that have been unilaterally nephrectomised (Sakemi and Baba 1993), and lower susceptibility to age dependent declines in renal function in females than in males (Munger and Baylis 1988). Cortisol affects the kidneys by inducing changes in sodium transport similar to the action of aldosterone on the distal tubule (Campen *et al.* 1983, Funder 1993, Funder *et al.* 1990).

In many wild mammals, crowding stress can significantly impact on survival, and one of the pathological changes observed in many species is renal failure. Woodchucks (*Marmota monax,* Christian *et al.* 1965), lemmings (*Lemmus trimucronatus,* Andrews *et al.* 1975) wild rats (*Rattus norvegicus*, Andrews *et al.* 1972), and long-haired rats (*R. villosissimus*, Barnett *et al.* 1975) all have large renal components in the initiation of death following prolonged stress. In the tree shrew (*Tupaia belangeri*), the renal failure and structural disintegration have been directly attributed to the actions of cortisol (von Holst 1972a).

The changes in GFR in male *A. stuartii* may be a response to the seasonal changes in plasma free hormones. Therefore, to determine whether testosterone or cortisol, or both, are causing a change in GFR, these hormones were administered to male *A. stuartii* at a time when

native free hormone levels are low. In May, plasma free concentrations of testosterone and cortisol are low and do not begin to rise until mid-June (Bradley *et al.* 1980). Sperm are not produced until early July and do not appear in the urine (spermatorrhoea) until after this time (Kerr and Hedger 1983, Woolley 1966). The GFR was measured in May before hormone administration and then approximately 4 weeks later. The exposure to concomitant high cortisol and testosterone levels resembles that seen in the wild. The aim of this chapter is to determine whether the seasonal changes in GFR can be mimicked by hormone administration, thus elucidating whether low GFR is induced by high plasma concentrations of testosterone or cortisol or a combination of both.

6.2 Materials and methods

6.2.1 Animals

Twenty-four male *A. stuartii* were trapped from the New England Tablelands, N. S. W. in late April (see Chapter 2 and Appendix II). In May, *A. stuartii* are adult body mass although they are sexually immature. *A. stuartii* were caged individually and provided with nest boxes with nesting material. They were given water *ad libitum* and fed with at least 20g of "Whiskas" tinned cat food just before dusk each day. Animals were housed under natural photoperiod at 22 ± 3°C.

6.2.2 Body mass

Body mass was measured on capture and weekly throughout the experiment, as an indication of condition. However, only body mass of animals prior to the first GFR measurement (initial captivity measurement) and final body masses are presented.

6.2.3 Determination of glomerular filtration rate

GFR was measured using the single injection method (Hall *et al.* 1977, Stacey and Thorburn 1966), as described in Chapter 2.

6.2.4 Haematocrit

Haematocrits were measured during the GFR experiments, both before and after hormone or saline injection.

6.2.5 Plasma samples and electrolytes

Plasma samples were collected after sacrifice and analysed for sodium and potassium as described in Chapter 2.

6.2.6 Urine and faecal collections

Urine and faecal samples were collected directly from the urogenital sinus when the animals were picked up for venipuncture and other handling procedures. All animals had urine and faeces collected in a specially manufactured escape-proof metabolic chamber placed over liquid paraffin (see Chapter 2). Animals were left overnight for a known time without food or water in these chambers. They were placed in the chamber just before dusk and released at least two hours after dawn the following morning. Activity recordings using infrared detectors were in place for another study and it was found that the period of urine collection encompassed the majority of the daily activity patterns for *A. stuartii* (Körtner, G. personal communication). Urine and faeces were frozen after retrieval and urine contaminated with faeces was discarded for electrolyte analysis after the urine volume was recorded. Overnight urine and faecal collections were made twice, before hormone administration, and approximately 4 weeks after hormone administration began.

6.2.7 Urine electrolytes

Urine potassium and sodium were analysed using a Corning 405 flame photometer with an internal lithium standard. Chloride was measured using a CMT10 chloride titrator. Urea was determined using the enzymatic UV test/GIDH method in a Cobas Bio Centrifugal analyser (Hoffman La Roche). Urine osmolality was measured using a Wescor 5500 vapour pressure osmometer (see Chapter 2). Total urine electrolytes excreted in the overnight collections were calculated by multiplying the concentration (mmol·L^{-1}) by the urine volume (L) to give total mmol. This was done for urinary sodium, potassium, chloride and urea.

6.2.8 Faecal samples

Faecal samples were weighed and then dried at 80°C until the mass stabilised. Percentage of faecal water was calculated from the

dry and wet masses. Further analyses of faecal electrolytes were not attempted because, while some information concerning electrolytic changes had been obtained during the seasonal studies, use of the ICP analyser was expensive and the information gained was not as helpful as other procedures in determining the physiological changes in *A. stuartii*.

6.2.9 Food and Water consumption

Food and water consumption were measured as an indirect indication of any metabolic changes. Food was weighed before placing in the cages and any left over at the following feeding time was weighed. A separate dish of food was weighed and placed into an empty cage at each feeding time and weighed at the following feeding time to determine the amount of desiccation occurring each day. An allowance for this was made in determining the daily food consumption. Food consumption was averaged every three days for each animal because of the disturbance to each animal of measuring GFRs and urine production. Water bottles were weighed each day to determine daily water consumption. Values were averaged for every three days.

6.2.10 Experimental Procedure

Animals were divided into four groups of six individuals. Animals caught at the same trap site were spread across groups to eliminate the possibility of siblings being in the same group. After 5 days of acclimatisation to captivity, the animals' GFR was measured and the urine was collected overnight within 1 or 2 days. Following GFR and urine collections, the animals were given an intramuscular injection of either saline, testosterone (150 $mg \cdot kg^{-1}$ "Durateston", injection of mixed testosterone esters, Intervet), cortisol (75 $mg \cdot kg^{-1}$ "Depredil", injection of methylprednisolone acetate, Ilium) or both testosterone and cortisol together. Methylprednisolone acetate was used because it is a potent glucocorticoid, with little mineralocorticoid action, similar to cortisol (Bennington 1984, Tepperman and Tepperman 1987). "Depredil" is a depot form of methylprednisolone acetate, acting over a two week period. "Depredil" was chosen for animal ethics considerations, following the guidlines of the Animal

Ethics and Care Committee, University of New England. In the next three chapters "cortisol administration" refers to the administration of "Depredil". The cortisol dosages were based on the data of Bradley *et al.* (1975), to give an estimated daily release of 5 mg·kg^{-1} per day. The testosterone dosages were based on data from McDonald *et al.* (1981) and Bradley *et al* (1980), to give an estimated dose of 10 mg·kg^{-1} per day. A second injection was given 15 days later. Between 25 and 30 days after the initial hormone injections, the GFRs and urine collections were again performed on the animals. Two cortisol treated individuals died before the experiment was completed, and one cortisol plus testosterone individual was sacrificed because of a deterioration of its condition. Another cortisol plus testosterone individual escaped and was not recaptured.

6.2.11 Statistical methods

All preinjection data were subtracted from post-injection data and two-way analyses of variance were used with presence and absence of testosterone and presence and absence of cortisol as separate groupings. These were followed by a Fisher PLSD test (Haycock *et al.* 1992, Zar 1984). Percentage data were arcsine transformed before analysis (Zar 1984). Where the assumption of equal variances was not met, data were transformed to give equal variances and then analysed (Zar 1984). However means ± standard errors of untransformed data are presented in the results.

Food and water consumption data were analysed by two-way analysis of variance with time and treatments as the variables. These were followed by a Fisher PLSD (Haycock *et al.* 1992, Zar 1984). Data presented in tables as pre-injection means ± standard errors of the mean include all individuals, and post-injection means ± standard errors of the mean include all animals alive at the end of the experiment. The difference data do not include the pre-injection data from individuals who escaped or died before the post-injection measurements could be obtained.

6.3 Results

6.3.1 General description

On capture the external appearance of all males was similar and all were similar to the males captured in May in the seasonal study. Animals had well-groomed fur, continuous over the whole body and the tendons of the tail were intact. All animals were in good health although many had external parasites.

