Chapter 1

Introduction

It is the purpose of this thesis to define some of the physiological changes that accompany the unusual life history of the small insectivorous marsupial *Antechinus stuartii* (Marsupialia: Dayuridae). This small mammal has a highly synchronised reproductive cycle, with all individuals in any population exhibiting the same stage of testicular or ovarian development at the same time of year (Dickman 1982, Lee and McDonald 1985, Woolley 1966). A brief monoestrous mating period occurs in late winter or early spring, depending on the locality (Dickman 1982, Lee *et al.* 1982, Woolley 1966).

Mating is followed by complete male mortality (Lee *et al.* 1982, Woolley 1966). Death of the males in this genus is believed to be caused by a failure of the glucocorticoid feedback mechanism (McDonald *et al.* 1981, McDonald *et al.* 1986), and is associated with higher plasma cortisol concentrations and outward manifestations of the "general adaptive syndrome" defined by Selye (1950). Most physiological studies on *A. stuartii* have concentrated on defining the changes in the reproductive cycle, and the hormonal changes associated with the male mortality (Bradley *et al.* 1980, Lee *et al.* 1977, McDonald *et al.* 1981, Woolley 1966). Other studies on small mammals with stress as a major contributor to their life cycle have shown that there is a large renal component to their demise (Andrews *et al.* 1972, Barnett *et al.* 1975, Christian *et al.* 1965). Renal structure and function have not been explored in *A. stuartii*.

The present study will concentrate on this aspect of the physiology of *A. stuartii*. A description of the reproductive system in males will parallel the renal work to associate the renal study with the known biology of *A. stuartii*. Firstly, however, a description of the biology and ecology of *A. stuartii* will be presented. This will be followed by a description of reproductive, adrenal, and renal function in marsupials and other mammals.

1.1 *Antechinus stuartii*: General Biology

Antechinus stuartii is widely distributed throughout forests and woodlands of south eastern Australia, from sea level to 2000 metres (Braithwaite *et al.* 1978, Dickman 1989, Dickman *et al.* 1983). *A. stuartii* is crepuscular and mostly terrestrial (Dickman 1989, 1991, Hall 1980, Lazenby-Cohen 1991, Wood 1970). They nest communally in trees with up to 20 other individuals (Lazenby-Cohen 1991). Nests appear to be a focus for the social system of *A. stuartii* (Cockburn and Lazenby-Cohen 1992, Lazenby-Cohen 1991). Animals leave the communal nests to forage, and during the mating season, to mate (Cockburn and Lazenby-Cohen 1992).

The sex ratio of *A. stuartii* does not change within populations, but can differ between populations (Cockburn 1990, Cockburn *et al.* 1985a). Male *A. stuartii* disperse after weaning (Cockburn *et al.* 1985b). It is believed 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 (Cockburn and Lazenby-Cohen 1992). Under more favourable conditions females will produce more daughters who stay close to the mother (Cockburn and Lazenby-Cohen 1992).

In the mating season the large communal nests serve as "leks", where the males wait for visits from the females (Lazenby-Cohen and Cockburn 1988). However, as the mating season progresses, males may travel large distances in search of females (Wood 1970). The most successful males are those that travel these large distances and some females will mate with more than one male (Scott and Tan 1985). The communal nests are abandoned during the period of lactation when young are detached from the teat, during which time the females nest alone (Lazenby-Cohen 1991).

Other behavioural observations from captive animals have shown that male, but not female, aggression increases over the annual cycle (Braithwaite 1974). In wild *A. stuartii*, dominant males were found to have home ranges in better habitat areas, and were larger individuals than their subordinates (Braithwaite 1979). There was little change in the home ranges of individuals over the year, although the males moved about considerably during the breeding season (Braithwaite 1979, Wood 1970).

A. stuartii interact with other closely related species and their behaviour is also modified by environmental conditions. The larger, closely related *A. swainsonii* will exclude *A. stuartii* from preferred habitats and population growth is limited by the presence of *A. swainsonii* (Dickman *et al*. 1983, Dickman 1986). In tropical rainforests the presence of other larger mammals causes *A. stuartii* to be strictly arboreal (Laurance 1992), and when the forest floor structure is more complex they will decrease arboreal activity (Dickman 1986).

A. stuartii also exhibit daily torpor. The presence of daily torpor was influenced more by body mass than season (Geiser 1988). There is a significant increase in diurnal activity and body temperature during the mating period in males, even when agonistic and mating encounters are absent (Körtner and Geiser 1995). The increase in activity found by Körtner and Geiser (1995) in the laboratory corresponds closely to the field studies of Braithwaite (1979), Cockburn and Lazenby-Cohen (1992) and Wood (1970). The higher activity of male *A. stuartii* during the mating period is associated with their unusual reproductive life history.

1.2 Reproductive biology in *A. stuartii* and other marsupials

1.2.1 Control of reproduction

A. stuartii have an unusual reproductive cycle. All aspects of reproduction are highly synchronised within each local population (Dickman 1982, Lee and Cockburn 1985). The brief, monoestrous mating period is followed by complete male mortality (Lee *et al*. 1982, Woolley, 1966). All males are dead before the females give birth 27 days after spontaneous ovulation (Selwood, 1980, 1982, Woolley 1966). The male mortality has been studied extensively in the genera *Antechinus* and *Phascogale* (Bradley 1987, 1990a, Bradley *et al*. 1975, Bradley *et al*. 1976, McDonald *et al*. 1981, McDonald *et al*. 1986) and has been observed in the genera *Dasyurus* and *Parantechinus* (Dickman and Braithwaite 1992). It is believed to be caused by a failure of the glucocorticoid feedback mechanism (Bradley *et al*. 1980, Bradley 1990a, McDonald *et al*. 1981, McDonald *et al*. 1986), which will be discussed in a later section.

The high degree of synchronisation was first linked to the photoperiodic cycle by Braithwaite (1979). The control of reproduction by photoperiod has been discussed further by Dickman (1982), McAllan and Dickman (1986), McAllan *et al.* (1991), and Selwood (1985). Animals in each population mate within a one to three week period, which is at the same time at each geographical location every year. The mating period is shorter at higher latitudes and progressively lengthens the further north the populations are found. The mating period is also later in the south than in the north (Dickman 1982). This is consistent with the theory that the rate of change of photoperiod, and not photoperiodic length, is the main determinant of mating activity (McAllan and Dickman 1986).

The study by McAllan and Dickman (1986) was based on the breeding data available for almost 150 populations of *A. stuartii*. This study suggested that the single species may be two separate species, one from southern N. S. W. and another from further north. Dickman *et al.* (1988) electrophoretically identified these two separate species at Kioloa, N. S. W., where both are reproductively isolated by the rate of change of photoperiod. The reproductive isolation has been challenged by McNee and Cockburn (1992) who believe that the cohabitation of males of both forms indicates that there is no reproductive isolation. Where *Antechinus* species cohabit, it has been noted that the larger *A. swainsonii* males will attempt to mate with *A. stuartii* females (Cockburn and Lazenby-Cohen 1992). However, cohabitation by males, but not females of two closely related species (McNee and Cockburn 1992) is not sufficient evidence to exclude reproductive isolation.

The study by McNee and Cockburn (1992) calculated a two week separation of reproductive activity using the formula of McAllan and Dickman (1986), but based their reproductive interpretations on pouch change and scrotal discoloration alone. Without other evidence, these are not reliable indicators of reproductive synchrony. Smears from the urogenital sinus of females must be used to determine oestrus, as cornified epithelial cells occur at no other time of the year except during the brief mating period (McAllan *et al.* 1991, Selwood 1985). Moreover, neither onset of spermatorrhea (sperm present in the urine, see Bolliger (1942) for a detailed description) nor

changes in the accessory reproductive glands were used as other indicators of male reproductive activity or senility. In closely related *Antechinus* species, spermatorrhea occurs in mid-late June in *A. stuartii* (McAllan *et al.* 1991, Woolley 1966), mid May in *A. swainsonii* (Dickman 1985) and in early May in *A. flavipes* (McAllan 1987). Other dasyurids also exhibit spermatorrhoea coincident with spermatogenic maturity (Fletcher 1985, Woolley 1988, 1991a,b). Finally, neither evidence of matings nor timing of births was monitored in their study so the true reproductive isolation was not tested by McNee and Cockburn (1992).

However, other factors besides photoperiod are also involved in reproductive synchrony. In some studies, animals exposed to unchanging photoperiods of different lengths continued to exhibit the reproductive changes observed in wild *A. stuartii*. In these studies synchronous mating was not always evident, and exposure to other animals or nesting material increased synchrony (Dickman 1985, Scott 1986).

A study delaying the yearly photoperiodic cycle by two months, beginning in either May or February, found that reproductive synchrony was delayed by two months in animals exposed to the different photoperiod in February, but the reproductive cycle of animals exposed in May was delayed, but unsynchronised (McAllan *et al.* 1991). Thus it appears that reproductive synchrony is maintained by the changing photoperiod modulating an underlying endogenous rhythm. It is also moderated by the intense social interactions observed in this mammal.

The control of reproduction by photoperiod has been observed in other marsupials (Curlewis and Loudon 1988, Curlewis *et al.* 1987, Gemmell 1987, Gemmell *et al.* 1985, Gemmell *et al.* 1993, Kerle and Howe 1992, Loudon and Curlewis 1987, 1989, Loudon *et al.* 1985). This has been studied best in the tammar (*Macropus eugenii*, Hearn 1972, Tyndale-Biscoe 1980). In tammars, the newly formed corpus luteum is inhibited by the frequent suckling stimulus provided by the young (Sharman 1955b, Sharman 1970, Tyndale-Biscoe and Renfree 1987). The quiescent period is known as embryonic diapause and is also induced by shortening photoperiod (seasonal diapause, Hearn 1972, Tyndale-Biscoe 1980, Tyndale-Biscoe 1986). Seasonal embryonic diapause is controlled by the pineal gland, and pineal

denervation abolishes seasonal diapause, but not lactational diapause (Renfree *et al.* 1981). Changes in both amplitude and duration of the pineal hormone, melatonin, have since been shown to be the main cue for the pituitary control of seasonal diapause (McConnell 1986, McConnell and Hinds 1985, McConnell and Tyndale-Biscoe 1985, McConnell *et al.* 1986). The melatonin cycle interacts with the diurnal prolactin surge, which inhibits the corpus luteum during seasonal diapause and must be abolished before the corpus luteum is reactivated (Hinds 1989).

In other dasyurid marsupials, the influence of photoperiod on reproduction has been studied in the seasonally polyoestrous *Sminthopsis crassicaudata*. Increased photoperiodic length induces reproductive activity in this marsupial. Refractoriness to long photoperiod occurs, although this can be curtailed by exposure to short photoperiods, with the return to the longer photoperiods again stimulating reproductive activity (Godfrey 1969, Smith *et al.* 1978). As discussed previously, in *A. stuartii* the photoperiodic cue appears to be changing photoperiod with a specific rate of change of photoperiod needed to induce ovulation and mating behaviour (McAllan *et al.* 1991). Oral administration of melatonin after the winter solstice does not abolish reproductive activity but the synchronisation of matings and births is completely abolished (McAllan 1987).

1.2.2 Reproduction in female marsupials

In female *A. stuartii* if mating occurs, the spontaneous ovulation is followed by pregnancy. The spontaneous ovulation can also be followed by a pseudopregnancy if females are unmated or matings are unsuccessful (McAllan 1987, Woolley 1966). The pseudopregnant female undergoes the enlargement of the shallow pouch and the formation of the pouch ridge and lengthening of guard hairs in exactly the same manner as the pregnant female (Woolley 1966). If birth does not occur, the pouch slowly regresses to its previous state (McAllan 1987, Woolley 1966). The progesterone levels in pregnant and pseudopregnant females are indistinguishable and 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). At this time corpora lutea are large, luteal cells are found throughout the corpus luteum,

and there is no invasion of connective tissue (Hinds and Selwood 1990). Pouch development would appear to depend on corpora luteal activity alone rather than pregnancy, further emphasising the spontaneous nature of reproduction in *A. stuartii*.

This contrasts with the findings for many macropods (Renfree 1983). The gravid endometrium of the uterus is morphologically different from the contralateral non-gravid uterus in many macropods (Owen 1834, Renfree 1972, 1983, Rose 1992, Shaw and Rose 1979) but not the opossum (Fleming and Harder 1981). The uterine differences are believed to reflect a maternal recognition of pregnancy in these marsupials (Merchant 1976, Renfree 1983, Rose 1992).

In *A. stuartii*, the histological changes that occur in the gravid and non-gravid uteri correlate with the developmental rate of the embryo, the circulating progesterone levels and with the formation of the corpus luteum (Cruz and Selwood 1993). It has been inferred that the corpus luteum controls the rate of embryonic development, and the progesterone regulation of this is mediated by the uterus (Cruz and Selwood 1993, Hinds and Selwood 1990, Woolley 1966).

The spontaneous monoestrous cycle of *A. stuartii* is similar to other *Antechinus* species (Calaby and Taylor 1981, Lee *et al.* 1982, Taylor and Horner 1970, Wilson 1986, Woolley 1966) and to other closely related dasyurids (Calaby and Taylor 1981, Taylor and Horner 1970, Woolley 1988, 199' a,b). The monoestrous cycle contrasts with many other marsupials, where ovulation can be followed by a second cycle if the litter is lost (Lee *et al.* 1982), or polyoestry occurs, either seasonally or continuously (Aslin 1975, Fleming 1973, Fletcher 1985, Godfrey 1975, Lee *et al.* 1982, Sharman 1970, Tyndale-Biscoe and Renfree 1987). The oestrous cycle of macropods is uninterrupted by pregnancy and parturition and the cessation of lactation will initiate a resumption of the oestrous cycle (Lee *et al.* 1982, Sharman 1970, Tyndale-Biscoe and Renfree 1987).

The hormonal changes during pregnancy and parturition have been best studied in the macropods. In both the tammar (*Macropus eugenii*) and the quokka (*Setonix brachyurus*) after ovulation a transient rise in circulating oestradiol-17ß occurs, coinciding with the growth of the corpus luteum and the blastocyst (Bradshaw and Bradshaw 1992, Flint and Renfree 1982). Then a second rise in

oestradiol-17ß occurs just before parturition and the post-partum oestrus (Flint and Renfree 1982). Plasma progesterone levels rise during pregnancy and drop rapidly on parturition (Harder *et al.* 1985, Hinds and Tyndale-Biscoe 1982a, Tyndale-Biscoe *et al.* 1983). Similar plasma progesterone changes have been found in other marsupials (Cake *et al.* 1980, Curlewis *et al.* 1987, Fletcher 1989a, Muths and Hinds 1996, Shorey and Hughes 1973).

In other marsupials the corpus luteum is essential for the development of the embryo beyond the blastocyst stage or is essential for parturition (Bryant and Rose 1986, Tyndale-Biscoe 1986). Unlike the situation for *A. stuartii*, more is known about the role of the Graafian follicle and the corpus luteum in the cycling of progesterone and oestradiol and the hormonal feedback for luteinising hormone (LH) and the postpartum oestrous in the macropods (Fletcher 1989b, Fletcher and Renfree 1988, Harder *et al.* 1985, Hinds *et al.* 1992, Shorey and Hughes 1973, Sutherland *et al.* 1980). Prostaglandins and prolactin are important in initiating luteolysis and parturition in the tammar (Hinds 1990, 1991, Hinds and Tyndale-Biscoe 1982b, Renfree *et al.* 1994). Little is known about these events in female *A. stuartii*.