Treatment with cortisol only or testosterone plus cortisol caused some individuals initially to acquire an accumulation of fat around their lower abdomen. Later in the experiment the weight accumulation was lost in most individuals and, on dissection, if present, the subcutaneous material had a watery consistency. The males treated with testosterone plus cortisol appeared larger than other males although, on sacrifice, there was little internal evidence of fat. Usually, overweight *A. stuartii* accumulate fat subcutaneously around the abdomen or intra-abdominally. In the testosterone only males there was little evidence of fat on dissection. Some individuals exhibited fur loss and/or brittle tail tendons. In some individuals the spleen was enlarged, and, in many individuals, parasites were observed in the liver, lungs or gut. Animals that died, or were killed before the completion of the experiment, had ulcerated and bleeding gastrointestinal tracts and blood in their urine.

There were no pathological changes in males treated with saline, although liver parasites were observed in two individuals. In testosterone only treated males some fur loss and extensive parasitism in the liver was observed in one individual, whereas all others were indistinguishable from the saline treated males.

Changes in the external reproductive tract will be discussed in Chapter 8.

6.3.2 Body mass

There were significant differences in body masses of groups after treatment with testosterone (testosterone $P=0.01$). Testosterone significantly increased body mass although the addition of cortisol to testosterone administration appeared to reduce the effect of testosterone (cortisol $P=0.087$, interaction $P=0.16$, Figure 6.1a, Table 6.1).

Table 6.1 Body mass (g) and haematocrit (%) in *A. stuartii* before and 25-30 days after hormone or saline injection. Plasma electrolytes ($mmol \cdot L^{-1}$) and osmolality ($mosm \cdot kg^{-1}$) are from animals at the end of the experiment. Data are means ± standard errors of the mean. Pre-injection means include all individuals, including animals who died or escaped before the post-injection values were collected. Significance levels at the base of the columns are for two-way ANOVAs performed on the differences between post-injection and pre-injection data.

	Body mass (g) pre-injection	Body mass (g) post-injection	Haematocrit (%) pre-injection	Haematocrit (%) post-injection	Plasma sodium ($mmol \cdot L^{-1}$)	Plasma potassium ($mmol \cdot L^{-1}$)	Plasma chloride ($mmol \cdot L^{-1}$)	Plasma Osmolality ($mosm \cdot kg^{-1}$)
Saline	25.8 ± 1.6	29.7 ± 1.2	50.9 ± 1.9	52.5 ± 0.9	122.5 ± 3.6	12.9 ± 0.9	115.1 ± 2.2	338.2 ± 7.9
Testosterone	26.7 ± 3.7	38.7 ± 1.7	49.3 ±1.4	59.1 ± 1.3	113.6 ± 5.0	11.4 ± 0.5	112.3 ± 2.5	330.6 ± 6.2
Cortisol	28.7 ± 2.7	29.5 ± 2.2	48.2 ± 1.6	47.9 ± 5.7	129.8 ± 9.6	12.8 ± 1.9	110.8 ± 1.8	328.8 ± 11.4
Cortisol and Testosterone	26.9 ± 1.1	31.9 ± 2.3	49.4 ± 1.5	49.3 ± 2.5	133.5 ± 9.8	18.2 ± 3.6	104.9 ± 2.8	332.3 ± 9.6
Testosterone	P=0.01		NS		NS	NS	P<0.09	NS
Cortisol	P=0.087		P=0.14		P=0.06	P<0.05	P<0.03	NS
Interaction	P=0.16		NS		NS	P<0.05	NS	NS

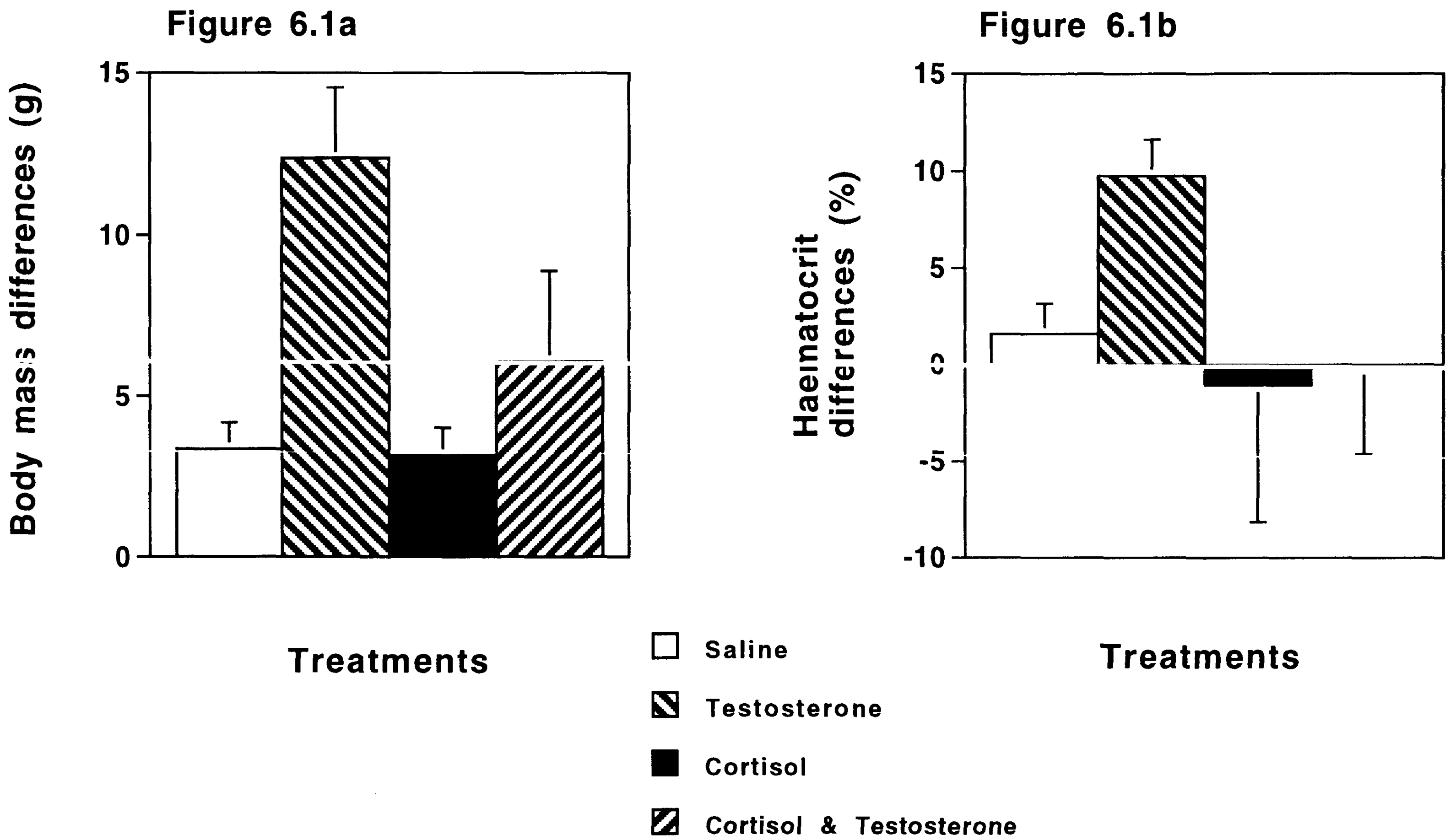


Figure 6.1 a) Differences in body mass (g) between preinjection and post-injection times in treatment groups and b) Differences in haematocrit (%). Values are means ± standard errors of the mean.

6.3.3 Glomerular filtration rate

GFRs for all animals prior to the hormone treatments were not significantly different from values collected from animals normally collected in May (see Chapter 3). Preinjection GFRs were not significantly different from one another. The differences in GFR showed a significant decrease between pre-injection and post-injection measurements in animals treated with testosterone ($P<0.02$, Figure 6.2, Table 6.2). Cortisol treatment did not not affect GFR (Figure 6.2, Table 6.2).

6.3.4 Haematocrit

Differences in haematocrit between pre-injection and post-injection values were not statistically significant between groups, (Figure 6.1b, Table 6.1).

6.3.5 Plasma electrolytes

Plasma sodium tended to be higher in both groups treated with cortisol, although this was not statistically significant (cortisol $P=0.06$, Figure 6.3a, Table 6.1). Plasma potassium was significantly higher in the animals treated with cortisol and testosterone but did not differ in the other groups (testosterone NS, cortisol $P<0.05$, interaction $P<0.05$, Figure 6.3b, Table 6.1). Plasma chloride was significantly different between treatment groups (testosterone $P<0.09$, cortisol $P<0.03$, interaction NS). Cortisol administration significantly lowered plasma chloride concentrations (Table 6.1, Figure 6.4a). Plasma osmolality did not differ between treatment groups (Table 6.1, Figure 6.4b).

6.3.6 Urine and faecal measurements

Urine volumes excreted overnight increased in animals treated with cortisol alone, although this was not significant (testosterone NS, cortisol $P=0.12$, interaction NS, Figure 6.5a, Table 6.2). Faecal percentage water content increased or was unchanged in all groups, although there were no significant differences between groups (testosterone $P=0.16$, Cortisol NS, interaction NS, Figure 6.5b, Table 6.2).

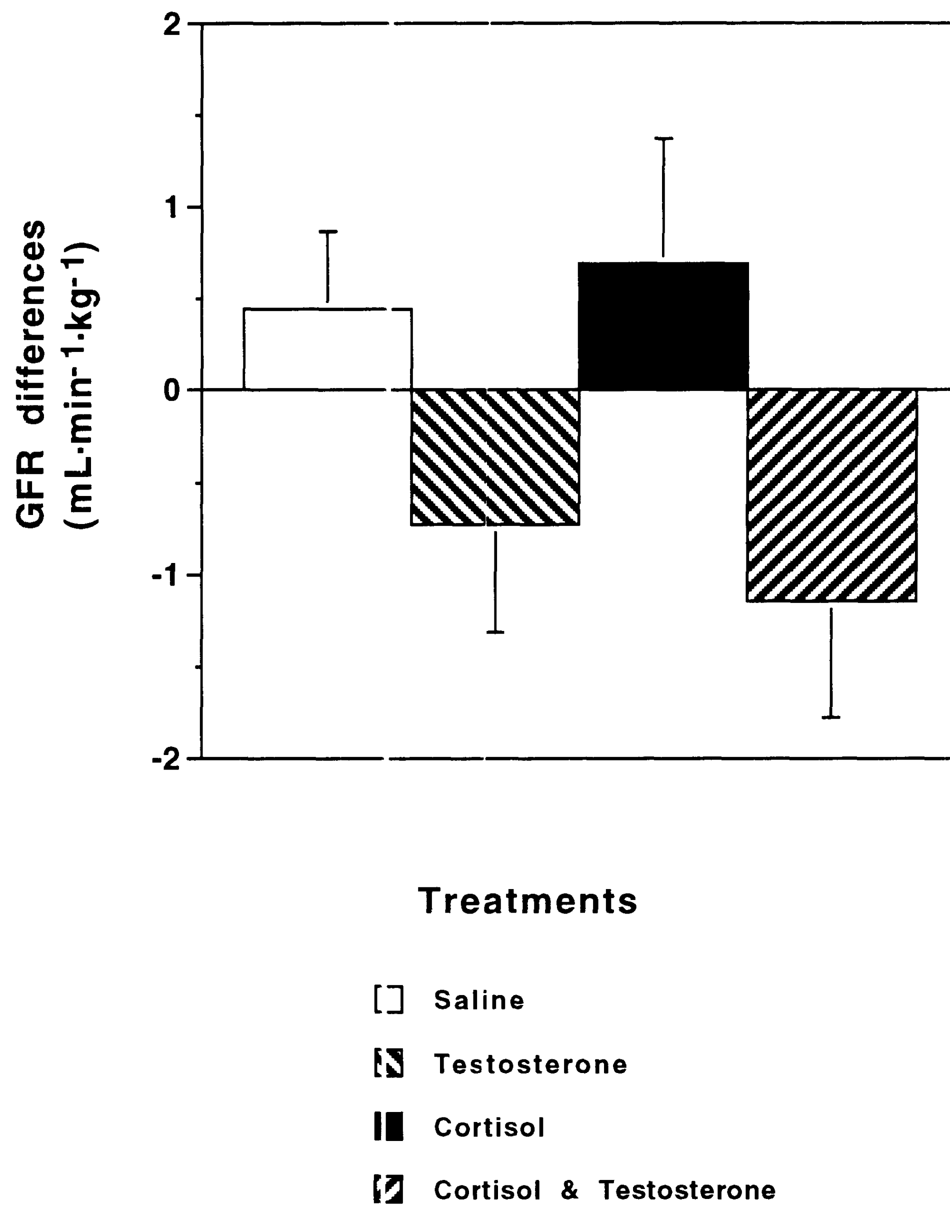
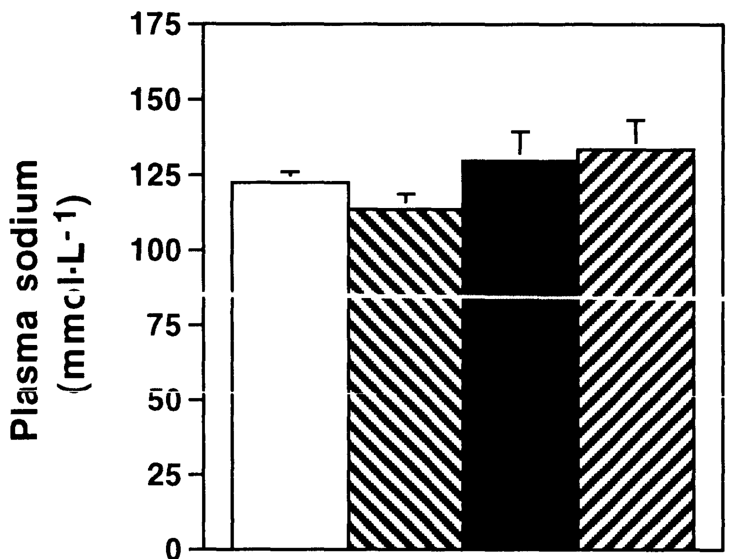


Figure 6.2. Differences in GFR (mL·min^{-1}·kg^{-1}) between treatment groups. GFR differences are post-injection GFR minus pre-injection GFR of each individual expressed as means ± standard errors of the mean.



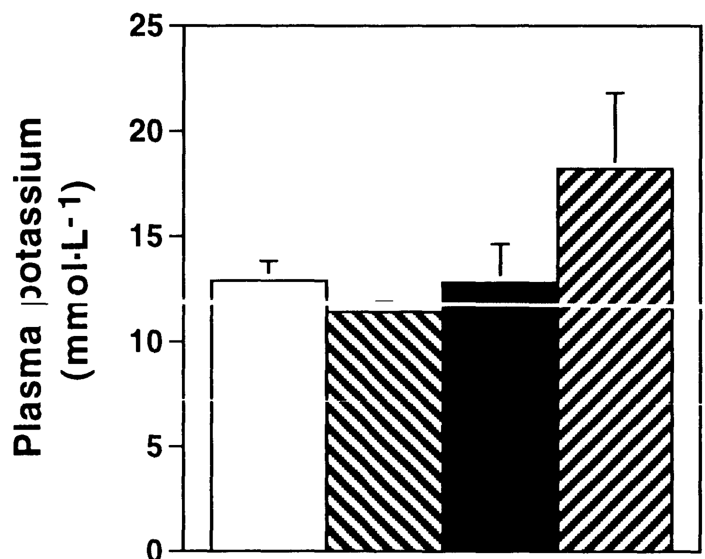
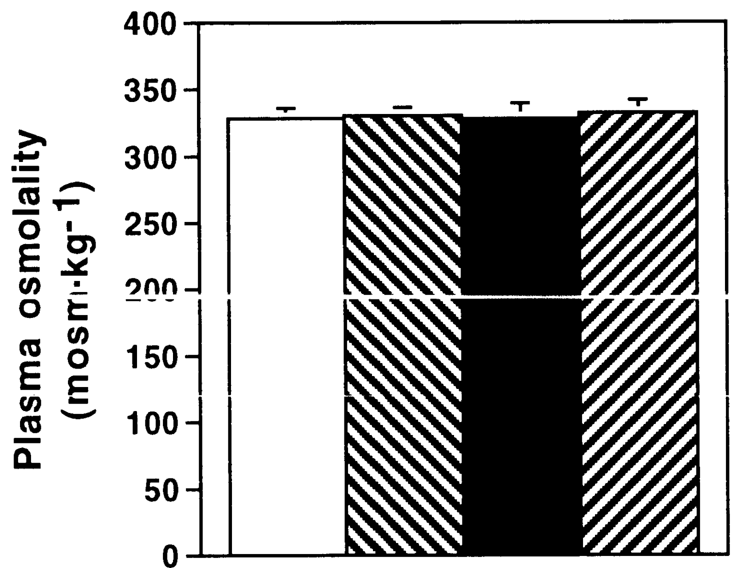
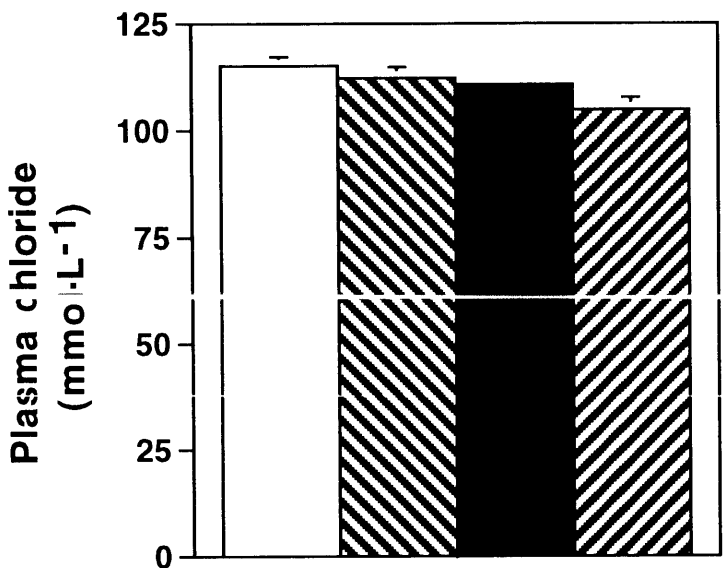