1.2.3 Reproduction in male marsupials

More information is available about reproduction in male *A. stuartii*. Changes in the seminiferous tubules of the testes are uniform between individuals at the same time of year and commonly only two or three different types of germ cells are found at one time, contrasting with the four to six found in other mammalian species (Kerr and Hedger 1983, Woolley 1966). Spermatogenesis is completed in late June (winter solstice, Kerr and Hedger 1983). *A. stuartii* also exhibit spontaneous spermatogenic failure, where, during development, the spermatogonia and early primary spermatocytes decline while more mature generations of germ cells continue their development (Kerr and Hedger 1983). The Sertoli and Leydig cells remain numerous and morphologically intact (Kerr and Hedger 1983).

Early epididymal differentiation precedes the completion of spermatogenesis in late June and is completed by mid-July (Taggart and Temple-Smith 1992). 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) and, coupled with the increases in plasma testosterone levels after spermatogenesis is completed, suggests that spermatogenesis is maintained by small amounts of locally acting hormones interacting between the Leydig and Sertoli cells in *Antechinus* (Kerr and Hedger 1983, Wilson and Bourne 1984).

The failure of spermatogenesis before the breeding season and the constant loss of sperm into the urine (spermatorrhea) and low sperm counts are reflected in the low numbers of sperm released per ejaculation. The structure of the caudal epididymis in the *Antechinus* genus may serve to ration the sperm released at each ejaculate (Taggart and Temple-Smith 1989, 1990, 1994, Woolley 1966). The increase in plasma testosterone levels is coincident with the cessation of spermatogenesis. The rise in plasma hormone levels begins at about the time of the winter solstice, suggesting that there may be a hypothalamic interaction with the peripheral endocrine glands by photoperiodic mechanisms. These override local testicular activity causing the subsequent failure to maintain spermatogenesis. The increase in plasma testosterone levels is coincident with the cessation of spermatogenesis (Kerr and Hedger 1983).

In *A. stuartii*, the plasma testosterone levels do not reach maximal levels until August, some two months after spermatogenesis has ceased (Bradley *et al.* 1980, Kerr and Hedger 1983). In May the plasma testosterone levels are 1 $ng \cdot mL^{-1}$ or 3.47 $nmol \cdot L^{-1}$, and at the end of June the levels are 3 $ng \cdot mL^{-1}$ or 10.4 $nmol \cdot L^{-1}$, and continue to rise, reaching a peak before the "die-off" of 8.2 $ng \cdot mL^{-1}$ or 28.4 $nmol \cdot L^{-1}$ (Bradley *et al.* 1980).

In other marsupials, the peak plasma testosterone levels are similar to those of *Antechinus* and to some eutherian values (Table 1.1). Moreover, like many eutherians, the plasma testosterone concentrations of the brush-tailed possum (*Trichosurus vulpecula*) have a diurnal rhythm of testosterone secretion, with a peak in the morning (Allen and Bradshaw 1980). There are seasonal changes in the plasma

Table 1.1 Plasma testosterone concentrations in *A. stuartii* and some other mammals. Asterisks indicate species that have male post-mating mortality as part of their life history. Some values have been recalculated from $ng \cdot mL^{-1}$

Species	Plasma testosterone concentrations	Authors
Antechinus stuartii * (brown Antechinus)	3.5 $nmol \cdot L^{-1}$ (May) to 28.4 $nmol \cdot L^{-1}$ (August)	Bradley *et al.* 1980
Antechinus swainsonii * (swamp Antechinus)	2-6 $nmol \cdot L^{-1}$ (non-breeding) 17.7 $nmol \cdot L^{-1}$ (August)	McDonald *et al.* 1981 McDonald *et al.* 1986
Antechinus flavipes * (yellow-footed Antechinus)	2-6 $nmol \cdot L^{-1}$ (non-breeding) 41.9 $nmol \cdot L^{-1}$ (August)	McDonald *et al.* 1981
Sminthopsis crassicaudata (fat-tailed dunnart)	16.3 ± 2.4 $nmol \cdot L^{-1}$ (non-breeding), 26.9 ± $nmol \cdot L^{-1}$ (breeding)	McDonald *et al.* 1981
Dasyurus viverrinus (eastern quoll)	17.3 $nmol \cdot L^{-1}$ (maximum)	Bryant 1986
Didelphis albiventris (white-belly opossum)	4.2 $nmol \cdot L^{-1}$ (non-breeding), 35.0 $nmol \cdot L^{-1}$ (breeding)	de Queiroz *et al.* 1995
Isoodon macrourus (bandicoot)	0.4 to 24.3 $nmol \cdot L^{-1}$	Gemmell *et al.* 1985
Trichosurus vulpecula (brush-tailed possum)	35-69 $nmol \cdot L^{-1}$ (maximum)	Gemmell *et al.* 1986
Phascolarctos cinereus (koala)	23.9 $nmol \cdot L^{-1}$ (maximum)	Cleva *et al.* 1994a
Macropus eugenii (tammar)	3 to 24 $nmol \cdot L^{-1}$	Inns 1982
Macropus giganteus (grey kangaroo)	7.2 to 29.2 $nmol \cdot L^{-1}$	Lincoln 1978
Macropus rufogriseus rufogriseus (Bennett's wallaby)	15 $nmol \cdot L^{-1}$ (maximum)	Curlewis 1991
Macropus rufus (red kangaroo)	15.4 $nmol \cdot L^{-1}$	Lincoln 1978
Thylogale thetis (pademelon)	4.0- 30.6 $nmol \cdot L^{-1}$	Lincoln 1978
Aepyceros melampus (impala)	37.8 $nmol \cdot L^{-1}$	Neaves and Bramley 1972
Lagostomus maximus maximus (vizcacha)	15.6 $nmol \cdot L^{-1}$	Fuentes *et al.* 1993
Meriones shawi (Moroccan gerbil)	0.5 to 5.0 $nmol \cdot L^{-1}$	Zaime *et al.* 1992
Homo sapiens (human)	23-25 $nmol \cdot L^{-1}$	Orth *et al.* 1992
Sus scrofa (pig)	20 $nmol \cdot L^{-1}$	Schwarzenberger *et al.* 1993
Spilogale gracilis (spotted skunk)	22.5 $nmol \cdot L^{-1}$	Kaplan and Mead 1993

testosterone levels in the tammar (*Macropus eugenii,* Inns 1982), and, similar to eutherians, the release of testosterone from the testes is controlled by the pituitary hormone, LH (Bryant 1992, Lincoln 1978).

Similar plasma testosterone concentrations to those of *A. stuartii* when spermatogenic activity is at its peak are, however, associated with hypogonadism in mice and humans (Crawford *et al.* 1993, Handelsman 1990), and low testicular size and activity in other mammals (Curlewis 1991, Fuentes *et al.* 1993, Inns 1982, Kaplan and Mead 1993, Weinbauer and Nieschlag 1990). In general, maximal spermatogenic activity corresponds with the highest plasma testosterone levels in mammals (Weinbauer and Nieschlag 1990). However, testosterone alone fails to reinitiate or maintain spermatogenic activity in many rodents and primates, and testosterone plus follicle stimulating hormone (FSH) is necessary for these to occur (Weinbauer and Neischlag 1990). The precise mechanism by which spermatogenesis is maintained with low plasma testosterone concentrations and then ceases before the breeding season when plasma testosterone concentrations are high has not been determined. The cessation of spermatogenic activity is not associated with a collapse in the interstitium of the testes but with a maintenance of cellular density and volume (Taggart *et al.* 1993, Woolley 1966).

In other marsupials there has been some suggestion that dihydrotestosterone may be an important metabolite in possums and some macropods, and the accessory reproductive glands are maintained by conversion of testosterone to dihydrotestosterone (DHT) by 5α-reductase activity (Cook *et al.* 1978, Curlewis and Stone 1985, Inns 1982, Jones 1989, Jones *et al.* 1988). The accessory reproductive glands in marsupials are sensitive to the effects of testosterone, and administration of testosterone to castrated animals causes an increase in weight and secretory properties of these glands (Cook *et al.* 1978, Curlewis and Stone 1985, Jones *et al.* 1988).

The accessory glands of marsupials were first described by Cowper (1704), in his dissection of a male Virginia opossum, presumably *Didelphis virginiana*. Cowper (1704) described two sets of glands surrounding the urogenital sinus, the anal or "odoriferous" glands, which after dissection of the skin away from the reproductive tract he was "finally freed of the ungrateful smell of it". These anal, or

paracloacal glands, have been observed in other marsupials (Bolliger and Whitten 1948, Green 1963, Heller-José and Freymüller 1995, Owen 1868, Rotenberg and Glauert 1928, Smith 1984, Sweet 1907). The glands appear to be for scent marking (Thomson and Pears 1962), and male *A. stuartii* have been observed marking in captivity by rubbing the cloacal region over objects during the breeding season (Braithwaite 1974). Either one or two pairs of glands are next to the urogenital opening (Bolliger and Whitten 1948, Green 1963, Heller-José and Freymüller 1995, Owen 1868, Rotenberg and Glauert 1928, Smith 1984, Sweet 1907).

The second set of accessory glands described by Cowper (1704), was the "four glandulous vesicles, two on each side", found at the base of the penis. These are known as Cowper's glands, and are associated with the bulbous corpora cavernosa (Cowper 1704, Owen 1868). Cowper (1704) demonstrated that the corpora cavernosa compressed the Cowper's glands when the penis was erected. Cowper (1704) observed two pairs of glands in *Didelphis virginiana*, although this was later revised to three by Chase (1939). The numbers of bulbourethral glands found in different marsupials are varied (see Table 1.2).

The first detailed histological study of the male accessory reproductive glands was performed by Rodger and Hughes (1973) on eight species of marsupials. They found that there were between one and three pairs of the Cowper's glands and that, in general, they consisted of branched tubules that are often considerably expanded with the lumina filled with secretory material (Rodger and Hughes 1973). The epithelium is usually columnar and occasionally pseudostratified (Rodger and Hughes 1973). The nature of the secretory material differed between species (Rodger and Hughes 1973). In *A. stuartii* there were three pairs of glands, of which 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 final accessory gland of the opossum described by Cowper (1704) was the prostate gland, which surrounded 7/8ths of the urethra between the bladder and penis. The long carrot-shaped

Table 1.2 Numbers of bulbourethral glands in some marsupials.

Species	Numbers of bulbourethral glands	Authors
Notoryctes typhlops (Marsupial mole)	0	Sweet 1907
Tarsipes rostratus (spencerae) (honey possum)	1	Woolley and Scarlett 1984
Cuscus phalangister (cuscus)	1	Cunningham 1882
Pseudocheirus peregrinus (ring-tailed possum)	1	Rodger and Hughes 1973
Thylacine cynocephalus (Tasmanian tiger)	2	Cunningham 1882
Sminthopsis crassicaudata (fat-tailed dunnart)	2	Rodger and Hughes 1973
Isoodon macrourus (common bandicoot)	2	Rodger and Hughes 1973
Permales nasuta (bandicoot)	2	Rodger and Hughes 1973
Petaurus breviceps (sugar glider)	2	Smith 1984
Gymnobelideus leadbeateri (Leadbeaters possum)	2	Smith 1984
Petaroides volans (greater glider)	2	Smith 1969
Didelphis albiventris (white-belly opossum)	3	Nogueira 1988
Didelphis virginiana (Virginia opossum)	3	Chase 1939
Antechinus stuartii (brown Antechinus)	3	Rodger and Hughes 1973
Dasyurus viverrinus (eastern quoll)	3	Fletcher 1985
Myrmecobius fasciatus (numbat)	3	Fordham 1928
Phascolarctos cinereus (koala)	3	Young 1879
Lasiorhinus latifrons (hairy-nosed wombat)	3	Brooks *et al.* 1978
Trichosurus vulpecula (brush-tailed possum)	3	Fraser 1918
Macropus eugenii (Tammar wallaby)	3	Rodger and Hughes 1973
"Hypsiprymnus" (possibly *Bettongia* spp or *Aepyprymnus rufescens*)	3	Owen 1868

prostate has been found in many other marsupials (Brooks *et al.* 1978, Chase 1939, Cook *et al.* 1978, Cunningham 1882, Owen 1868, Rodger and Hughes 1973, Rotenberg and Glauert 1928, Smith 1984, Temple-Smith 1984). The only exceptions are the koala *Phascolarctos cinereus*, and the bandicoot *Isoodon macrourus*, who have a heart shaped prostate (Owen 1868, Rodger and Hughes 1973, Todhunter and Gemmell 1987). The prostate is of a disseminate or diffuse nature, and is totally interstitial to the urethral muscular coat (Owen 1868, Rodger and Hughes 1973).

A. stuartii has a carrot shaped prostate, which, like the macropods and possums, is divided into three histological types (Rodger and Hughes 1973). In *A. stuartii* the anterior part is made up of simple branched tubules, lined with a single layer of columnar cells and secretes acid mucosubstances (Rodger and Hughes 1973). The posterior sections are i) internal posterior segment that surrounds the urethral epithelium and secretes proteinaceous, neutral and small quantities of acid mucosubstances, and ii) the external posterior segment whose proteinaceous secretions must pass along the internal segment to be secreted into the urethral lumen (Rodger and Hughes 1973). The internal posterior segment secretes neutral mucosubstances and proteinaceous material into the lumen of the urethra (Rodger and Hughes 1973).

Rodger and Hughes (1973) study on *A. stuartii* included only three previously deep-frozen specimens of an unknown age. However, other studies on 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, Todhunter and Gemmell 1987, Woolley 1966). Heller (1930, 1932) and Chase (1939) were the first to link the changes in the Cowper's glands to testis hormone production. The increase in weights of the prostate and Cowper's glands is directly related to plasma testosterone levels (Curlewis 1991, Curlewis and Stone 1985, Todhunter and Gemmell 1987), although this is believed to be due to conversion of testosterone to dihydrotestosterone by 5α-reductase activity (Cook *et al.* 1978, Curlewis and Stone 1985, Inns 1982, Jones 1989, Jones *et al.* 1988). However, in *A. stuartii*, the seasonal changes in both the prostate and the Cowper's glands have been addressed only briefly

(Bradley *et al.* 1980, Woolley 1966). The accessory glands show marked increases in weight as the animals mature, and the most rapid increase occurs before the breeding season (Bradley *et al.* 1980, Woolley 1966). The increase in size is presumably in anticipation of the breeding season, and at this point spermatogenesis is finished and the sperm have been stored, also in anticipation of the breeding season.

The limited sperm production in *A. stuartii* is not compensated for by efficient sperm storage in the male epididymis (Taggart and Temple-Smith 1989). The cell structure is similar throughout the epididymis, although the caudal region, the site of sperm storage in mammals, increases in epithelial height as well as tubule dimensions. It has been suggested that the sperm storage capacity is extremely limited (Taggart and Temple-Smith 1989).