Figure 6.3 a) Plasma sodium concentration ($mmol{\cdot}L^{-1}$) and b) plasma potassium concentration ($mmol{\cdot}L^{-1}$). Values are means ± standard errors of the mean.





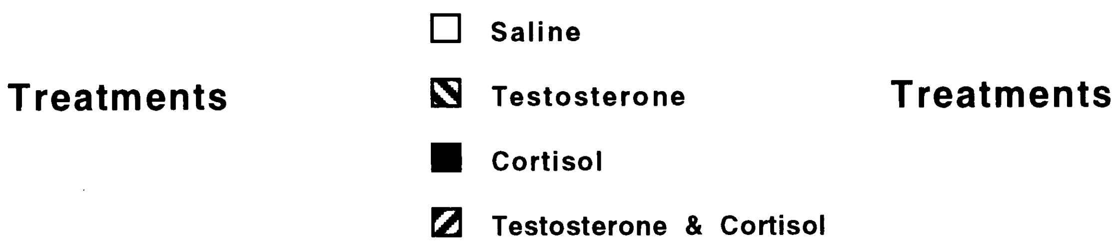


Figure 6.4 a) Plasma chloride concentration (mmol·L^{-1}) and b) plasma osmolality (mosm·kg^{-1}). Values are means ± standard errors of the mean.

Table 6.2. GFR ($mL \cdot min^{-1} \cdot kg^{-1}$), urine volumes (mL), and faecal water content (%) in *A. stuartii* before and 25-30 days after hormone or saline injection. Data are means ± standard errors of the mean. Pre-injection means include all individuals, including animals that died or escaped before the post-injection values were collected. Significance levels at the base of the columns are for two-way ANOVAs performed on the differences between post-injection and pre-injection data.

	GFR ($mL \cdot min^{-1} \cdot kg^{-1}$) pre-injection	**GFR ($mL \cdot min^{-1} \cdot kg^{-1}$) post-injection**	**Urine volumes (mL) pre-injection**	**Urine volumes (mL) post-injection**	**Faecal water content (%) pre-injection**	**Faecal water content (%) post-injection**
Saline	3.84 ± 0.47	4.28 ± 0.16	0.44 ± 0.06	0.32 ± 0.05	59.1 ± 7.4	74.8 ± 7.7
Testosterone	4.65 ± 0.27	3.93 ± 0.41	0.50 ± 0.09	0.45 ± 0.13	64.0 ± 8.7	66.8 ± 13.4
Cortisol	3.29 ± 0.30	3.79 ± 0.73	0.46 ± 0.07	0.76 ± 0.37	68.8 ± 5.5	80.4 ± 2.8
Testosterone plus Cortisol	3.96 ± 0.42	2.97 ± 0.48	0.59 ± 0.08	0.69 ± 0.19	71.0 ± 6.8	73.9 ± 5.3
Testosterone	$P < 0.02$		NS		$P = 0.16$	
Cortisol	NS		$P = 0.12$		NS	
Interaction	NS		NS		NS	

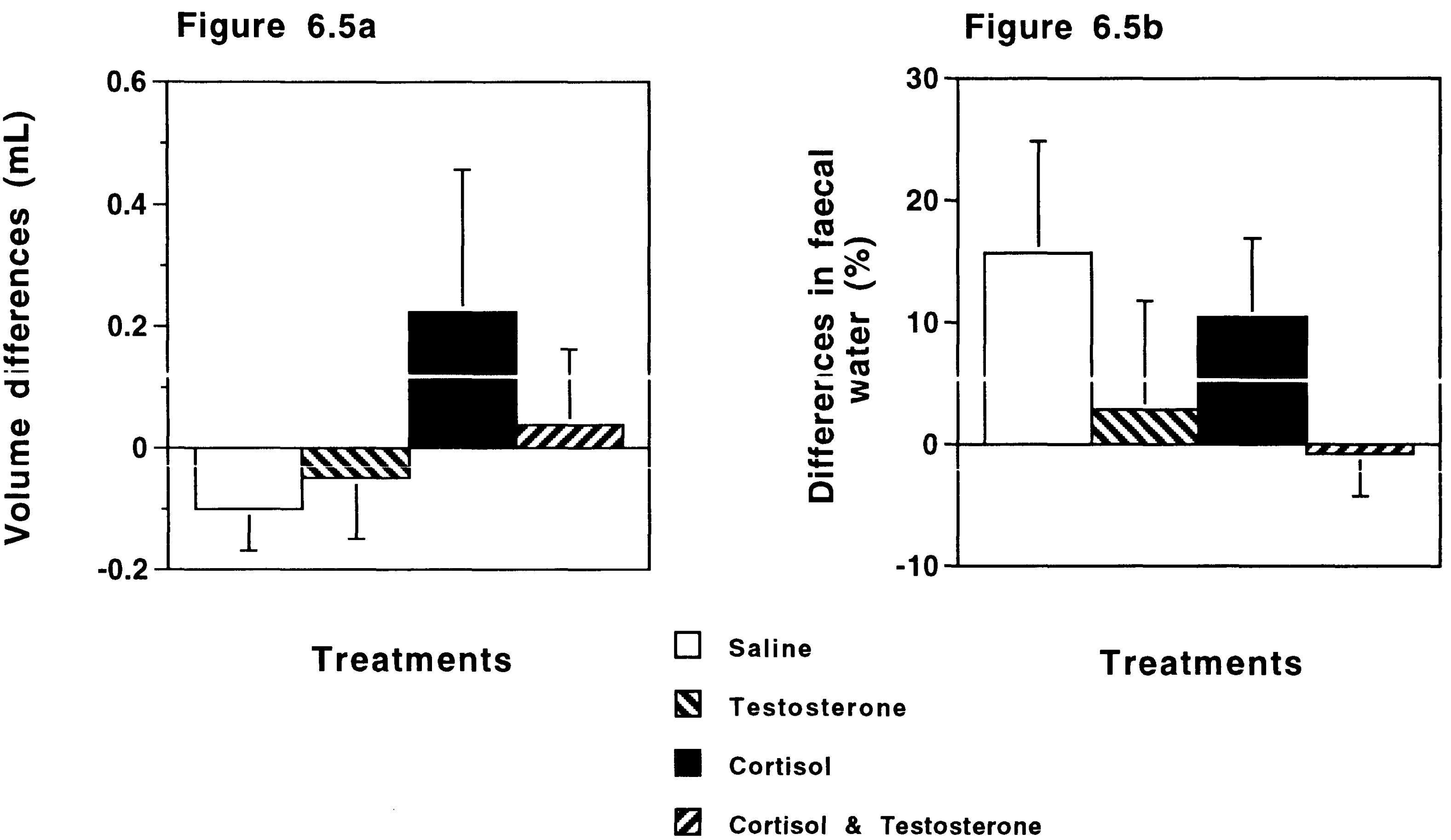


Figure 6.5. Differences between post-injection and pre-injection values for a) urine volumes (mL) and b) faecal water (%) collected overnight. Values are means ± standard errors of the mean.

6.3.7 Urine analysis

There were significant differences between pre-injection and post-injection concentrations for urinary sodium, potassium, chloride, and urea concentrations and urine osmolality (Figure 6.6a, 6.6b and 6.6c, Figure 6.7a and 6.7b, Table 6.3). In animals from both groups treated with cortisol, urinary sodium, chloride and osmolality decreased significantly (cortisol, at least P<0.01), but this effect was reversed by the addition of testosterone (testosterone NS, interaction, at least P<0.01, Figures 6.6a and b, 6.7a). Urinary potassium concentration decreased significantly in animals treated with cortisol (cortisol P<0.01, testosterone NS, interaction NS, Figure 6.6c). Urinary urea concentration decreased with cortisol treatment but this was negated by the addition of testosterone treatment to that of cortisol (cortisol NS, testosterone NS, interaction P<0.01, Figure 6.7b). None of the differences between pre-injection and post-injection values of total electrolytes or urea excreted were significantly different between groups (Table 6.4).

6.3.8 Food and water consumption

Water consumption changed significantly over the course of the experiment (time P<0.005, Figure 6.8), although no clear trend was observed from the post-hoc tests. Water consumption was significantly higher in cortisol only and cortisol plus testosterone treated animals than saline and testosterone only treated animals (treatment P<0.0001, interaction NS, Fisher's PLSD test P<0.0002, Figure 6.8). Water consumption of testosterone only males was significantly higher than the saline treated males (Fisher's PLSD test P<0.01, Figure 6.8). Food consumption varied significantly in all animals over the period of the experiment (time P<0.0001, Figure 6.9). However the food consumption of testosterone only animals was significantly higher than all other treatments (treatments P=0.01, interaction NS, Fishers PLSD test P<0.008, Figure 6.9).

6.4 Discussion

Glomerular filtration rate in male *A. stuartii* decreased as a result of testosterone administration. The seasonal change in GFR observed in natural populations occurred at a time when endogenous

Table 6.3 Urine electrolytes (mmol·L^{-1}), osmolality (mosm·kg^{-1}), and urea (mmol·L^{-1}) in *A. stuartii* before and 25-30 days after hormone or saline injection. Data are means ± standard errors of the mean. Pre-injection means include all individuals, including animals that died or escaped before the post-injection values were collected. Significance levels at the base of the columns are for two-way ANOVAs performed on the differences between post-injection and pre-injection data.

	Urine sodium (mmol·L^{-1}) pre-injection	**Urine sodium (mmol·L^{-1}) post-injection**	**Urine potassium (mmol·L^{-1}) pre-injection**	**Urine potassium (mmol·L^{-1}) post-injection**	**Urine chloride (mmol·L^{-1}) pre-injection**	**Urine chloride (mmol·L^{-1}) post-injection**	**Urine osmolality (mosm·kg^{-1}) pre-injection**	**Urine osmolality (mosm·kg^{-1}) post-injection**	**Urine urea (mmol·L^{-1}) pre-injection**	**Urine urea (mmol·L^{-1}) post-injection**
Saline	99.0 ± 14.8	171.0 ± 9.2	136.0 ± 33.9	209.3 ± 18.9	136.8 ± 33.5	148.3 ± 25.3	1819 ± 142	2770 ± 166	4175 ± 372	4843 ± 99
Testosterone	127.8 ± 19.9	136.7 ± 30.2	118.0 ± 23.7	178.8 ± 48.0	138.0 ± 32.1	145.0 ± 36.9	1962 ± 239	2079 ± 263	4370 ± 557	4198 ± 353
Cortisol	169.8 ± 13.0	85.3 ± 13.2	211.8 ± 11.7	122.8 ± 26.2	169.2 ± 13.4	69.2 ± 9.8	2425 ± 124	1176 ± 227	4947 ± 269	3486 ± 384
Testosterone plus Cortisol	117.0 ± 24.1	108.0 ± 12.5	147.5 ± 28.9	131.5 ± 4.9	143.5 ± 29.5	146.5 ± 20.7	1849 ± 311	1329 ± 145	3401 ± 357	3557 ± 300
Testosterone	NS		NS		P=0.18		NS		NS	
Cortisol	P<0.002		P<0.01		P<0.01		P<0.01		NS	
Interaction	P<0.005		NS		P<0.01		P<0.01		P<0.01	

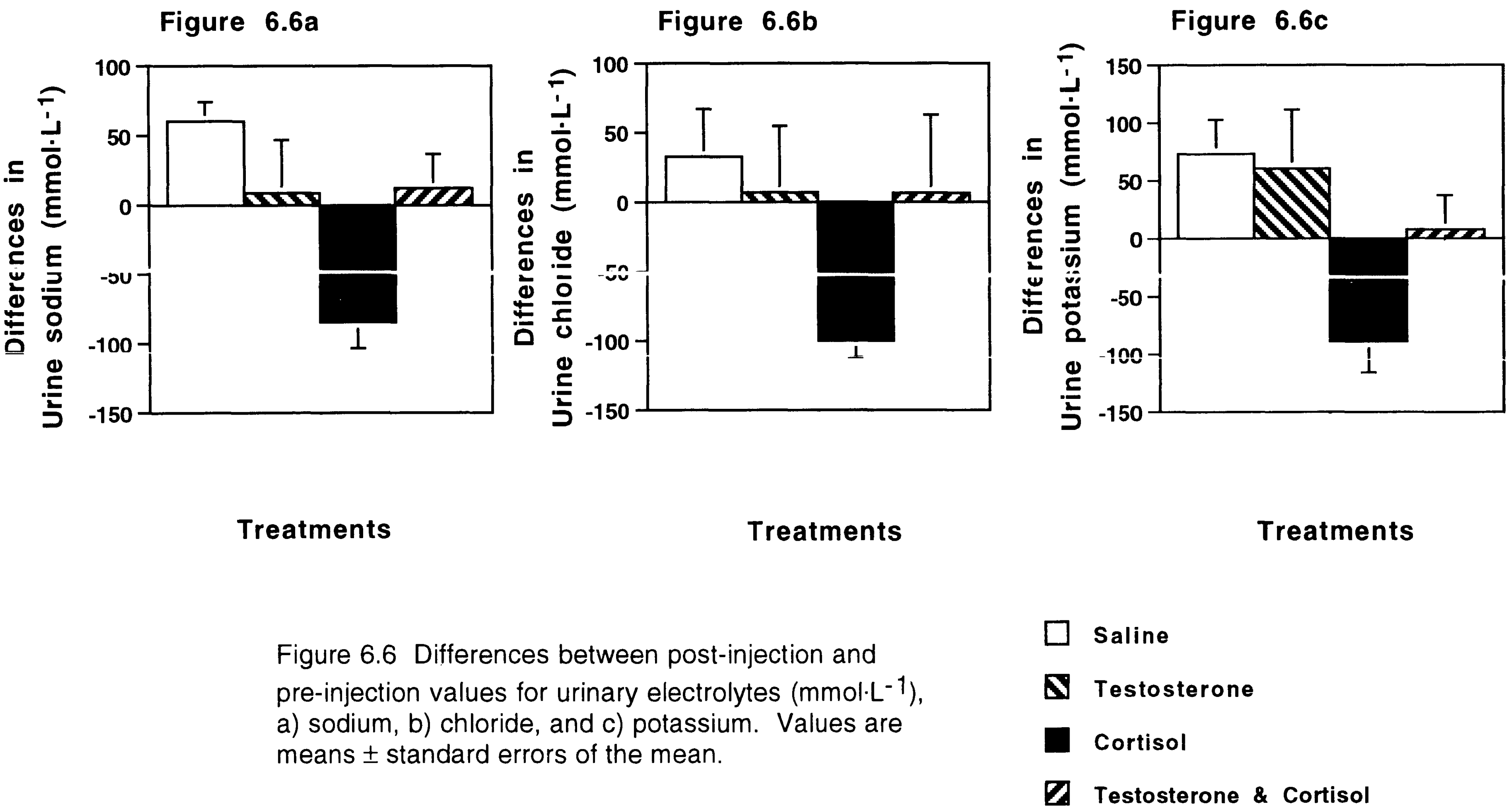
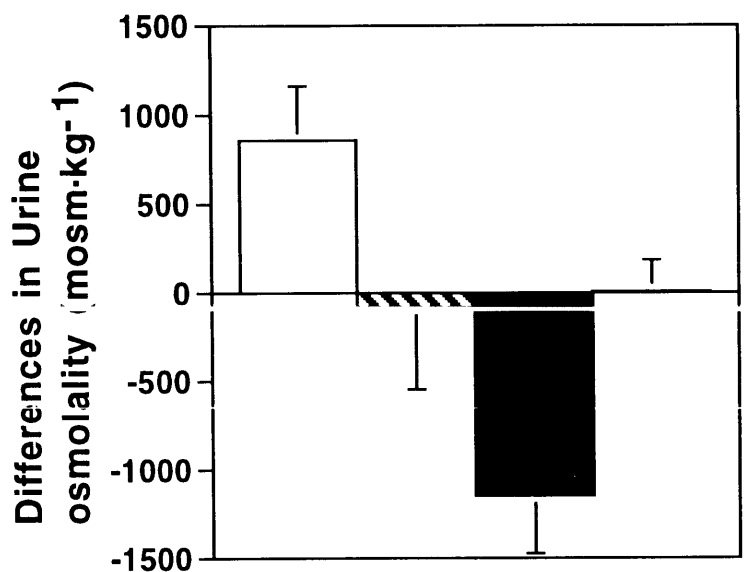