While sperm storage in the male appears to be limited, female *A. stuartii* are better equipped than males for sperm storage and maturation (Selwood and McCallum 1987, Taggart and Temple-Smith 1991). Sperm can be stored for up to 13 days in the isthmus of the oviduct and require some storage for viable fertilisation (Selwood 1983, Selwood and McCallum 1987, Taggart and Temple-Smith 1991). As males require one or two days to recover sperm numbers after a single mating (Taggart and Temple-Smith 1991) this may enable females to store some sperm from the mate of their choice before their spontaneous ovulation.

Following the intense mating period of *A. stuartii* there is complete male mortality. Social interactions are important in intensifying the "die-off" of the males (Scott 1987, Wood 1970, Woolley 1966). In captivity, males held alone or with females did not die as rapidly as those held with other males (Wood 1970, Woolley 1966) and the quicker death was the result of increased corticosteroid levels in males subjected to interactions with other males (Scott 1987). Antagonism is common in males involved in male-male interactions (Braithwaite 1974) and social factors such as population densities of *Antechinus* spp. may affect the speed at which males in the wild die (Bradley and Monamy 1991). Thus, the complex social interactions of *A. stuartii* are intertwined with the unusual endocrine changes that eventually kill all the males. The seasonal hormonal

changes that overtake the males will be discussed in the context of adrenal structure and function of marsupials in the next section.

1.3 Adrenal structure and function in mammals

1.3.1 Adrenal structure

The adrenal glands of mammals lie above and next to the kidneys and consist of a medulla surrounded by the cortex. The cortex is divided into three zones that secrete a variety of steroid hormones, based on cholesterol, and whose targets are found throughout the body (Bourne 1961, Fraser 1992, Greep 1961).

The zona reticularis lies close to the medulla and is poorly defined in many mammals (Fawcett 1986). This zone secretes the adrenal androgens, including dehydroepiandrosterone (DHEA) and androstenedione (Tepperman and Tepperman 1987). These have a weak androgenic effect compared with testosterone and dihydrotestosterone, but can be converted to testosterone by 17ß-ketosteroid reductase in peripheral tissues (Orth *et al.* 1992) and can have a considerable virilising effect *in utero* (Yalcinkaya *et al.* 1993). Oestrogens are produced from conversion of DHEA and androstenedione by the microsomal enzyme P-450 aromatase. However, most oestrogen produced through the aromatisation of DHEA and androstenedione occurs in peripheral tissues (Vermeulen and Rubens 1992).

The middle zone is the zona fasciculata that secretes the glucocorticoids, cortisol and corticosterone (Orth *et al.* 1992). The outer zone, the zona glomerulosa, secretes the mineralocorticoid aldosterone, which is important in maintaining electrolyte balance (Orth *et al.* 1992). Aldosterone is involved with salt retention and acts at the distal and connecting tubules and collecting ducts to promote transepithelial uptake of sodium and secretion of potassium (Funder 1993, Quinn and Williams 1992).

1.3.2 Control of adrenal secretion

In many mammals the regulation of cortisol is mediated by feedback mechanisms that involve the hippocampus, hypothalamus, pituitary and adrenal glands (Buckingham *et al.* 1992, Orth *et al.* 1992). The external stressor stimulates the release of corticotropin

releasing factor (CRF) from the hypothalamus, which stimulates the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary, which subsequently stimulates cortisol release from the adrenal gland (Orth *et al.* 1992, Sapolsky *et al.* 1986). Cortisol then feeds back to the hypothalamus and pituitary to inhibit further release of CRF and ACTH (Levin *et al.* 1988, Sapolsky *et al.* 1986). The strength of the stimuli and the time exposed to the stressor will affect the strength and persistence of the cortisol feedback response (Keller-Wood *et al.* 1984, van Dijken *et al.* 1993, Young *et al.* 1990).

The feedback mechanisms involve hormone receptors in the brain. The hippocampus and septum of the brain have mineralocorticoid (type I) and glucocorticoid (type II) receptors. The receptors for mineralocorticoids and glucocorticoids have a very similar structure (Arriza *et al.* 1987, Carson-Jurica *et al.* 1990). Receptors for mineralocorticoids and glucocorticoids have been found in a wide variety of tissues (Funder 1986, Funder *et al.* 1973b, Meyer and Schmidt 1994, Monder and Lakshmi 1990). The receptors are important for diurnal rhythms of cortisol and for responses to stress, and their interaction with cortisol feedback mechanisms may be to inhibit plasma cortisol levels (Casolini *et al.* 1993, Funder 1986, Feldman and Weidenfeld 1993).

The cortisol interactions with the limbic and autonomic nervous systems have important implications for CRF release. CRF release is modified by peripheral glucocorticoid secretion (Luo *et al.* 1995, Vale *et al.* 1981). CRF is believed to be a number of factors, of which the most important are a 41 amino acid polypeptide, CRF-41, and arginine vasopressin (AVP), acting together synergistically to stimulate ACTH release (Buckingham *et al.* 1992).

CRF stimulates ACTH secretion into the systemic blood through drainage to the dural sinuses and sinuses in the sphenoid bone (Fawcett 1986, Orth *et al.* 1992). ACTH is produced in the corticotrophs by proteolytic cleavage of the large precursor propiomelanocortin (POMC) and is secreted in equimolar amounts to the other products of POMC, β-lipotrophin and β-endorphin (Imura 1985, Rees and Lowry 1983, Vale *et al.* 1981).

Cortisol is produced from pregnenolone, which is converted to deoxycortisol in three steps in the smooth endoplasmic reticulum,

although the last step in the conversion to cortisol takes place in the mitochondria of the zona fasciculata cell (Funder 1993, James and Few 1985). Cortisol is transported in the blood by corticosteroid binding globulin (CBG) that binds cortisol with a high affinity and low capacity (Tepperman and Tepperman 1987). Androgens decrease CBG concentration and oestrogens increase CBG concentration (Mooradian *et al.* 1987, Riad-Fahmy *et al.* 1983). In the peripheral circulation cortisol exists in three forms, bound to CBG (80%), bound to albumin (10%) and as "free" steroid (10%) (Hsu and Kuhn 1988, Riad-Fahmy *et al.* 1983). Only the free steroid is metabolically active and available for uptake by target tissues (Riad-Fahmy *et al.* 1983). The predominant function of cortisol is carbohydrate metabolism, where hepatic glycogen stores are increased, gluconeogenesis from amino acids from peripheral tissues such as skeletal muscle and connective tissue, and lipolysis in adipose tissue are all increased (Orth *et al.* 1992). Suppression of immunological function and inflammatory processes also occurs (Orth *et al.* 1992).

The stimuli for aldosterone synthesis and release by the zona glomerulosa are angiotensin II, increased plasma potassium concentrations, decreased plasma sodium concentrations and permissive ACTH stimulation (Funder 1993, Orth *et al.* 1992, Quinn and Williams 1992, Tepperman and Tepperman 1987). In most mammalian species, the main stimulators of aldosterone release from the zona glomerulosa are angiotensin II and plasma potassium concentrations (Funder 1993, Guyton and Hall 1996). Aldosterone acts to retain renal sodium (Guyton and Hall 1996).

In the kidney, the juxtaglomerular complex is found adjacent to the afferent arterioles of the glomeruli. Low concentrations of sodium in the distal tubule or low glomerular filtration rate stimulate the secretory organelles in the macula densa that are directed towards the juxtaglomerular cells (Guyton and Hall 1996). This is believed to provoke the juxtaglomerular cells of the afferent arterioles to release renin into the peripheral circulation where angiotensinogen is converted to angiotensin I (Ganong 1993). Angiotensinogen is a glycoprotein secreted into the circulation by the liver (Ganong 1993, Sernia *et al.* 1992).

The decapeptide angiotensin I is converted to the active octapeptide angiotensin II by angiotensin-converting enzyme, which is found in blood vessel walls, the circulation, and many tissues (Ganong 1993, Henderson *et al.* 1993). Angiotensin II has been found in large concentrations in the lactotrophes and corticotrophes of the pituitary, indicating a role in ACTH and prolactin release (Ganong 1993), although this has been disputed (Kemppainen *et al.* 1993). However, the main role of angiotensin II is to stimulate the zona glomerulosa cell to synthesize and release aldosterone (Thornhill 1987). Aldosterone then binds with the tight junctioned epithelia of the distal tubule, collecting tubule and collecting duct of the nephron, causing increased sodium reabsorption from the tubule lumen and increased potassium and hydrogen ion secretion by the tubule (Rossier *et al.* 1992). High plasma concentrations of potassium also increase aldosterone activity (Rabinowitz 1989), although this is believed to by-pass the renin-angiotensin II mechanism (McCabe *et al.* 1993).

Aldosterone has many similarities to the product of the zona fasciculata, cortisol. Aldosterone is produced in the zona glomerulosa from the C_{21} steroid precursor by conversion of a methyl group to an aldehyde group at C_{18} (Fraser 1992, Funder 1993). This is done by the mitochondrial enzyme, 18-methyloxidase (P-4501B2) which is confined to the zona glomerulosa (Funder 1993). However, the mitochondrial enzyme 11ß- hydroxylase (P-4501B1) which catalyses the ß-hydroxylation at C_{11}, is present in both the zona fasciculata and the zona glomerulosa (Funder 1993, Orth *et al.* 1992). It is believed that the operation of 18-methyloxidase is one of the two main methods by which aldosterone can maintain some specificity for its target cells in the face of much higher circulating glucocorticoid concentrations (Funder 1993).

The regulation of the secretion of the adrenal androgens, DHEA and androstenedione from the zona reticularis is not as well understood as for the other products of the adrenal cortex, although ACTH has a role as circadian rhythms of plasma DHEA, androstenedione and testosterone, parallel those found for cortisol (Orth *et al.* 1992). Certainly the secretion of DHEA and androstenedione result in conversion to either testosterone or oestrogen in the peripheral tissues, notably adipose and muscle (Orth

et al. 1992, Vermeulen and Rubens 1992). Adrenal androgens are important in the maintenance of pregnancy and have been implicated in the reduction of atherosclerosis (Tepperman and Tepperman 1987).

1.3.3 Corticosteroid and mineralocorticoid receptors

The competition by cortisol for aldosterone target cells is well recognised (Beaumont and Fanestil 1983, Funder *et al.* 1973a,b, Lee *et al.* 1983, Strum *et al.* 1975). Aldosterone binds with the type I glucocorticoid receptors (Funder 1986). In peripheral tissues these are found in the distal tubule of the kidney, the gut and the salivary glands. The brain binding sites appear to be important for feedback mechanisms that control the secretion of aldosterone by pituitary factors (Rossier *et al.* 1992).

The specific binding properties of the type I receptor sites are, however, due to enzyme specificity within the receptor cell (Funder *et al.* 1988). The enzyme, 11ß- hydroxysteroid dehydrogenase (11ß-OHSD, E. C. 1.1.1.146), is found in many receptor sites, including hypothalamus, hippocampus, cerebral cortex, kidney, parotid, male reproductive tract, and colon (Funder *et al.* 1988, Michael and Cooke 1994, Moisan *et al.* 1990, Monder and Lakshmi 1990, Monder *et al.* 1994a). 11ß-OHSD catalyses the conversion of C_{21} steroids, and the biologically active cortisol is oxidised at carbon 11 to the inactive cortisone, thus enabling aldosterone access to the type I receptor (Lakshmi and Monder 1985, Stewart and Whorwood 1994).

The renal collecting ducts cells are a major site of aldosterone action, although high levels of 11ß-OHSD have not been localized in these cells, but are found in the cells of the proximal tubule of the renal cortex and in the interstitial cells of the medulla (Náray-Fejes-Tóth *et al.* 1991). Because the type I receptors and 11ß-OHSD activity do not coincide, it was suggested that there was more than one type of dehydrogenase in the kidney (Krozowski *et al.* 1990, Mercer and Krozowski 1992). This has since been confirmed and localised to the distal convoluted tubules and collecting ducts (Cole 1995, Low *et al.* 1993, Náray-Fejes-Tóth and Fejes-Tóth 1994, Náray-Fejes-Tóth *et al.* 1994a,b). A third receptor, the type III receptor, has also been isolated and is believed to have CBG-like properties (Marver 1992).

In eutherians functional blurring between the corticosteroid and mineralocorticoid actions has been observed (Funder 1993, Marver 1992). Deoxycorticosterone has an affinity similar to aldosterone for Type I receptors, and progesterone is 15-20% as effective a mineralocorticoid as aldosterone (Funder 1993). Moreover, the binding of glucocorticoids to all parts of the nephron and the production of sodium transport changes by glucocorticoid administration has led to the suggestion that type II stimulation can be indistinguishable from aldosterone actions on the nephron (Funder 1993, Funder *et al.* 1973b, Funder *et al.* 1990, Náray-Fejes-Tóth and Fejes-Tóth 1990).

1.3.4 Adrenal medulla

The adrenal medulla is part of the sympathetic nervous system and the chromaffin cells of the medulla secrete epinephrine and norepinephrine in response to splanchnic nerve activity (Carmichael 1986, Hinson 1990). The interrelationships between the two components of the adrena gland are not fully understood (Charlton 1990, Hinson 1990), although there is evidence of both paracrine and neural interactions between the medulla and the cortex (Unsicker 1993, Charlton 1990) and for initiation of secretory responses by stimulation of hypothalamus, medulla oblongata, pons and midbrain (Matsui 1981).

1.4 Adrenal structure and function in *A. stuartii* and other marsupials.

1.4.1 Adrenal structure and secretion

The adrenal glands of marsupials are organised in essentially the same manner as those of eutherian mammals (Bourne 1961), although there is considerable interspecific variation in the ratio of the cortex to the medulla (Barbour 1977 . The cortical zones are clearly defined in macropods (McDonald and Martin 1989). There are, however, some unusual anatomical anomalies found in the adrenal gland of the brush-tailed possum (*Trichosurus vulpecula*, Bourne 1934). There is a hypertrophied area between the main cortical region and the medulla in adult females that increases dramatically in size during pregnancy (Bourne 1934). This has been called the special zone (Weiss 1984, Weiss and Carson 1987) and is believed to be analogous to the X zone

found in pre-pubertal mice (Vinson *et al.* 1971). Testosterone administration completely removes the X zone in pre-pubertal male mice (Chester-Jones 1949). The X zone in mice is believed to be an alteration of the zona reticularis, and to be highly active in the synthesis of androgens (Bourne 1961, Chester-Jones *et al.* 1964, Ross 1967).

The special zone is undetectable in males and immature female brush-tailed possums, and hormonal differences can be attributed to the presence and activity of the special zone in the adrenals of possums (Kerr and Weiss 1991, Weiss and Ford 1977, 1982). *In vitro* incubation studies of the adrenal cortex of female brush-tailed possums found that the special zone produced significant amounts of testosterone, which may be related to an *in vivo* plasma testosterone concentration of 175 nmol·l^{-1} (Chester-Jones *et al.* 1964, Vinson *et al.* 1971). In males, the principal products from whole adrenal homogenates were cortisol and corticosterone, but in females the major conversion products from incubation with [^{14}C]progesterone were 5α-reduced metabolites (Weiss 1975, Weiss and Ford 1977, 1982). The androstane and pregnane based metabolites may have either some regulatory control of the true adrenal cortex or have some important adaptive role for the adult female possum (Weiss and Ford 1977, 1982).