Figure 6.6 Differences between post-injection and pre-injection values for urinary electrolytes (mmol·L^{-1}), a) sodium, b) chloride, and c) potassium. Values are means ± standard errors of the mean.



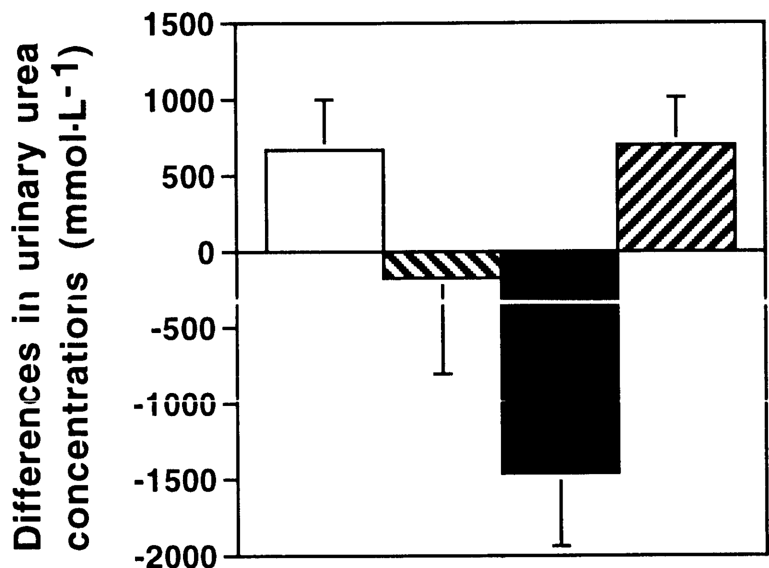


Figure 6.7 Differences between post-injection and pre-injection for a) urinary osmolality (mosm·kg^{-1}) and b) urinary urea (mmol·L^{-1}). Values are means ± standard errors of the mean.

Table 6.4 Total electrolytes and urea excreted (μmol) overnight in *A. stuartii*. Data are means ± standard errors of the mean. Pre-injection means include all individuals, including animals who died or escaped before the post-injection values were collected. Significance levels at the base of the columns are for two-way ANOVAs performed on the differences between post-injection and pre-injection data.

	Urine sodium (μmol) pre-injection	Urine sodium (μmol) post-injection	Urine potassium (μmol) pre-injection	Urine potassium (μmol) post-injection	Urine chloride (μmol) pre-injection	Urine chloride (μmol) post-injection	Urine urea (μmol) pre-injection	Urine urea (μmol) post-injection
Saline	42.2 ± 7.1	52.0 ± 5.9	57.4 ± 12.0	56.6 ± 6.3	36.7 ± 9.0	42.4 ± 7.3	1858 ± 331	1507 ± 222
Testosterone	59.1 ± 12.9	71.6 ± 20.4	59.0 ± 18.0	92.0 ± 28.8	63.2 ± 14.8	74.2 ± 24.5	2039 ± 297	1773 ± 509
Cortisol	68.2 ± 10.1	53.9 ± 16.3	96.6 ± 15.2	82.9 ± 23.0	77.5 ± 10.5	51.5 ± 21.2	2050 ± 368	2543 ± 932
Testosterone plus Cortisol	69.8 ± 14.2	69.0 ± 13.6	78.0 ± 7.7	90.8 ± 24.7	77.3 ± 11.8	105.3 ± 37.0	1934 ± 231	2352 ± 522
Testosterone	NS		NS		NS		NS	
Cortisol	NS		P=0.14		NS		NS	
Interaction	NS		NS		NS		NS	

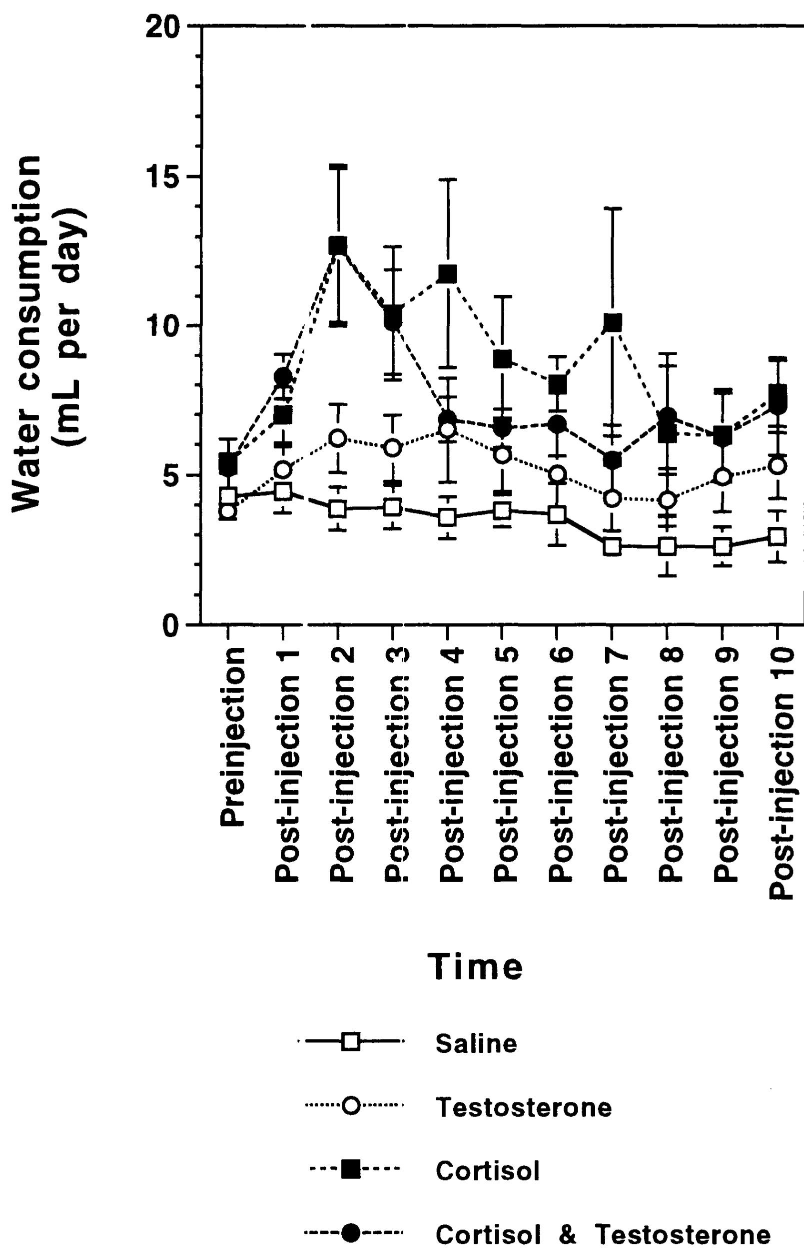


Figure 6.8. Water consumption (mL drunk per day) throughout the course of the experiment. Each time period represents the average of three days consumption, with one pre-injection period and 10 post-injection periods. Data are the mean and standard errors of the mean.

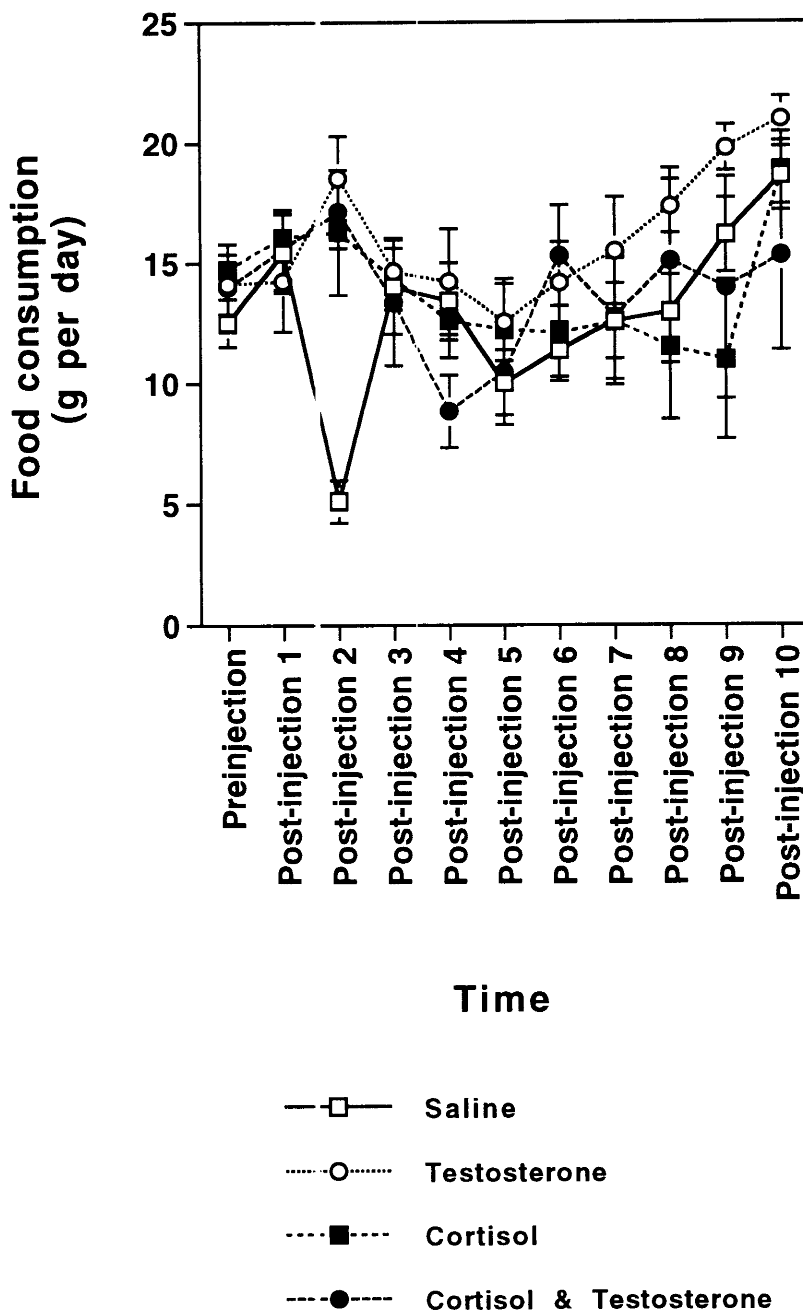


Figure 6.9. Food consumption (g eaten per day) throughout the course of the experiment. Each time period represents the average of three days consumption, with one pre-injection period and 10 post-injection periods. Data are means and standard errors of the mean.

testosterone was also known from other studies to be high. This suggests that the significant decrease in GFR observed in these populations is a result of high testosterone levels. In other mammals, GFR decreases in animals exposed to water stress (Bakker and Bradshaw 1983, Denny and Dawson 1977, Grenot 1992) and a decline in GFR has been observed in aging mammals (Corman *et al.* 1985, Haley and Bulger 1983). The age-related decline in GFR is exacerbated in males (Munger and Baylis 1988).

However most research on the effects of testosterone on renal factors has concentrated on structural changes. Testosterone causes hypertrophy rather than hyperplasia in the kidney (Bardin and Catterall 1981). The hypertrophy observed in testosterone treatment of castrate animals or females does not appear to be localised, with the glomeruli, proximal and distal tubules, and enzymatic gene expression all responding to testosterone (Baik *et al.* 1992, Catterall *et al.* 1986, Crabtree 1941b, Corman *et al.* 1985, Daigeler 1981, Mills and Bardin 1980, Oudar *et al.* 1991, Scheibler and Danner 1978, Selye 1939). Few renal function studies have accompanied the structural studies, and those that have suggest a complex series of events that are not easily separated from the effects of aging on the kidney (Bardin and Catterall 1981, Corman *et al.* 1985).

In the present study, there also were effects of cortisol on the renal function in *A. stuartii*. The concentration of electrolytes and urea in urine decreased with cortisol treatment, although this was frequently reversed by the addition of testosterone to the cortisol treatment. Lower osmolalities were observed in animals treated with cortisol only, and this, too, was reversed by the addition of testosterone to the cortisol administration. The changes in electrolyte concentrations contrast with the seasonal study, where no significant changes were observed. This was, in part, due to the incomplete collections of urine samples over longer periods of time in the seasonal study. Sampling of urine in specially sized metabolic chambers overnight was not always available in the seasonal study and many of the earlier collections were based on urine collected upon handling of the animals during the GFR experiments. While urine volume was measured, complete excretion over longer periods of time was not known. This accounts for many of the differences between the

seasonal and the hormone studies in the total electrolyte excreted data. The method employed in the present study has enabled a better estimation of urine electrolyte excretion over time.

While mindful of the limitations of the seasonal data, it is noted that many of the effects of cortisol on urinary electrolytes were negated by the addition of testosterone. The urinary concentrations of electrolytes in cortisol and testosterone treated males were similar to those found in males in July and both sampling periods in August (see Tables 3.2 and 3.3). It would appear that, while administration of cortisol only produces copious amounts of dilute urine, the addition of testosterone to cortisol administration, with its effect of slowing glomerular filtration, allows more water and solutes to be resorbed by the tubules.

The interaction of testosterone and cortisol can also be seen in some of the other parameters. The addition of cortisol to testosterone treatment accentuated the drop in GFR (see Figure 6.2). Urine volume, while not statistically significant, showed a positive mean difference in the testosterone and cortisol group, although this was much lower than in the cortisol only group (Figure 6.5a).

Moreover, the increase in haematocrit in testosterone only treated males was not observed with the addition of cortisol, although the large variation in both the cortisol treated groups precluded any consistent findings. The haematocrit values in the seasonal study increased significantly in males in July and August (see Table 3.1). This increase contrasts with another study where a drop in haematocrit was observed just before death in the males (Barker *et al.* 1978). While males in the seasonal study were sacrificed before their natural death occurred, some of the males in the present study were measured in the throes of the induced "die-off" phenomenon, thus contributing to the within group variation. Indeed, two males died before the completion of the experiment. The ailing males, like those described by Barker *et al.* (1978) and Moore (1974), had evidence of gut ulceration and bleeding, low haematocrits, and opportunistic growth by parasites in the lungs, gut and liver on autopsy. Most of the males in the seasonal study were in good general health, even though massive proliferation of hookworms was frequently observed on autopsy, and worms were sometimes seen exuding from the

urogenital sinus on handling. It would appear that the higher haematocrit values observed in the seasonal study are mimicked by treatment with testosterone (a 10% increase was observed), although the final drop observed in the testosterone and cortisol males and in other studies, would most likely be induced by the elevated cortisol levels causing gut ulceration and bleeding.