There is some evidence that development of the special zone could be induced in immature females by injection of pregnant mare's serum (PMS) plus human chorionic gonadotrophin (hCG), or by FSH (Weiss 1984, Weiss and Carson 1987) or oestradiol (Risbridger and Weiss 1985). ACTH, however, did not cause any increase in size of the special zone (Chester-Jones *et al.* 1994).

The special zone has not been observed in other marsupials (Barbour 1977, Hall and Leslie 1990). Few studies have explored the changes to the adrenal gland in *Antechinus*. Adrenal weight has been found to change over time (Barnett 1973), although Moore (1974) found that adrenal weight did not vary when corrected for body weight. Moreover, Barnett (1973) used an average body weight rather than individual body weights.

The zones of the adrenal cortex change with the yearly reproductive cycle (Barnett 1973, Moore 1974, McAllan and Firth

unpublished). The zona reticularis and zona glomerulosa increase in size and activity when the animals are sexually mature and during the mating season. In surviving females, these cortical layers revert to pre-breeding levels (McAllan and Firth unpublished). An increase in the zona fasciculata was evident during the mating period and there is a significant loss of lipid inclusions during this time (Barnett 1973, McAllan and Firth unpublished). The changes are coincident with the dramatic endocrine changes observed in these mammals (Bradley *et al.* 1980).

The steroidal activity of the adrenal gland of *A. stuartii* has not been studied, however, the steroidal products of the adrenal cortex of many other marsupials have been investigated (Weiss 1980). An ultrastructural study on the newborn bandicoot, *Isoodon macrourus*, found cells with large amounts of smooth endoplasmic reticulum and mitochondria with cristae (Gemmell *et al.* 1982). The steroid secreting nature of the cells was confirmed by cortisol radioimmunoassay (Gemmell *et al.* 1982). Conversion studies *in vitro* using [^{14}C]-progesterone and stimulation of the adrenal by infusion of ACTH *in vivo* have helped determine the metabolic and biosynthetic activity of the adrenal cortex in marsupials (Weiss 1980). The brush-tailed possum (Chester-Jones *et al.* 1964, Vinson *et al.* 1971, Weiss and Ford 1977, 1982), the kangaroo (species unnamed) and *Antechinus swainsonii* have been examined (Weiss 1980). Cortisol, and to a lesser extent corticosterone, were the main conversion products from [^{14}C]-progesterone in the latter species and between 0.1-0.2% of the yields were aldosterone (Weiss 1980).

The biosynthesis of corticosteroids in adrenal homogenates or slices does produce some different metabolites to those from eutherians. Frequently 17ß-hydroxyprogesterone, 11ß-deoxycortisol, and 21ß-deoxycortisol are found in marsupial preparations (Weiss 1968, 1980, Weiss and McDonald 1967). It appears that the 11ß-hydroxylase system of marsupials differs from that of eutherians (McDonald and Martin 1989, Weiss 1980). The main pathway for cortisol biosynthesis appears to be from 11ß-hydroxyprogesterone via 21ß-deoxycortisol then to cortisol with 17ß-hydroxylation preceding 21ß-hydroxylation (McDonald and Martin 1989, Weiss 1980). The higher plasma, homogenate and cortical slice concentrations of the

cortisol precursors 17ß-hydroxyprogesterone, 11ß-deoxycortisol, and 21ß-deoxycortisol are explained by the less complex enzyme system found in marsupial adrenocortical biosynthesis (McDonald and Martin 1989, Weiss 1980).

In the plasma of many marsupials the main steroid recovered from plasma was cortisol (Johnston *et al.* 1967, Oddie *et al.* 1976, Weiss and McDonald 1966a,b, Weiss and Richards 1971). Corticosterone is the major product in the koala (*Phascolarctos cinereus*) and cortisol is only produced after ACTH stimulation (Oddie *et al.* 1976). In stressed quokkas (*Setonix brachyurus*), the plasma glucocorticoids rise from similar resting concentrations of cortisol and cortisone to cortisol levels relatively higher than the plasma cortisone concentration (Ilett 1969, McDonald and Bradshaw 1977).

ACTH stimulation of the adrenal gland has been studied in many marsupials. In opossums, ACTH infusion increased the output of cortisol in dexamethasone treated animals (Johnston *et al.* 1967), although results from other marsupials were more equivocal (Cooley and Janssens 1977, Martin and McDonald 1986, McDonald and Bradshaw 1977, Oddie *et al.* 1976, Vinson 1974, Weiss and McDonald 1966b, 1967, Weiss and Richards 1970, 1971).

Stimulation by ACTH did not cause an increase in plasma cortisol concentration in some species, and in others there was an increase in only a few individuals (Weiss and McDonald 1966a,b, Weiss and Richards 1970, 1971), although this has been contested for the koala and common wombat (Coghlan and Scoggins 1967, Oddie *et al.* 1976). In other marsupials the response to ACTH is similar to that of eutherian mammals, with a rise from pre-stimulation levels (Cooley and Janssens 1977, Martin and McDonald 1986, McDonald and Bradshaw 1977, Thomas and Bradley 1990, Vinson 1974). However, with the exception of the brush-tailed possum (*Trichosurus vulpecula*, Than and McDonald 1974a), the increase of plasma cortisol concentrations in response to ACTH was much lower than that of many eutherian mammals (Martin and McDonald 1986, McDonald and Bradshaw 1977, Thomas and Bradley 1990).

There are other differences between marsupials and eutherians in their adrenal physiology. Some marsupials are resistant to the general effects of glucocorticoid excess. Normally glucocorticoids

affect carbohydrate metabolism, by both stimulating hepatic gluconeogenic enzymes and by mobilizing tissue alpha-amino nitrogen. This can cause muscle wastage, an increase in urinary nitrogen and a negative nitrogen balance (Guyton and Hall 1996, Tepperman and Tepperman 1987). However, when cortisone was injected into red kangaroos (*Macropus rufus*) plasma glucose levels were not altered (Griffiths *et al.* 1969). The resistance to the diabetogenic and nitrogen mobilizing action of cortisol has been observed in other marsupials (Martin and McDonald 1985, McDonald and Bradshaw 1981, Thomas and Bradley 1990).

This contrasts with the high sensitivity to the actions of glucocorticoids in the brush-tailed possum (*Trichosurus vulpecula*) and in *Antechinus* spp. (McDonald *et al.* 1981, McDonald *et al.* 1986, Than and McDonald 1974a,b). Diabetogenic effects of long term cortisol administration were also evident in the tammar (*Macropus eugenii*, Janssens and Hinds 1981). It would appear that there are two groups of marsupials one that is sensitive to glucocorticoid action, and another that is relatively insensitive to the actions of glucocorticoids.

1.4.2 Adrenalectomy studies

The apparent dichotomy in glucocorticoid sensitivity has also been observed in adrenalectomy studies in marsupials. One of the first adrenalectomy studies was performed by Silvette and Britton (1936) on the opossum (*Didelphis virginiana*), where survival time of untreated animals was a mean of 6 days, and serum sodium and chloride rose significantly in all animals after adrenalectomy. This contrasted with eutherian studies where serum sodium and chloride fell significantly after adrenalectomy (Silvette and Britton 1936).

The relative resistance to adrenalectomy in the opossum was not observed in later studies on Australian marsupials. Survival of the quokka (*Setonix brachyurus*) following adrenalectomy was 48 hours or less, and survival could not be extended by the administration of salt, adrenocortical extract, or deoxycorticosterone (Buttle *et al.* 1952). A later study found that the quokka could be maintained indefinitely if corticosteroids were administered, but not if only a high salt diet was available (McDonald and Bradshaw 1993). In contrast to the

opossum, and similar to many eutherians, both studies found that plasma sodium fell significantly in untreated adrenalectomised quokkas (Buttle *et al.* 1952, McDonald and Bradshaw 1993).

The importance of the adrenal gland in maintaining electrolyte and glucose balance in marsupials was confirmed by other adrenalectomy studies. Reid and McDonald (1968a) found that untreated adrenalectomy in the brush-tailed possum (*Trichosurus vulpecula*) resulted in death within 3 days, with plasma urea and potassium concentrations rising, plasma sodium, chloride and bicarbonate falling, and a rise in urinary sodium excretion. The signs of adrenal insufficiency could be arrested by corticosteroid administration (Reid and McDonald 1968a). Even with access to saline drinking water, adrenalectomy without corticosteroid treatment led to death within 48 hours in the tammar (Janssens and Tyndale-Biscoe 1982). The sensitivity to adrenalectomy was less obvious in the red kangaroo (*Macropus rufus*), where not only corticosteroid administration but also self administration of normal saline drinking water maintained electrolyte balance (McDonald 1974). However, when both treatments were removed animals lost condition and died within a few days (McDonald 1974).

1.4.3 Adrenocorticoid activity and renal function

Adrenocorticoid activity is clearly essential for survival in marsupials, although there are differing sensitivities to its actions between species, even within the same genus. The adrenocorticoid action has been explored also in relation to renal function. Aldosterone secretion is very responsive to sodium depletion in the environment and plasma sodium concentration fluctuations are minimised by an increase in aldosterone output in the quokka (*Setonix brachyurus*, Miller and Bradshaw 1973), the wombat (*Vombatus ursinus*) and the grey kangaroo (*Macropus giganteus*) (Blair-West *et al.* 1968, Coghlan and Scoggins 1967, Scoggins *et al.* 1970). The changes found in the wombat and grey kangaroo were also observed in eutherians sharing the same environments (Blair-West *et al.* 1968, Scoggins *et al.* 1970).

Some interactions between the glucocorticoids and mineralocorticoids in electrolyte balance have been suggested for marsupials (McDonald 1980). The results from the adrenalectomy

studies seem to support this notion, where electrolyte balance was not significantly impaired in corticosteroid treated quokkas (McDonald and Bradshaw 1993), or brush-tailed possums (Reid and McDonald 1968a).

While the renin-angiotensin system is similar to that of eutherians (Blair-West and Gibson 1980, Johnston *et al.* 1967, Reid and McDonald 1969, Simpson and Blair-West 1971), the renal renin concentrations in the eastern grey kangaroo (*Macropus giganteus*) and the wombat (*Vombatus ursinus*) were less than 1% of the concentrations found in the sheep (*Ovis aries*). Plasma renin concentrations are also lower than has been found in some eutherians (Simpson and Blair-West 1971).

Angiotensin II receptors have been found in the liver and adrenal of brush-tailed possums (*Trichosurus vulpecula*), although they were completely absent in the brain (Sernia *et al.* 1990). This contrasts with the study of Findlay *et al.* (1980) who found that the intracranial infusion of angiotensin II stimulated drinking in the opossum (*Didelphis virginiana*). As has been found for eutherians, the subfornical organ was found to be a site for angiotensin II activity, although other sites of action were not excluded (Findlay *et al.* 1980). The dipsogenic action of angiotensin II was seen in another study on the brush-tailed possum although the physiological significance of this was unclear, as drinking untreated brush-tailed possums were never observed (Young and McDonald 1978).

In a study on adult female *A. stuartii,* large doses of angiotensin II (30 μg) had to be administered before experimental animals drank more than the control individuals (Blair-West *et al.* 1983). Injections of 0.1 IU of sheep renin had no effect on water intake, however angiotensin II did increase arterial blood pressure (Blair-West *et al.* 1983). Drinking did increase after water deprivation although drinking was rarely observed (Blair-West *et al.* 1983).

It would appear that, while the renin-angiotensin II system is present and functional in marsupials, the dipsogenic action of angiotensin II is less obvious than in eutherian mammals. This contrasts with the studies on mammals from sodium depleted environments where the alterations to plasma aldosterone concentrations and the juxtaglomerular apparatus confirmed the importance of the renin-angiotensin II system in sodium balance in

marsupials (Blair-West *et al.* 1968, Johnston *et al.* 1967, Reid and McDonald 1969). It would appear that the mineralocorticoid action of aldosterone is an important part of electrolyte homeostasis in marsupials.

1.4.4 Adrenal function and *A. stuartii*

The suggestion that the mineralocorticoids and glucocorticoids may interact in electrolyte balance in marsupials (McDonald 1974) may have special significance for *A. stuartii*, where cortisol plays an important role in the life of males. The endocrine changes in the males are centred on the androgenic and glucocorticoid responses. Barnett (1973) found that the plasma corticosteroid concentrations in males rose twofold from late July to August, which are the weeks preceding the mating period. The levels continued to rise until death, a few weeks after the mating period. The mean total plasma values are 96.6 $nmol \cdot L^{-1}$ in March rising to 165.5 $nmol \cdot L^{-1}$ in August (Bradley *et al.* 1980). In females the levels are higher than the males before July, but while they increase (to 110.3 $nmol \cdot L^{-1}$) in the month before mating they return to lower levels after the mating period (Barnett 1973, Bradley *et al.* 1975). These compare and contrast with the plasma cortisol concentration in some eutherians (table 1.3).

Mean plasma corticosteroid concentrations in other marsupials without the male mortality as part of their life history strategy are at the low end of the eutherian range (Weiss 1980, table 1.3). Seasonal changes have been found in corticosteroid secretion in some marsupials (Bradley and Stoddart 1992, Miller and Bradshaw 1979). Miller and Bradshaw (1979) observed that plasma corticosteroid concentration rose significantly during the end of the dry season into the wet season in the quokka (*Setonix brachyurus*). During this period, when body condition is lost, there is no fresh water and their diet is nitrogen deficient, and plasma corticosteroid levels rise from 30.1 ± 2.4 $nmol \cdot L^{-1}$ to 40.6 ± 3.9 $nmol \cdot L^{-1}$ (Miller and Bradshaw 1979). Bradley and Stoddart (1992) concluded that the seasonal changes found in the sugar glider *Petaurus breviceps*) seemed to depend on population density at the time of breeding. The effect of social interactions on cortisol secretion has been well documented in baboons (Sapolsky 1985, 1993).

Table 1.3 Plasma glucocorticoid concentrations in some marsupials and placentals. Asterisks indicate species with post-mating mortality. Some values were recalculated to nmol·L^{-1} from ng·mL^{-1}.