The interactions between cortisol and testosterone are also seen in the general indicators of metabolic rate, food and water consumption. Food consumption increased over time in all groups. However, in testosterone only animals, this increase was significantly higher than for all other groups. Addition of cortisol appeared to cancel out this effect of testosterone. The food consumption of the saline, cortisol only, and testosterone plus cortisol groups of *A. stuartii* were similar to laboratory values found by Nagy *et al.* (1978), whose animals consumed 37% of their own body mass in food each day. However, the testosterone treated animals consumed about 50% of their own body mass in food daily, similar to the field data collected in July (pre-mating) by Nagy *et al.* (1978). This suggests that the metabolic effects of testosterone only treatment are similar to those found in untreated *A. stuartii* under pre-mating conditions.

The metabolic effects of cortisol plus testosterone are reflected in the changes in body masses, where body mass increased in all groups over the course of the experiment, but testosterone only treated animals were significantly larger than all other groups (see Figure 6.1a). Addition of cortisol appeared to negate the anabolic effects of testosterone. In the seasonal study, there were no body mass changes after May, when all males had reached adult body mass (see Table 3.6). Testosterone administration alone produced an inflated body mass compared to other treatments, although this was not higher than the normal mean body mass of male *A. stuartii* in August.

The effect of hormone treatment on body mass and food consumption contrasts to the combined effects of testosterone and cortisol on water consumption. Water consumption remained stable throughout the experiment for both saline and testosterone only groups, although there was a sharp increase in water consumption in both groups treated with cortisol. The consumption in the cortisol only

treated animals remained higher than all groups for most of the experiment, but dropped from an early peak in the animals treated with both testosterone plus cortisol. The water consumption of the testosterone plus cortisol group still remained significantly above the saline and testosterone only groups, although the addition of testosterone reduced the effect of cortisol.

The water consumption of the saline treatment animals was similar to that of *A. stuartii* studied by Blair-West *et al.* (1983), where 4.3 ± 0.4 mL were drunk over a 24 hour period. Blair-West *et al.* (1983) found that *A. stuartii* was insensitive to the administration of angiotensin II in inducing the thirst mechanism. The study of Blair-West *et al.* (1983) was performed when the animals were more than 9 months old, as in the present study, well before the period of acute glucocorticoid activity. However, the present study does demonstrate a cortisol induction of the thirst mechanism in *A. stuartii*. Nagy *et al.* (1978) discussed the high water fluxes found in *A. stuartii* in August, and believed them to be the result of drinking excess rain water available at that time as the high intake was not needed to maintain water balance. It is possible that the high water fluxes are the result of cortisol action in wild *A. stuartii*.

McDonald *et al.* (1986) demonstrated unequivocally that the "die-off" phenomenon was the result of a failure of the glucocorticoid feedback mechanism in this genus. Other studies have found that adrenalectomised marsupials require only cortisol or deoxycortisone to maintain plasma electrolytes, and aldosterone administration only does not redress the fall in plasma sodium concentration (Janssens and Hinds 1981, Reid and McDonald 1968a). In the present study, plasma sodium concentrations rose in both groups treated with cortisol, and the urine concentrations of sodium dropped in the cortisol only group. Plasma potassium concentration rose in testosterone and cortisol treated animals, although urinary potassium concentration decreased in both cortisol treated groups. This suggests that cortisol administration was affecting renal handling of sodium and potassium, although the addition of testosterone to cortisol administration indicated that the ion exchange does not appear to be simply a distal nephron uptake of sodium and excretion of potassium.

The raised plasma potassium concentrations in cortisol and testosterone treated animals contrast with the seasonal study, where the plasma potassium concentrations were lower and plasma sodium concentrations were higher in July and August than February and May (see Table 3.6). Cortisol affects the plasma sodium concentrations by elevating them, much as would be expected if renal mineralocorticoid receptors were activated by excess cortisol (Funder 1993, Funder *et al.* 1990). In the present study, however, the results of hormone treatment were equivocal, with raised plasma potassium concentrations in cortisol and testosterone treated animals and raised sodium concentrations in both cortisol treated groups.

The concentrations of plasma potassium, however, were very high, with the normal eutherian parameters (Guyton and Hall 1996) and the values for *A. stuartii* found by a previous study (up to a mean of 6.05 mmol·L^{-1}, Barnett 1973) being far exceeded by the present study. In the study of Barnett (1973) sodium values dropped significantly prior to male death but this was not found in either the seasonal or the hormonal studies. The differences between the present studies and that of Barnett (1973) are not easy to reconcile.

The raised plasma potassium concentrations may be due to the method of collection, where contamination with intracellular fluid may contribute to the plasma values. However, some mammalian studies, where samples were obtained by other bleeding methods, have recorded high plasma concentrations of potassium, some of which are as high as those found for the saline, testosterone only and cortisol only treated animals (Bakko 1975, Buffenstein 1984, Collins 1978, Purohit 1971, Urison and Buffenstein 1994, and see Table 3.7). Many of the large changes observed in plasma electrolyte concentrations are a response to water restriction or diet changes (Bakker and Bradshaw 1983, 1989, Vogel and Vogel 1972, Yaakobi and Shkolnik 1974). However, the higher values in the large range in plasma potassium found by Bakko (from 4.4-27.0 mmol·L^{-1} 1975, from 5-18.2 mmol·L^{-1} 1977) are not dissimilar to the present study. The low plasma sodium concentrations and high plasma potassium concentrations measured in the present study are more likely to be a result of the method of collection, as the values obtained from the orbital sinus by Barnett (1973) were closer to the eutherian norm. However, while

there may be contamination from the intracellular space causing inflated absolute concentrations, the significant alterations to these concentrations as the result of hormone treatments cannot be discounted.

Normally hyperaldosteronism produces an increase in fluid retention along with the sodium (Guyton and Hall 1996). However, after maximum retention the increase in blood pressure causes a pressure diuresis and natriuresis, returning plasma sodium levels closer to normal (Guyton and Hall 1996). In the cortisol only treated group diuresis was observed, although this was not seen in the testosterone plus cortisol group. The combination of cortisol plus testosterone appeared to produce a paradoxical response, low GFR with sodium and potassium retention. In other mammals, GFR usually rises with cortisol treatment, and plasma potassium concentration drops as it is exchanged for sodium (Baylis and Brenner 1978, Marver 1992). There are sex differences in GFR and renal structure, with GFRs higher and more labile in males than females (Bardin and Catterall 1981, Crabtree 1941b, Munger and Baylis 1988, Remuzzi *et al.* 1988, Selye 1939). The low GFRs found in both testosterone treated groups is contrary to that finding. Moreover, the lower GFR induced by testosterone administration is not ameliorated by cortisol administration. Normally glucocorticoid excess induces an increase in GFR (Baylis and Brenner 1978). This suggests, that in *A. stuartii,* any effects of cortisol are secondary to that of testosterone and are an adjunct to the effects of testosterone.

Other marsupials lower GFR in response to dehydration and low protein stress, thus conserving water and recycling urea (Bakker and Bradshaw 1983, 1989, Denny and Dawson 1977). Perhaps the anabolic effects of testosterone in *A. stuartii* create such a demand for protein that even increasing food intake is insufficient to completely satisfy this demand, and the animals respond by lowering their GFRs. This may be compounded by the pressure diuretic effect of cortisol. The distal tubules may be unable to respond to the increasing loss of water, and this may initiate a thirst response (as seen by this study and indirectly by Nagy *et al.* 1978), and perhaps a lowering of GFR to conserve water.

Moreover, in the wild, animals frequently reduce time spent feeding during the mating period, when they intensively compete for mates (Lazenby-Cohen and Cockburn 1988), exacerbating the nitrogen deficit observed in males (Woollard 1971). In captivity this is alleviated by readily available food and by separate housing, which reduces the behavioural stress normally experienced by *A. stuartii* in the wild.

While the metabolic changes in mating and post-mating males may exacerbate any renal modification by testosterone and cortisol, clearly the seasonal changes in renal function are mimicked by hormone administration. In many instances the mineralocorticoid actions of cortisol administration are negated by the presence of testosterone. The relationship between hormone administration and structural changes will be explored in the next chapter.