Species	Plasma glucocorticoid concentration	Authors
Antechinus stuartii * (brown Antechinus)	male non-breeding 10.4 nmol·L^{-1} male breeding 110.3 nmol·L^{-1}	Bradley *et al.* 1980
Antechinus flavipes * (yellow-footed Antechinus)	non-breeding 40 to 69 nmol·L^{-1} male breeding 165 nmol·L^{-1}	McDonald *et al.* 1981
Antechinus swainsonii * (swamp Antechinus)	non-breeding 17 to 51 nmol·L^{-1} male breeding peak 163 ± 30 nmol·L^{-1} female breeding peak 207 ± 39 nmol·L^{-1}	McDonald *et al.* 1981
Phascogale calura * (red-tailed phascogale)	male non-breeding 72 ± 13 nmol·L^{-1} male breeding 148 ± 20 nmol·L^{-1} female < 100 nmol·L^{-1}	Bradley 1987
Dasyurus geoffroyii (western quoll)	49.7 nmol·L^{-1}	Oddie *et al.* 1976
Sarcophilus harrisii (Tasmanian devil)	121 nmol L^{-1}	Weiss and Richards 1971
Sminthopsis crassicaudata (fat-tailed dunnart)	15 to 58 nmol·L^{-1}	McDonald *et al.* 1981
Petaurus breviceps (sugar glider)	males non-breeding 35.7 ± 5.6 nmol·L^{-1} males breeding 152.6 ± 14.2 nmol·L^{-1} females non-breeding 34.2 ± 6.1 nmol·L^{-1} females breeding 137.1 ± 19.8 nmol·L^{-1}	Bradley and Stoddart 1992
Setonix brachyurus (quokka)	dry season 30.1 nmol·L^{-1} wet season 40.6 nmol·L^{-1}	Miller and Bradshaw 1979
Thylogale billardierii (Tasmanian pademelon)	male 229.0 nmol·L^{-1} female 209.7 nmol·L^{-1}	Martin and McDonald 1986
Vombatus ursinus (wombat)	male 30.2-38.6 nmol·L^{-1} female 85.5 nmol·L^{-1}	Weiss and McDonald 1966a
Homo sapiens (human)	331.1 nmol·L^{-1}	Guyton and Hall 1996
Macaca fascicularis (rhesus macaque)	"unstressed" 234.5 nmol·L^{-1}	Norman 1993
Phocid seals	1350-2380 nmol L^{-1}	Liggins *et al.* 1993
Cervus eldi thamin (Eld's deer)	30.1 to 40.8 nmol·L^{-1}	Monfort *et al.* 1993
Vulpes vulpes (red fox)	63.5 nmol·L^{-1}	Oddie *et al.* 1976
Ovis aries (sheep)	14.3 nmol·L^{-1}	Oddie *et al.* 1976

In another marsupial with post-mating mortality, the red-tailed phascogale, *Phascogale calura*, plasma corticosteroid changes are seen which are similar to those in *A. stuartii* (Bradley 1987, table 1.3). Coincident with the corticosteroid changes in male *P. calura* and *A. stuartii* are the approximately eightfold rise in plasma androgens and the drop in plasma concentrations of CBGs (Bradley 1987, Bradley *et al.* 1980). These are not evident in the females, moreover, the maximum cortisol binding capacity remains high in females, except for the duration of the mating period (Bradley 1987, Bradley *et al.* 1980, Lee *et al.* 1977).

McDonald *et al.* (1981) found that in *Antechinus swainsonii* plasma corticosteroid levels did not appear to be greatly affected by the stress of capture until just before the mating period. They concluded that the secretion of ACTH was unaffected by feedback mechanisms. Using the dexamethasone suppression and ACTH stimulation tests, McDonald *et al.* (1986) found that male mortality was induced by a failure of the glucocorticoid feedback mechanism. The dexamethasone suppression test is used to determine whether the excess corticosteroids secreted in some disorders are produced because of pituitary or adrenal dysfunction (Bennington 1984). When dexamethasone is administered to a normal individual, the free cortisol secretion is reduced to less than 50% of baseline plasma concentrations. However, when adrenal hyperactivity is present, free cortisol is suppressed to less than 70% of the baseline concentrations (Bennington 1984). When pituitary dysfunction is present, such as in ACTH producing tumors, plasma cortisol concentrations remain high (Bennington 1984). The failure of the glucocorticoid feedback mechanism has also been confirmed for *Phascogale calura* (Bradley 1990a).

The systemic effects of the glucocorticoids, or "stress" hormones, are well known (Orth *et al.* 1992, Tepperman and Tepperman 1987) and many have been observed in the genus *Antechinus*. One of the most obvious is immunosuppression and invasion by parasites including *Listeria monocytogenes* and *Babesia* spp. (Barker *et al.* 1978). Worm burdens were found to increase throughout the year and are significantly higher in males during the

breeding season, coincident with the higher plasma concentrations of glucocorticoids (Beveridge and Barker 1976).

There are reports of focal hepatic necrosis, severely involuted splenic follicles, gastrointestinal haemorrhage from duodenal and gastric ulcers, haemoglobinuria, lymphopenia and neutrophilia (Barker *et al.* 1978, Bradley *et al.* 1980, Cheal *et al.* 1976). Anaemia in males is present in the last days before death, presumably because of the ulceration and parasite burdens on male *A. stuartii* (Cheal *et al.* 1976). Many of these changes have also been observed in other dasyurids that exhibit this life history (Bradley 1987, 1990b, Bradley and Monamy 1991, Monamy 1991, Poskitt *et al.* 1984).

Plasma glucose levels drop and liver glycogen stores rise coincident with the changes in plasma cortisol (Barnett 1973). Males lose weight and there is a negative nitrogen balance in the males but not the females (Wood 1970, Woollard 1971, Woolley 1966). This is associated with changes in the adrenal glands of *A. stuartii* (Barnett 1973).

The glucocorticoid action during the mating period has devastating effects on the males. Corticosteroid action can involve the kidney, therefore the role of cortisol in the renal function and electrolyte balance in *A. stuartii* should be considered. Do excess glucocorticoids also affect the renal tubules in males? This is an especially important question when the clear nitrogen mobilising and gluconeogenic responses of male *A. stuartii* are recalled. However, the renal structure and function of mammals, and then renal structural-functional relationships will be examined, before considering the scope of the thesis.

1.5 Renal Structure and Function in Mammals

1.5.1 General structure

The kidney functions to conserve water in the body, maintain plasma electrolyte concentrations, and remove the nitrogenous waste product, urea (Schmidt-Nielsen 1990). The mammalian kidney functions in essentially the same manner as all vertebrate kidneys, by ultrafiltration of the plasma through a semipermeable membrane so that water and small molecules pass through, but proteins and large molecules remain in the blood. There is also active transport,

secretion, and reabsorption of products, thus preserving important molecules such as glucose, amino acids, vitamins, and in certain circumstances electrolytes (Schmidt-Nielsen 1990).

In mammals, the kidney is divided into a cortex and medulla. The cortex lies on the outer edge of the sectioned kidney (Jamison and Kriz 1982). The renal medulla is cone shaped, and is often divided into two visibly discernible sections. These are the outer medulla, which is closest to the cortex, and the inner medulla that includes the papilla. The papilla extends into the renal pelvis, an expansion of the ureter (Jamison and Kriz 1982, Kriz and Bankir 1988). The outer medulla may be divided into the outer stripe, which is closest to the cortex, and the inner stripe (Kriz and Bankir 1988).

The essential functional unit of the kidney is the nephron. There are both long and short-looped nephrons in the mammalian kidney, however, the following description is of the long-looped nephron. The nephron consists of a renal corpuscle where afferent arterial vessels form a capillary tuft, or glomerulus, which is expanded into a blind end of the tubules to become the Bowman's capsule (Jamison and Kriz 1982, Kriz and Kaissling 1992). The efferent arterioles drain the glomeruli. The tubular section of the nephron exits at the opposite pole to the arterioles and becomes the proximal convoluted tubule (PCT), which lies within the cortical region of the kidney (Kriz and Bankir 1988, Kriz and Kaissling 1992). The cells of the PCT are easily distinguished by their tall epithelium and their extensive brush border and are associated with high absorption of both organic solutes and electrolytes (Kriz and Kaissling 1992).

The proximal straight tubule (PST) exits the cortex and is found in the outer stripe of the outer medulla. The tubule narrows significantly into the descending thin limb of the loop of Henle (TDLH) which descends into the inner medulla before turning 180^{o} into the ascending thin limb of the loop of Henle (TALH) (Kriz and Bankir 1988, Kriz and Kaissling 1992). The cells of the thin loops of Henle are flattened and interdigitate in a simple or complex manner, depending on the species (Kriz and Kaissling 1992). In general, the cells of the TDLH have a thicker epithelium, although the luminal diameter is smaller than the TALH (Jamison and Kriz 1982, Kriz and Kaissling 1992). The cells of the TDLH are water and urea permeable, but are

not involved in active transport or in sodium reabsorption (Jamison and Kriz 1982, Kriz and Kaissling 1992). This contrasts with the cells of the TALH that are not permeable to water, weakly permeable to urea, and are weakly permeable to sodium and chloride (Guyton and Hall 1996, Jamison and Kriz 1982, Schmidt-Nielsen 1990).

The thin limb changes abruptly into the distal straight tubule, or thick ascending limb (DST or TAL) at the inner stripe of the outer medulla and returns to the cortical region of the kidney. The DST cells may have varying epithelial heights, although generally the height increases as the tubule approaches the cortex. Microvilli numbers increase as the tubule approaches the junction with the distal convoluted tubule (Kriz and Kaissling 1992). The plentiful cytoplasmic material is involved in active reabsorption of HCO_3^- and NaCl (Jamison and Kriz 1982). The DST is a major site of urine dilution and is impermeable to water, involved in the active transport of ammonium across the luminal membrane (Good 1994, Jamison and Kriz 1982).

The distal tubule then convolutes (distal convoluted tubule, DCT). At the angle where the DST becomes the DCT lies the macula densa. The macula densa is a plaque of large nucleated cells in the distal tubule and is a part of the juxtaglomerular apparatus (JGA), lying at the vascular pole of the glomerulus (Kriz and Bankir 1988, Kriz and Kaissling 1992). The JGA also includes the extraglomerular mesangial cells. The extraglomerular mesangial cells surround the arterioles and extend into the glomerular tufts becoming the intraglomerular mesangium (Kriz and Bankir 1988, Kriz and Kaissling 1992). The terminal portion of the afferent arteriole contains the electron dense, irregularly shaped granular cells that produce the enzyme renin (Kriz and Kaissling 1992).

The cells of the DCT increase in epithelial height, nuclei lie closer to the luminal cell membrane, and cells are packed with mitochondria and smooth endoplasmic reticulum. The DCT transforms to the connecting tubule (CNT) which is differentiated from the DCT by the rounding of the luminal surface and an apparent reduction in intracellular components (Kriz and Kaissling 1992).

The CNT then joins the collecting duct (CD), which has been divided into a cortical collecting duct (CCD), an outer medullary collecting duct (OMCD) and an inner medullary collecting duct (IMCD)

by histologists. The division between the OMCD and IMCD is based only on their relative positions within the kidney. The division between these sections of the CD and the CCD is based on cellular differences (Kriz and Bankir 1988). The cells of the CCD have a simple polygon shape and have much cytoplasmic free basal infolding in the basal third of the cell and secrete potassium (Kriz and Kaissling 1992, Wade *et al.* 1992). The cells of the OMCD demonstrate basal infolding and increased size and this continues throughout the IMCD to the papilla (Kriz and Kaissling 1992). The intercalated cells are found scattered throughout the DCT, CNT and CD and are distinguished histologically by their darker staining and because they bulge further into the lumen than do the CD cells (Kriz and Kaissling 1992, Wade *et al.* 1992).

The intercalated cells stain positively for carbonic anhydrase, which, by the catalysing of the reversible hydration of CO_2 to form HCO_3^- and H^+, buffers the urine (Wade *et al.* 1992). There are two types of intercalated cells α and ß cells. The α -intercalated cells secrete H^+ ions and reabsorb HCO_3^- in exchange for Cl^- and the ß-intercalated cells secrete HCO_3^- in exchange for Cl^- and reabsorb H^+ (Kaissling and Stanton 1992).

The CD cells are often called principal cells by some histologists and are lightly stained with few cytoplasmic organelles (Jamison and Kriz 1982, Kriz and Kaissling 1992). The CD cells are responsive to anti-diuretic hormone (ADH) which increases the water permeability of the apical membrane to water (Jamison and Kriz 1982, Kaissling and Stanton 1992). Functional studies have demonstrated the morphological response by the CD cells to ADH (Kaissling and Stanton 1992). In ADH treated rats, hypertrophy of the CD cells is observed and in some cases the cells of the DST were also hypertrophied (Kaissling and Stanton 1992, Trinh-Trang-Tan *et al.* 1987, Wade *et al.* 1992).

Other components of the medulla are the interstitial cells of the medulla and the renal blood supply. The interstitial cells lie between the renal tubules throughout the medulla. They have lipid droplets throughout their cytoplasm and support the renal tubules and vasculature, and all exchanges are mediated through the interstitium (Jamison and Kriz 1982, Lemley and Kriz 1987).

The blood supply of the kidney enters the kidney through the hilar tunnel, where the renal hilus is the flattened side of the kidney and within which the renal sinus is found. The expansion of the ureter to the renal pelvis occurs within the renal sinus (Jamison and Kriz 1982). The renal artery branches within the sinus and the branches enter the renal tissue at the corticomedullary border. These branches, the arcuate arteries, follow this border around the kidney and then branch out into the cortex where they branch further to become the afferent arterioles of the glomeruli.

The efferent arterioles drain the glomeruli and then take one of two courses on their way out of the kidney. The efferent arterioles of the juxtamedullary glomeruli descend into the medulla where they divide to form the descending vasa recta that appear as bundles of blood vessels. At various levels throughout the medulla the capillary plexi leave the vasa recta and perfuse the medullary region before draining back as the ascending vasa recta where they join the veins that drain back by the same route that the arteries entered the kidney (arcuate veins) (Jamison and Kriz 1982, Kriz and Kaissling 1992, Plakke and Pfeiffer 1964). The place at which they rejoin the larger vessels is back within the cortex, often in peritubular capillaries so that blood that perfuses the medulla also perfuses the cortical tubules (Kriz and Kaissling 1992).

The efferent arterioles that drain the superficial glomeruli extend to the surface of the kidney before dividing to form a capillary plexus throughout the cortex (Kriz 1970). They then rejoin to form the interlobular veins near the surface of the kidney and then drain down into the arcuate veins. The complex draining pattern is sometimes modified to increase perfusion contact (Kriz 1970, Plakke and Pfeiffer 1964). The arrangement of the nephrons and the complex anastomosing of the renal vasculature help to concentrate the urine of mammals. Urine concentrating mechanisms in mammals will be discussed in the next section.

1.5.2 General function

The passage of the filtrate through the nephron begins at the glomeruli. Blood from the afferent arteriole is passed through the semipermeable membranes of the glomerular basement membrane and

the plasma filtration is maintained by the blood pressure (Guyton and Hall 1996, Kriz and Kaissling 1992). The filtrate passes out of the urinary pole of the glomerulus to the proximal tubule. Here up to 65% of water and solutes are reabsorbed (Guyton and Hall 1996) and almost all amino acids are reabsorbed (Dantzler and Silbernagl 1993). The filtrate passes down to the loop of Henle where water leaves the descending limb and solutes leave the ascending limb. While some of the changes are due to active transport across the tubule, some are due to the passive changes in solutes. The passive passage of solutes through the tubular membrane into the blood supply or into the interstitial tissue is due to the concentration gradient that is maintained in the mammalian kidney by the countercurrent mechanism produced by the vasa recta and the closely associated loops of Henle (Kriz 1970, Schmidt-Nielsen 1990).

The countercurrent mechanism of urine concentration was first discussed theoretically by Kuhn and Ryffel in 1942 (in Schmidt-Nielsen 1995). These authors proposed that the single effect of concentration is multiplied many times by the concentration gradient from the cortex to the tip of the medulla. The gradient is maintained by the blood in the highly permeable blood vessels flowing in one direction and the filtrate in the parallel selectively permeable nephron flowing in the other direction (in Schmidt Nielsen 1995).

The osmolality of the blood circulating in the cortex is 300 $mosm \cdot L^{-1}$ and as the blood circulates through the medulla it becomes increasingly concentrated, up to 1200 $mosm \cdot L^{-1}$ in humans, as NaCl and then urea is taken up by the blood. As the blood supply ascends through the medulla, initially urea, and then NaCl, progressively leave the blood vessel, which returns the osmolality to 300 $mosm \cdot L^{-1}$ as the blood re-enters the cortex (Bankir and de Rouffignac 1985, de Rouffignac 1990). The interstitial fluid has a similar osmotic gradient from the cortex to the medulla. The osmolality of the contents of the TDLH is also 300 $mosm \cdot L^{-1}$ as it leaves the outer medulla.

Water leaves the TDLH and osmolality reaches that of the interstitium (up to 1200 $mosm \cdot L^{-1}$ in humans), and the active reabsorption of sodium, potassium and chloride from the DST means that, as the electrolytes leave the lumen of the DST, the increasing osmotic pressure in the interstitial fluid causes passive readjustments

of water flow from the TDLH. The electron neutral transport of potassium back into the lumen of the DST depends on the transport of sodium and chloride, and is sensitive to the changes in potassium balance (Unwin *et al.* 1994). As the DST turns up into the cortex tubular osmolality is again low, because of the removal of sodium from the DST (Bankir and de Rouffignac 1985, de Rouffignac 1990). The DCT has the same properties as the DST, active reabsorption of sodium, potassium and chloride while remaining impermeable to water and urea (Guyton and Hall 1996).

Along with the exchanges of positive ions are the exchanges of negative ions. Ammonium transport by the PCT and DST is an important buffering mechanism for the excretion of acid (Hamm and Simon 1988). It is not an effective buffer at physiological pH, but it limits the fall in pH necessary to excrete acid (Hamm and Simon 1988). Transport of NH_3 occurs into the tubules where an H^+ ion attaches and the impermeable NH_4^+ is formed to be excreted (Good 1994, Hamm and Simon 1988). Actively transported absorption of NH_3 by the DST allows the DST to contribute to the urinary ammonia excretion when acidic urine needs to be buffered (Good 1994). Thus NH_3 can be reabsorbed from the TDLH, can accumulate in the medullary interstitium where it can reenter the TALH or the CD, and can be used for proton buffering in the DST and the CD and then excreted (Hamm and Simon 1988).

Along with ammonium transport occur bicarbonate exchanges to maintain electroneutrality (Good 1994, Wang *et al.* 1993), where HCO_3^- is reabsorbed by the distal tubule, in exchange for Cl^- ions (Wang *et al.* 1993). The bicarbonate buffering system is dependent on Cl^- secretion by the tubule, which in turn is affected by pH changes in the CD (Ishikawa *et al.* 1993, Wang *et al.* 1993). The intercalated cells of the CD are important for the exchange of Cl^- and HCO_3^- (Náray-Fejes-Tóth *et al.* 1994a, Wang *et al.* 1993).

The CD is the site for water reabsorption and urea reabsorption. Urea is synthesised from ammonia and carbon dioxide and phosphate to form carbamyl phosphate that then enters the ornithine cycle for synthesis into urea (Lehninger 1982). Thus high concentrations of the potentially toxic ammonium ions are neutralized before excretion (Schmidt-Nielsen 1990). In some desert mammals, nitrogenous waste

can be further modified to form allantoin, a precipitate end product of purine metabolism that saves three times as much water as excreting urea alone (Buffenstein *et al* 1985, Downs and Perrin 1991, Lehninger 1982).

Water leaves the CD, TALH, and DST under the influence of the osmotic gradient and ADH (de Rouffignac *et al.* 1993). Permeability of the collecting duct to water is controlled by ADH and the water that is reabsorbed is transported out of the papilla by the vasa recta (Bankir and de Rouffignac 1985, Breyer and Ando 1994). Urea is reabsorbed by the CD and recycled through the loops of Henle by the interstitium in the inner medulla passing back through the distal tubule to be concentrated in the CD (Bankir and de Rouffignac 1985, de Rouffignac *et al.* 1981). The kidney is the main site of urea excretion (Bankir and de Rouffignac 1985). Thus the water reabsorption and the excretion of urea along the CD concentrate the urine as it leaves the kidney.

There is an inverse relationship between urea and electrolyte concentrations, where urea is excreted in a smaller volume of water than an equiosmolar concentration of electrolytes (Schmidt-Nielsen *et al.* 1961). Schmidt-Nielsen *et al.* (1961) proposed that the collecting ducts were differentially permeable to urea depending on the urea load. The inverse relationship between electrolytes and urea concentrations on the urine has been recorded in other studies, especially in water deprivation studies on desert mammals (Goyal *et al.* 1988, Haines 1964, Kam and Degen 1983, Schmidt-Nielsen and Haines 1964).

However, this has not always been observed, and it appears to depend on the salt intake of the mammal under study and the constraints of the renal medullary structure (Pfeiffer 1968, Schmidt-Nielsen *et al.* 1961, Zahn 1968) The concentration of sodium and potassium in the urine also depends on the plasma concentrations that can be dependent on the diet of the mammal. High salt diets can result in high sodium excretion as well as high urea excretion. The efficient concentration of electrolytes and urea in the urine has allowed many mammals to exist on salty diets without free water, or in environments with little free water (Buffenstein *et al.* 1985, Carpenter 1968, 1969, Grenot 1992, Haines 1964, Lee 1963, MacMillen 1972, Schmidt-Nielsen *et al.* 1948).

The excretion of urea, however, is strongly dependent on the amount of protein in the diet and the necessity to maintain blood urea levels at a low concentration. The relationship between high protein diets and increased urine osmolality due to high urinary urea is well established (Edwards *et al.* 1983, Leon *et al.* 1983, McFarland and Wimsatt 1969, Nagy *et al.* 1976, Vogel and Vogel 1972). Low protein diets or starvation normally experienced in the life cycle induce a lower excretion of urea as the urea is recycled around the loops of Henle to help maintain the medullary concentration gradient (Choshniak and Arnon 1985, Kooyman and Drabek 1968, Schmidt-Nielsen 1990, Wallace *et al.* 1984).

The recycling of urea, reabsorption of water and the production of urine significantly more concentrated than plasma is one of the main reasons vertebrates have been able to successfully inhabit a wide variety of environments. One of the mechanisms by which this is achieved is to slow the filtration rate through the nephron. Glomerular filtration rate (GFR) is often used as a measure of renal function and is a parameter frequently used to determine any changes in whole kidney function in mammals. Glomerular filtration is the process by which the plasma is filtered through the capillary tufts into the tubules (Guyton and Hall 1996). The rate per unit of time at which a compound is filtered out of the plasma, unaffected by tubular reabsorption, is the GFR (Schuster and Seldin 1992). In some mammals GFR is normally kept constant by the autoregulation of the renal blood flow through changes in the afferent arteriolar resistance (Guyton and Hall 1996, ter Wee and Donker 1994). The GFR is also regulated by tubuloglomerular feedback where a fall in GFR causes decreased sodium and chloride concentration at the macula densa due to the diminished flow through the tubules. This causes the afferent arterioles to dilate and efferent arterioles to constrict, restoring GFR (ter Wee and Donker 1994 .

1.5.3 Glomerular filtration rate in mammals

GFR can be altered by many environmental and physiological conditions. Body size and metabolic rate (Yokota *et al.* 1985), diet (Bakker and Bradshaw 1983), hibernation (Brown *et al.* 1971, Zatzman and South 1972), age, sex, and changes in environmental conditions

(Blantz *et al.* 1988, Denny and Dawson 1977, Remuzzi *et al.* 1988) all affect GFR.

Changes in GFR are often associated with dietary changes and water deprivation. Labile GFRs have been observed in desert mammals, which are able to reduce GFR in response to low water availability (Denny and Dawson 1977, Etzion and Yagil 1986, Grenot 1992, Maloiy 1972). These reductions are usually a response to an immediate environmental challenge, and the low GFRs are reversed by hydration.

In other mammals high protein diets raise GFR and excreted urea (Alvestrand and Bergström 1989, Ladd *et al.* 1951), and low protein diets induce a reduction in GFR and excreted urea (Bakker and Bradshaw 1983, Choshniak and Arnon 1985). The recycling of urea to maintain the concentration gradient for effective urine concentration and water conservation has been linked to the lowering of GFR to help achieve a low water loss (Bakker and Bradshaw 1983). GFR is raised following a proteinaceous meal and the increased GFR is followed by higher urea and solute excretion (Alvestrand and Bergeström 1989, Hiatt and Hiatt 1942, Malvin and Rayner 1968).

In marsupials there have been few studies of GFR, although, several studies on water metabolism and urine concentrating ability have been conducted. GFR in the brush-tailed possum (*Trichosurus vulpecula*) was reported to be similar to that of eutherian mammals (Reid and McDonald 1968b) although the GFRs of the red kangaroo (*Macropus rufus*) and the euro (*M. robustus*) were reported to be low compared to similar sized eutherian mammals (Denny and Dawson 1977). Dehydration reduced the GFR of both species of kangaroo (Denny and Dawson 1977) Low GFRs for size were also reported for the wombats (*Lasiorhinus latifrons* and *Vombatus ursinus*), and GFR was not reduced further by water restriction (Barboza 1993).

Two other species have been studied in some detail, the spectacled hare-wallaby *Lagorchestes conspicillatus*, Bakker and Bradshaw 1983), and the quokka (*Setonix brachyurus*, McDonald and Bradshaw 1993). The spectacled hare-wallaby has a variable GFR, with low protein diets inducing a drop in GFR of more than half of the GFR produced on a high protein diet with dehydration. Dehydration caused a significant drop in GFR when the animals were fed a high

protein diet, but only a slight drop on a low protein diet. Exposure to both a low protein diet and dehydration initiated GFRs of one third the value of those of animals exposed to a high protein, hydrated regime (Bakker and Bradshaw 1983). A drop in urine volume and sodium and urea excretion paralleled the changes in GFR (Bakker and Bradshaw 1983). These authors concluded that compensation for the low protein diet, a natural occurrence for these wallabies, was by lowering the GFR and enhancing nitrogen balance by urea "sparing" (Bakker and Bradshaw 1983). The fluctuations in urine production and urea output of the spectacled hare-wallaby were correlated with the protein and water content of the plants in their diet (Bakker and Bradshaw 1989). The conservatory nature of this strategy was also confirmed, where water turnover was found to be extremely low, about 5% of total body water per day (Bakker and Bradshaw 1989), compared to about 8% for the red kangaroo (*Macropus rufus*), another arid zone dwelling marsupial (Denny and Dawson 1975).

The quokka normally has a GFR similar to the hydrated, high protein fed spectacled hare-wallaby (McDonald and Bradshaw 1993). In adrenalectomized quokkas this GFR could only be maintained by administering aldosterone and cortisol acetate, although plasma concentrations of electrolytes and urea were maintained by all combinations of corticosteroid supplementation (McDonald and Bradshaw 1993). The adrenal gland appears to be essential for the maintenance of renal filtration and perfusion in the quokka (McDonald and Bradshaw 1993).

Although renal function studies involving GFR estimations are uncommon in marsupials, there have been many studies concerning water balance and urinary concentrating ability. The quokka has been examined extensively, and early studies noted that this species could effectively concentrate urine and reduce urine volume during the dry season and after dehydration or starvation, and could also tolerate saline drinking water (Bentley 1955, Bentley and Shield 1962). The quokka is adapted to the water restricted environment on Rottnest Island in Western Australia, where in summer little or no fresh water is available (Jones *et al.* 1990). The dehydrated condition of these animals in summer is reflected in higher plasma lysine vasopressin

(antidiuretic hormone, LVP) concentrations, a more concentrated urine, and lower urine production (Jones *et al.* 1990).

The tammar (*Macropus eugenii*) also has been studied in some detail. Like the quokka, plasma ADH levels in the tammar rose with water deprivation, although concentrations were not as high as for the quokka (Bakker and Bradshaw 1978). The ability to concentrate urine occurs at about 20 weeks of pouch life and, although there is LVP in the pituitary of week old pouch young, adult plasma concentrations are not achieved before exit from the pouch at 28-30 weeks of age (Wilkes and Janssens 1986). This seems to be more a consequence of an inadequate medullary concentrating gradient, especially for urea, than an undeveloped response to LVP (Wilkes and Janssens 1986).

The tammar is able to eat dry food and drink sea water for up to 25 days before losing body condition (Kinnear *et al.* 1968, Purohit 1971). This adaptation to little or no fresh water is a response to its harsh environment on islands off the coast of Western Australia and the adaptation to low available water is reflected in the high urine osmolalities of above 3000 mosm·L^{-1} (Kinnear *et al.* 1968, Purohit 1971). The tammar also can tolerate high plasma concentrations of sodium (176 mmol·L^{-1}) and potassium (7.2 mmol·L^{-1}, Purohit 1971). Salt water is tolerated by many mammals, and a number of small mammals must survive for part of the year with only salty water or no water available (Carpenter 1968, Grenot 1992, Hudson 1963, Lee 1963, MacMillen 1972, MacMillen and Lee 1967).

Saline drinking water does not affect nitrogen balance in the tammar (Hume and Dunning 1979). Furthermore, low nitrogen diets had a similar effect to that in the spectacled hare-wallaby, with less urea excreted although urine output remained similar between hydrated and dehydrated tammars (Barker *et al.* 1970). This contrasted with similar studies in the red-necked pademelon (*Thylogale thetis*), where exposure to saline drinking water induced a large negative nitrogen balance and excessive drinking by these rainforest dwelling wallabies (Hume and Dunning 1979). Urea recycling to the gut and retention of urea in the kidneys, evident in the tammar, was not exhibited by the red-necked pademelon that live in a mesic environment (Chilcott *et al.* 1985, Dellow and Hume 1982, Lintern and Barker 1969). Water restriction was found to severely compromise the red-necked

pademelon and, while urea recycling was unchanged, excretion increased (Chilcott *et al.* 1985).

The comparative studies of marsupials from habitats with differing water restrictions have included other macropods (Denny and Dawson 1977, Ealey *et al.* 1965, Kennedy and Heinsohn 1974). Water turnover is lower than in eutherian mammals and dehydration raises urine concentration, and lowers urine output (Denny and Dawson 1977, Ealey *et al.* 1965, Kennedy and Heinsohn 1974). Lower water turnover has been postulated to be closely correlated with the lower metabolic rates of marsupials (Denny and Dawson 1977). However, a closer analysis revealed that it was affected more by habitat than by phylogenetic association (Nicol 1978).

Water turnover, urine concentrating ability, and ability to withstand water deprivation, have been studied in many non-macropodid marsupials. In bandicoots (*Isoodon macrourus*), water turnover was lower without free drinking water (Hulbert and Gordon 1972). Bandicoots from different environments were all able to lower water turnover and concentrate their urine when water intake was restricted, although the desert dwelling bilby (*Macrotis lagotis*) was able to do this to a greater extent than the other species studied (Hulbert and Dawson 1974a,b). This was, in part, due to lower metabolic rates in the desert bilbies, a phenomenon seen in another arid zone bandicoot, the golden bandicoot (*Isoodon auratus*, Withers 1992).

In many dasyurids, water balance appears to be maintained by their carnivorous diet (Green and Eberhard 1983, Haines *et al.* 1974, Morton 1980, Nagy *et al.* 1978). Both evaporation rates and urine concentrating ability are related to habitat differences, with the desert dwellers able to concentrate urine, lower water turnover and survive on diets with no free water (Haines *et al.* 1974, MacMillen and Dawson 1986, Morton 1980, Schmidt-Nielsen and Newsome 1962). This was not evident in dasyurids that live in more mesic habitats (Kennedy and MacFarlane 1971, Morton 1980). While there are some studies in marsupials that have dealt with renal function and urine concentrating ability, few have dealt with the relationship between renal structure and function. The next section will discuss the structure-function correlates of the kidney in mammals.

1.5.4 Structure and functional correlates of the kidney

In mammals, the unique development of juxtamedullary nephrons and elaborate countercurrent mechanisms has led to the ability to produce extremely concentrated urine in some species (Jamison and Kriz 1982, Schmidt-Nielson and O'Dell 1961). The first identification of the linkage between urine concentrating ability and the length of long-looped nephrons was made by Sperber (1944). In an extensive study of the renal morphological characteristics of mammals, Sperber (1944) concluded that the medullary thickness was directly related to the number of long-looped nephrons and that the medullary thickness, once adjusted for kidney size, was a response to the different environmental pressures experienced by the mammals studied. Sperber (1944) quantified this by measuring the relative medullary thickness (RMT), where medullary thickness (MT, in mm) was divided by the kidney size multiplied 10 X. The kidney size (mm) was defined as $\sqrt[3]{(\text{length} \times \text{breadth} \times \text{width})}$.

Following the study by Schmidt-Nielson and O'Dell (1961) the indices of RMT and MT have been used widely as indicators of kidney concentrating ability. However in recent times other predictors have been considered more useful as predictors of urine concentration. These include percentage medullary thickness (PMT, Heisinger and Breitenbach 1969), relative medullary area (RMA, Brownfield and Wunder 1976) and ratios of inner medulla to cortex length and outer medulla to cortex length (IM/C, OM/C, Geluso 1978).

The continuing attempts to quantify the length of the long loops of Henle and their relationship with urine concentration are due to the importance of the long loops in the counter-current multiplier system present in the mammalian kidney. Increasing the length of the nephrons, and their blood supply, helps to produce an even more concentrated urine (Greenwald 1989, Greenwald and Stetson 1988). In mammals from xeric conditions this will help to conserve water. However, the correlation of medullary length with concentrating ability is not always consistent (see Beuchat 1990a, 1993).

The determination of the important parameters for predicting urine concentrating ability has not been limited to medullary indices. Beuchat (1990a) found that body size was another important

component of urine concentrating ability. Recently, the scaling of renal indices against body mass has been used as an indicator of urine concentrating ability (Beuchat 1990a, Blake 1977, Brooker and Withers 1994, Edwards 1975, Greegor 1975). Most studies have been restricted to taxonomic subsets of the data for mammals (Blake 1977, Brooker and Withers 1994, Edwards 1975, Greegor 1975). Beuchat (1990a) has made the most extensive survey of the literature to date, and found that the ability to produce concentrated urine declined slightly with body size, as did RMT, whilst MT increased with body mass. RMT was a reasonable predictor of urine concentration, while MT alone could not explain urine concentration.

Other studies on mammals using smaller ranges of body mass have shown similar exponents for the scaling of RMT against body mass to those found by Beuchat (1990a) (Blake 1977, Brooker and Withers 1994, Calder and Braun 1983, Greegor 1975). While many studies have discussed the apparent connection between water availability in the mammal's environment and renal indices, few studies have examined the habitat correlates with renal indices (Bassett 1986, Brooker and Withers 1994). Brooker and Withers (1994) used kidney size, body mass, phylogenetic relationships and climatic factors to try to predict renal indices of the dasyurid marsupials from a wide variety of habitats. Kidney size was found to increase with body mass, and medullary thickness indices decreased with body mass. The medullary thickness indices were correlated with climatic, but not phylogenetic factors (Brooker and Withers 1994). Prothero (1984) correlated body mass with kidney mass and compared terrestrial and marine mammals, however, more specific habitat or phylogenetic separations were not attempted.

Studies using limited ranges of examples have linked the ability to concentrate urine with higher renal indices in mammals from more xeric environments (for example, see Brooker and Withers 1994, Greegor 1975, Heisinger and Breitenbach 1969). Further simple analyses of renal indices (once allowances have been made for body weight) between phylogenetic groups and environmental groups, may help to confirm the tenet that renal indices alter with environmental conditions.

Besides the broad linkage between environment and renal indices, there have been many studies examining the roles of environmental and physiological constraints on individual components of the nephron. Correlations have been found between number of vasa recta and concentrating ability (Munkácsi and Palkovits 1977), high dietary potassium and CD hypertrophy, high dietary sodium and DCT hypertrophy (Kaissling and Stanton 1992), high temperature and tubular ischemia (Kasiske *et al.* 1988) and ADH with hypertrophy and hyperplasia of DST (Bankir *et al.* 1988, Trinh-Trang-Tan *et al.* 1987).

There are hormonal effects on renal structure. The mineralocorticoid aldosterone affects renal morphology. Aldosterone secretion is stimulated by a high potassium or a low sodium diet and this transforms the CD cells (Kaissling and Stanton 1992, Wade *et al.* 1992). High K^+ diets cause hypertrophy of the CD cells and a proliferation of the folding of the basolateral membrane (Kaissling and Stanton 1992). Conversely, a high Na^+ diet causes similar changes to the DCT (Kaissling and Stanton 1992).

In marsupials, few studies have addressed the structure-function relationship in the kidneys. The gross morphological differences of kidneys in dasyurids have been related to habitat aridity (Brooker and Withers 1994). In macropods, similar relationships have also been found (Yadav 1979). In the wombats (*Lasiorhinus latifrons* and *Vombatus ursinus*) the gross and microscopic morphology of the kidney was related to the greater capacity of *L. latifrons* to conserve water in its arid environment (McAllan *et al.* 1995). The renal vasculature of *Sminthopsis dolichura* and *S. crassicaudata* has been found to be similar to the rabbit, and the long looped nephrons found in these dasyurids have been related to their urine concentrating ability (Brooker *et al.* 1995).

In eutherians, there have been some studies on the effects of both stress and the sex hormones on the kidneys of mammals. Benda (1887) first noted that the proximal tubule epithelium proliferated past the tubular end of the glomerulus in mice, demonstrating the continuity of the glomerulus with the proximal tubule. The same phenomenon was later observed in old mice of both sexes (Crabtree 1940, 1941a, Selye 1939). Testosterone was found to promote this phenomenon

and the hypertrophy of the proximal tubule in castrates of both sexes (Crabtree 1941b, Selye 1939).

The lysosome pattern of the proximal tubule changes with testosterone treatment. More lysosomes are evident in the earlier part of the proximal tubule of males, and more in the later part of the tubule in females (Schiebler and Danner 1978). These sex differences remain, although the differences are reduced, in rats castrated as adults (Zabel and Schiebler 1980). There were also changes to the proximal tubule after hypophysectomy causing an atrophy of the proximal tubule cells (Daigeler 1981, Evan *et al.* 1972). The changes may be related to the absence of other hormones besides the sex hormones, because urine volume increased (Daigeler 1981, Evan *et al.* 1972), although proteinuria was maintained if the anterior pituitary was retained (Daigeler 1981 .

Other studies have shown that testosterone administration in female or castrate male mice increases the rate of synthesis of the lysosomal enzyme α-glucuronidase in the renal proximal tubules and this can be potentiated by the action of progestins (Bullock *et al.* 1978, Mowszowicz *et al.* 1974). The rate of excretion of α-glucuronidase into the urine is also increased (Bullock *et al.* 1978). Activity of other renal enzymes has also been found to be stimulated by testosterone, including alcohol dehydrogenase and ornithine decarboxylase (Koibuchi *et al.* 1993, Mills and Bardin 1980, Swank *et al.* 1978).

The increase in enzymatic activity is a direct result of RNA stimulation, but not DNA activity (Baik *et al.* 1992, Catterall *et al.* 1986, Mills *et al.* 1979). The lack of DNA synthesis evident in the kidney indicates that the increase in tubular mass is not cellular proliferation, but an increase in cell size (Bardin and Catterall 1981, Berger and Watson 1989, Mills *et al.* 1979). This contrasts with the hyperplasia, hypertrophy and DNA activity seen in the male reproductive tract (Bardin and Catterall 1981, Berger and Watson 1989).

The hypertrophy seen in the proximal tubules is reflected in the larger cortical region in male rats and conflicting reports of larger kidney weight relative to body weight (Kochakain *et al.* 1948, MacKay and MacKay 1927, Oudar *et al.* 1991). However, in females, the

epithelial volumes of the DCT were larger than in males although the urine concentrating ability was not different between the sexes (Oudar *et al.* 1991). The sex differences in renal structure correlate with functional differences, with females able to withstand the age dependent declines in renal function and less susceptible to renal blood flow fluctuations than males (Corman *et al.* 1985, Munger and Baylis 1988, Remuzzi *et al.* 1988).

Renal hypertrophy has been linked to the increase in reabsorptive work, which can be induced by unilateral nephrectomy (Fine 1986, Fine and Bradley 1985). There is glomerular as well as proximal tubule hypertrophy and a long term product of nephrectomy is glomerular sclerosis and proteinuria that can result in protein casts in the kidney tubules (Andrews 1981, Arataki 1925, Fine 1986, Sakemi and Baba 1993). Absence of testosterone, due to castration, prevents the glomerular sclerosis and tubular damage associated with long term survival after unilateral nephrectomy (Sakemi and Baba 1993).

Glomerular and tubular damage have also been reported in wild rodent populations. These are believed to be the result of increased stress during certain phases of their population cycles, usually when population numbers increase (Andrews 1968, Andrews *et al.* 1975, Christian *et al.* 1965). The renal pathology observed in the deer mouse (*Peromyscus maniculatus*), the lemming (*Lemmus trimucronatus*), the Tupaia (*Tupaia belangeri*), wild Norway rats (*Rattus norvegicus*), and the long-haired rat (*Rattus villosissimus*) are all similar, and include increasing sclerosis of the glomeruli, loss of tubular epithelium, hyalinization of the glomeruli, dilated DCT and even disintegration of the tubules (Andrews *et al.* 1972, Andrews and Belknap 1979, Barnett *et al.* 1975, von Holst 1972a). The extent of the damage was correlated with the number of social interactions and with population density (Andrews and Belknap 1979, Barnett *et al.* 1975). Adrenal secretory reactivity was correlated with the extent of the damage in the deer mouse and the lemming (Andrews and Belknap 1979).

Clearly, the hormonal actions of both the adrenal gland and the testes have implications for renal structure and function. In *A. stuartii,* the endocrine changes that occur in the life history of this species could alter renal structure and function in the males. Analysis of any

seasonal changes in males and females of renal structure and function may contribute to the understanding of male mortality in this species. Morphometric analysis of the kidneys will clarify any changes. In recent years, the science of morphometry has been altered by the introduction of new techniques that enable histologists to reduce the time spent analysing tissue, while retaining the integrity of sampling procedures.

Morphometrics has been described as the quantitative description, analysis and interpretation of shape and shape variation in biology (Rohlf 1990). For histologists, one of the main objectives of morphometric analysis is to obtain information about cells that can be related to cell function, thus selecting appropriate variables for analysis (Lauder 1990, Rohlf 1990). 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, Lauder 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 regarding 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 that were useful for estimating volumes for bulk parameters, but often had inbuilt biases that had to be overcome (Weibel 1979). Errors due to differences in fixation shrinkage 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). This has been summarised in Appendix I.

1.6 Renal and reproductive structure and function in *Antechinus stuartii*

The present summary has indicated that there are pronounced seasonal changes in the physiology of *A. stuartii*. Previous studies have not assessed renal structure and function in *A. stuartii*. The life history of male *A. stuartii* suggests that these animals are sensitive to the actions of both testosterone and cortisol. The high endogenous plasma concentrations of testosterone experienced by male *A. stuartii*, are well known for their effects on CBGs, and thus circulating cortisol levels. It is possible that they also contribute to any effects of cortisol on renal structure and function. In other mammals there are patterns of hormone interaction with renal structure and function that indicate an involvement of reproductive and adrenal hormones in renal activities. This may also be true for *A. stuartii*.

The renal structural and functional relationship will be explored by determining kidney function and any alterations that may contribute to male mortality. Therefore GFR, urine potassium, sodium, chloride, urea, urine osmolality, plasma potassium sodium and chloride, and faecal electrolytes will be measured to establish any seasonal and sex differences in renal function in *A. stuartii*. The causes of any seasonal changes in renal structure and function will be assessed by administration of cortisol and testosterone to determine the possible role of these hormones.

Changes in the reproductive tracts will also be monitored as an indicator of hormonal changes. Some of the gross and microscopic anatomical changes associated with the seasonal physiological changes in *A. stuartii* have been discussed in other studies. 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, 1994, Taggart *et al.* 1993) have concentrated on electron microscopic studies of the epididymis. Seasonal morphological changes in the accessory reproductive tract have not previously been examined. In the present study on *A. stuartii,* light microscopic studies of the reproductive tract and kidneys will be assessed using morphometric methods.

Thus the aim of this thesis is to determine any seasonal changes in the renal structure and function of *A. stuartii* and to correlate them

with endogenous hormonal changes and with parallels in the reproductive system. Glomerular filtration rate, and renal morphometrics will be determined throughout the year and an attempt will be made to elucidate the relative contributions to renal changes by testosterone and cortisol.

Chapter 2

General Materials and Methods

2.1 Introduction

This chapter outlines the general experimental procedures undertaken for the collection and analysis of data for this thesis. Methods specific to particular studies are described in the respective chapters.

2.2 Field work and animals

Antechinus stuartii were captured from the wild for all experiments under licence from New South Wales National Parks and Wildlife Service (license number 318) and Animal Ethics approval from the University of New England. Animals were trapped from the New England Tablelands from non-service areas around Walcha and Dorrigo, and along highways throughout the tablelands. Elliott collapsible traps were supplied with plastic fibre bedding and a bait mixture of peanut butter, rolled oats, bacon pieces and mince beef. On wet nights, or in damp areas, plastic bags were placed over the traps.

Traps were placed in undergrowth, under logs and beside trees in trap lines chosen for their habitat suitability for *A. stuartii* rather than in a systematic grid more commonly used for ecological studies. Traps were placed in the field just before dusk and were checked after dawn the following day. Some animals were collected from wooden nest boxes placed along the highways to Dorrigo, Walcha, and Guyra N.S.W. for a study on feathertail gliders (*Acrobates pygmaeus*) by Dr F. Geiser, Department of Zoology, University of New England.

Animals for the seasonal studies were collected over two and a half years. In the first six months animals were collected from nest boxes. The following years trapping with Elliot traps and collections from nest boxes were employed. In the first year of trapping mainly males were captured, and, to avoid removing too many breeding females from the population, every second female was released. In the subsequent year of trapping females were again scarce and as few

as possible were removed from the trapping areas, while adding to the sample sizes for each season collected. Male numbers were also supplemented in this second year. This reluctance to denude the areas of potential breeding females has led to small numbers of females collected for each season, although male numbers are ample for each collection period. Field data, and information on other species caught while trapping for *A. stuartii*, are presented in appendix II and sample sizes are presented in chapters 3, 4 and 5.

Male *A. stuartii* for the testosterone and cortisol administration experiments were collected in a manner identical to that for the seasonal experiments. However, more females than males were captured in the final year. These females were released at the capture site. Field data are presented in appendix II and sample sizes are presented in chapters 6, 7 and 8.

The seasonal studies on renal function in *A. stuartii* were compared to the renal function of a small eutherian mammal, the laboratory mouse (*Mus musculus*). Mice were obtained from the animal house at the University of New England.

2.3 Animal maintenance in captivity

A. stuartii were caged individually in plastic rat cages 30 x 60 x 20 cm with "Fibresorb" organic animal litter (Australian Fibresorb Pty. Ltd.) placed in the bottom of the cage. They were also given nest boxes with nesting material. Animals were given water *ad libitum* and fed with at least 20g of "Whiskas" tinned cat food (Uncle Ben's of Australia Limited) just before dusk each day. The food was a mixture of beef, chicken, mutton, and rabbit meat, gel, flavour, food colouring, minerals and vitamins and contained 9.0 % crude protein, 6.0 % crude fat, 0.4 % naturally occurring salt, and 1.0 % crude fibre.

M. musculus were housed in same sex pairs in plastic rat cages 30 x 60 x 20 cm with "Fibresorb" organic animal litter placed in the bottom of the cage and given nesting material. Water and commercial mouse chow (Goodman Fielders Industries Limited) were given *ad libitum*.

2.4 Renal function determination

2.4.1 Measurement of Glomerular filtration rate (GFR)

Animals were weighed and GFR was measured using the single injection method (Hall *et al.* 1977, Stacey and Thorburn 1966). A pre-injection blood sample was taken and then 1 MBq of ^{51}Cr-EDTA was injected intraperitoneally. Subsequent blood samples were obtained at 20, 40, 60, 80, 100 and 120 minutes after the injection. Blood samples before 20 minutes were not taken as repeated venipuncture of these small mammals (males = 35g, females = 20g) can be difficult and it was thought better to estimate the slow exponential component of the two component equation usually derived from the semilog transformation of the disappearance of the radioactivity from the plasma.

Approximately 5μL of whole blood was taken from the lateral tail vein or outer leg vein. Haematocrits were measured for plasma estimation and then whole blood was suspended in 0.5 mL of sterile distilled water and radioactivity was counted with a 1470 Wallac Wizard automatic γ-counter (Wallac Oy, Finland, distributed by Pharmacia Australia Proprietary Limited) to less than 1% error. Counts per minute (CPM) were then adjusted for the different sample volumes and CPM per mL of plasma calculated. The CPM per mL were $\log_n$ transformed and regressed against time of collection. GFR was calculated from the equation

$$\text{GFR (mL·min}^{-1}) = \frac{\text{Total quantity of marker injected}}{\text{Antilogarithm of the Y-intercept}} \times \text{slope of the regression line}$$

where slope is the absolute value of the slope of the regression equation, and the antilogarithm of the Y-intercept gives the Y-intercept in CPM (Hall *et al.* 1977, Stacey and Thorburn 1966). The GFR is then adjusted for body mass to give GFR (mL·min^{-1}·kg^{-1}).

2.4.2 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. Some animals had urine and faeces

collected in a specially manufactured escape-proof metabolic chamber placed over liquid paraffin. This chamber was a piece of plastic pipe 13 cm in diameter with a screw-on plastic lid. The bottom of the chamber was made of stiff thick wire mesh. The plastic lid also had a wire mesh top, and a hole was cut in the side and covered in mesh so that the animal could see its environs, and thus reduce stress. An animal was placed in the chamber, and then the chamber and the liquid paraffin dish were placed back in the animal's home cage. Animals were left overnight for a known time without food or water in these chambers. Urine and faeces were frozen at -20° C after retrieval and urine samples contaminated with faeces were discarded for electrolyte analysis.

2.4.3 Urine electrolytes

Urine potassium and sodium were analysed using a Corning 405 flame photometer with an internal lithium standard (Corning Limited, United Kingdom). Chloride was measured using a CMT10 chloride titrator (Radiometer Limited, Denmark). Urea was determined using the enzymatic UV test/GLDH method in a Cobas Bio Centrifugal analyser (Hoffman La Roche Proprietary Limited). Urine osmolality was measured using a Wescor 5500 vapour pressure osmometer (Wescor Incorporated, U. S. A.). Urine electrolyte concentrations were expressed in $mmol \cdot L^{-1}$.

2.4.4 Faecal electrolytes

Faecal samples collected during renal function experiments were analysed for sodium, potassium, calcium, magnesium and iron using the sealed chamber digestion method (Anderson and Henderson 1986). Faecal samples were dried in an oven at 80° C until a constant weight was obtained, pre-digested with perchloric acid and hydrogen peroxide and then analysed with an inductively coupled plasma atomic emission spectrophotometer, ARL3560B ICP analyser. Percentage of faecal water was calculated before digestion analysis.

2.4.5 Total electrolytes

The concentrations of the faecal and urine electrolytes were multiplied by the urine volume to give the total excreted in mmol.

Values in the seasonal study were calculated from samples collected over known time periods, such as the GFR studies, and from overnight collections. However, not all animals from the seasonal study had urine collected overnight. Thus, although calculated, the total electrolyte data for the seasonal study are not truly representative of total electrolytes excreted over longer periods of time. This applies mostly to data collected early in the study prior to the manufacture of the overnight chambers.

The total electrolyte data for the hormone administration study were calculated from overnight collection data only. The animals were left overnight in the collecting chambers, and the times of collection ranged from 13 to 15 hours. However, the period of time during which the animals were in the collection chamber encompasses the main period of activity in *A. stuartii* in captivity (G. Körtner personal communication). Thus the overnight collections may indicate a full 24 excretion of electrolytes in the hormonal study.

2.5 Tissue and blood collections

Initially it was anticipated that electron microscopy would be performed on some of the tissues. Therefore selected tissues were processed accordingly. Moreover, because of the efficiency of using *in vitro* autoradiography of many hormones on the same individual, the *in vitro* autoradiography method was attempted during the thesis. Use of *in vitro* autoradiography to determine binding sites of cortisol in *A. stuartii* was unsuccessful. The remainder of the stored frozen tissues are available for other studies. Tissue collections for both electron microscopy and *in vitro* autoradiography are described here as this was how all animals were processed after they were euthanased.

2.5.1 Animal euthanasia

All animals were given an overdose of "Nembutal" pentobarbitone sodium (60 mg per mL, Boehringer Ingelheim), based on the rate for anaesthesia for dogs (2.3 mL per 5 kg). When the animals were deeply anaesthesised they were quickly decapitated and trunk blood was drained into heparinised tubes.

2.5.2 Plasma samples

After animal sacrifice, blood was kept on ice while tissues were collected for other studies. Blood was spun down at 4°C and plasma was pipetted off and was stored at -80°C until use.

2.5.3 Haematocrit

Haematocrits were obtained from every animal when GFR experiments were performed. Blood was collected in dried heparinised tubes, sealed at one end with plasticine and centrifuged at 13,000 g for 5 minutes in a Microhematocrit centrifuge (Hawksley and Sons Limited, United Kingdom). Haematocrit was calculated by dividing the length of the tube filled with red blood cells by the total part of the filled tube and multiplying by 100 to get a percentage value.

2.5.4 Plasma electrolytes

Plasma sodium and potassium were analysed using a Corning 405 flame photometer with an internal lithium standard. Chloride was measured using a CMT1C chloride titrator (Radiometer Limited, Denmark). Plasma osmolality was measured using a Wescor 5500 vapour pressure osmometer (Wescor Incorporated, U. S. A.). Plasma electrolyte concentrations were expressed in $mmol \cdot L^{-1}$.

2.5.5 General procedure for tissue collections

After blood collection, animals were dissected for tissue collection. The brain was dissected out of the skull by cutting through the occipital, parietal and frontal bones to a point at the base of the nasal bones. This part of the skull was then peeled away carefully, in an attempt to preserve the choroid plexus of the third ventricle. The whole brain was carefully excised and placed immediately in fixative for either light microscopy or for frozen sections. The pituitary was then removed from the pituitary fossa and placed in fixative for either electron microscopy or for frozen sections.

Following treatment of the head, the body of the animal was placed on its dorsal surface. An incision was made in the midline of the ventral surface and the skin and muscle removed to expose the internal organs. The animals left adrenal gland was quickly excised, weighed, and placed into fixative for either electron microscopy or for

frozen sections. The animals left kidney was quickly excised, weighed, the length, breadth and width measured with Vernier calipers and placed into fixative for either electron microscopy or for frozen sections. The same procedure was followed for the right kidney and adrenal gland with the exception that the tissues were placed in fixative for light microscopy.

The complete reproductive tract of females was excised and the left ovary fixed for either electron microscopy or frozen sections and the remainder of the reproductive tract fixed for light microscopy. These tissues from females will be used in later studies. The scrotal widths of the males were first measured with Vernier calipers and then dissected out of the scrotal sac and carefully separated into two testes and epididymis. The left half was placed into fixative for either electron microscopy or frozen sections and the right half was preserved for light microscopy. The prostate and bladder were then excised and cut sagitally in half, with the left half fixed for electron microscopy or frozen sections and the right half preserved for light microscopy. The bulbourethral glands of the males were then excised from beside the base of the tail and the dorsal surface of the erector muscles. The left glands were removed and preserved for either electron microscopy or frozen sections and the right glands were preserved for light microscopy.

Following these dissections, a piece of the small intestine was removed and placed in fixative for light microscopy. A piece was removed from below the pyloric sphincter and extending about 3 cms along the duodenum and the jejunum. After immersion in fixative for 1-2 days the gut was then cut transversely into several smaller pieces and re-immersed in formalin. The total time from sacrifice until the immersion of the gut in fixative was between 20 and 25 minutes duration.

2.5.6 Histological procedure for light microscopy.

Tissues were placed in at least 20 times volume of 10% Neutral Buffered Formalin, pH 7.0, osmolality 595 mosm·kg^{-1}. Tissues remained in fixative for 7-10 days, which is the optimum time for formalin fixation (Drury and Wallington 1980). The tissues were transferred to 30 % ethanol for 4-6 hours. They were then dehydrated

and processed through to melted paraffin using a histokinette (Thomas Optical and Scientific Company, Australia). Vacuum embedding was then employed until no bubbles rose from the tissue and then the tissues were placed in casts and the paraffin quickly solidified by placement on ice. Appendix III contains a detailed description of all histological methods.

Sections were cut at 5 μm for all tissues on a Leitz 1516 microtome (Ernst Leitz GMBH Wettzlar, Germany). Serial sections of adrenal, kidney and female reproductive tissues were taken. For the testes, the blocks were sectioned for a random number of sections until the material was well exposed. A total of ten slides, each containing several sections were obtained for the testes of each male. The bulbourethral glands and the prostate of sexually mature males were sectioned serially, although only every alternate five sections were placed on microscope slides. The bulbourethral glands and the prostate of sexually immature males were serially sectioned.

Sections were stained for general observation with Haemotoxylin and Eosin or Masson's trichrome. Glycogen and mucopolysaccharides were detected by staining with Periodic acid - Schiff - Alcian blue (pH 1.0 or pH 2.5) method (PAS-Alcian blue). These methods are described in appendix III.

2.5.7 Histological procedure for electron microscopy.

The preparation of tissues for electron microscopy was performed on each individual when sacrificed. However, the electron microscopy was not included in the body of the thesis and the methods employed are thus in appendix III.

2.5.8 Histological procedure for frozen sections.

Initially one of the aims of the thesis was to determine the sites of cortisol action by using *in vitro* autoradiography with tritiated cortisol. While this was attempted, it was unsuccessful, and the methods and the results for this initial section of the thesis are presented in appendix IV. However, samples for frozen sections were collected from all animals in both seasonal and hormonal studies, and *in vitro* autoradiography methods were attempted on *M. musculus* and

A. stuartii (see appendix IV . These frozen samples are available for other studies.

2.6 Morphometric analysis

Several renal parameters were measured for *A. stuartii* using the methods described in appendix I. In addition, the reproductive tract was measured morphometrically to determine seasonal changes in cell size. The specific methods are described in the relevant chapters (Chapters 4 and 5).

2.7 Statistical methods

2.7.1 Renal function experiments

GFR data from the seasonal study were separated into sex and season and compared by using a two-way analysis of variance (two-way ANOVA). If significant, this was followed by Fisher's protected least significant difference pairwise test (PLSD test) using the Statview® statistical program (Haycock *et al.* 1992). Significance levels were $P < 0.05$ (Zar 1984). GFR data from the hormone administration study were separated into two groups, administration of testosterone, and administration of cortisol. Data were then analysed by two-way ANOVA followed by Fisher's PLSD test if appropriate.

Urine and faecal electrolytes were analysed using only samples collected over a known time period, either during GFR measurements, or from an overnight collection. When data were collected over a known time, they were analysed separately from other urine collected, using a two-way ANOVA followed by Fisher's PLSD test. Significance levels were $P < 0.05$. Data from the hormone administration study were separated into two groups, administration of testosterone, and administration of cortsiol. Data were then analysed by two-way ANOVA followed by Fisher's PLSD test if appropriate.

Before analysis, the percentage haematocrit data were transformed to approximate normality (Zar 1984). The percentages were changed to a decimal fraction of one, the square root of each was taken, and then arcsine transformed (Zar 1984). These values were then analysed by two-way ANOVA, followed by Fisher's PLSD test. Significance levels were $P < 0.05$. Data from the hormone administration study were separated into two groups, administration of

testosterone, and administration of cortisol. Data were then analysed by two-way ANOVA followed by Fisher's PLSD test if appropriate.

Plasma electrolytes were separated into sex and season and compared by using a two-way ANOVA. If significant, this was followed by Fisher's PLSD test. Significance levels were $P < 0.05$ (Zar 1984). Data from the hormone administration study were separated into two groups, administration of testosterone, and administration of cortsiol. Data were then analysed by two-way ANOVA followed by Fisher's PLSD test if appropriate.

2.7.2 Morphometric data

Morphometric data were analysed by taking the mean of each parameter for each individual and then dividing data from individuals into sex and season for the seasonal study, or hormonal treatment for the testosterone and cortisol experiment and performing a two-way analysis of variance on each parameter. If significant, this was followed by Fisher's PLSD test (Haycock *et al.* 1992, Zar 1984). Significance levels were $P < 0.05$.

Sections stained with PAS-Alcian blue were analysed as follows. The data for each component were assigned ranks for each stain where absence = 0, very weak presence = 1, presence = 2, strong staining = 3. The ranks of each stain were summed for each component and then Kruskall-Wallis analyses of variance were performed to determine differences in sex or season and were followed by pairwise Dunn's least square difference tests. Significance levels were $P < 0.05$.