The mating system and reproduction in the honey possum, *Tarsipes rostratus*: a life-history and genetical perspective

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This thesis is presented for the degree of Doctor of Philosophy of Murdoch University, 2004.
I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

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Abstract

The honey possum *Tarsipes rostratus*, a marsupial endemic to south-western Australia, feeds exclusively upon nectar and pollen. It is one of the smallest marsupials, with adult females (8-12g) significantly larger than adult males (6-9g). Honey possum males have the longest sperm (356µm) recorded for any mammal and the testes represent 4.2% body weight, amongst the largest recorded for mammal species. These features suggest that sperm competition is an important part of the mating system. This study used a combination of field based studies, DNA analysis and histological examination of the female reproductive tract to investigate the life history, multiple paternity and reproduction of the honey possum in natural populations in the Fitzgerald River National Park (FRNP), on the south coast of Western Australia.

This study drew upon earlier work on the honey possum in the FRNP in order to describe its life-history. The honey possum is short-lived (1-2 years), and attains sexual maturity whilst still growing. All four teats are occupied after birth, but the litter is reduced to 2 or 3 young during pouch life. The young have a relatively slow rate of growth. Breeding occurs continuously throughout the year, but is affected by the flowering phenologies of its foodplants. The greatest proportion of females with pouch-young occurs in winter; there are fewest pouch-young in autumn, a time of year when there is a dearth of flowers. Honey possums are essentially solitary animals, with no structured social unit, and male and female home ranges overlap. In captivity they are largely tolerant of one another, but larger females are behaviorally dominant to smaller females and to males.

The densities and structure of the honey possum populations in the FRNP were analyzed from trapping data collected over 19 years. Population densities fluctuated significantly from season to season throughout the year, with changes in the flowering food resources available. There were also year-to-year differences in the intensity of those fluctuations, and these were significantly associated with rainfall in the previous year, and probably mediated through a lag effect in the flowering of the honey possum’s foodplants. The greatest densities of animals occurred over winter. In years following high rainfall, mean winter densities reached 88 individuals per
hectare. The lowest densities occurred in spring, and in years following low rainfall mean spring densities fell to 8 individuals per hectare. Even at these lowest densities, there is still the potential for interaction between males and females. A succession from high to low, then back to high densities was seen during the three years of the present study (2000-2002) and this shadowed a similar succession of changes in rainfall.

The proportion of females with pouch-young was significantly affected by the season, and by rainfall in the previous year. Years following low rainfall had a lower proportion of females in a condition to breed. The autumn dip in breeding that occurred in all years was exacerbated following dry years. Of those females that did breed in 2001, a time of low resources, there was no difference in the size of the litter compared to 2000 and 2002, times of higher resource availability. The sex-ratio of pouch young was at parity, but there was a slight bias towards males among both juveniles (56%) and adults (58%). This was probably due to the greater movements shown by males. Sex ratios were not affected by changes in rainfall and density. Male-biased dispersal was detected using genetic data and the movement patterns of males showed that they moved greater distances than females during their normal activity.

Analysis of four microsatellite loci revealed extremely high levels of variation, with 28 to 50 alleles per locus and a mean expected heterozygosity of 0.95. These are amongst the highest seen in any microsatellite study of vertebrates. There was multiple paternity in 86% litters, using a minimum number of sires per litter method, and in 95% litters, using an estimated number of sires method based upon the relatedness of litter males. This indicates that multiple mating is frequent in female honey possums and is evidence for sperm competition. The estimated number of sires in a litter was often three or four. In 41% of cases, the number of sires was less than the number of young in the litter, indicating that some males were more successful at siring offspring than others. Nevertheless, no more than two offspring in a litter were known to have been sired by the same male. Despite marked fluctuations in density from high in 2000, to low in 2001, then high again in 2002, the level of multiple paternity remained equally high in all years.
Embryonic diapause and female reproduction was investigated in the honey possum. All adult females examined, both with and without pouch-young, were either close to oestrus, had ovulated or were carrying conceptuses. The honey possum has a post-partum oestrus and it was evident that this occurs approximately 2-4 days after birth. Cleavage and formation of the unilaminar blastocyst appears to occur rapidly over approximately 5 days. Embryonic diapause proceeded in a two phase manner similar to other small possum species. The unilaminar blastocyst expanded rapidly at first; and then, from about 18-20 days after birth, the diameter of the blastocyst remained constant at approximately 1.2-1.8mm. No growth or development beyond the unilaminar stage was observed during pouch-life. The first signs of reactivation occurred during lactation, after pouch exit, and expansion of the blastocyst only occurred in one post-lactational female. The development of the corpus luteum appeared different to patterns described for other marsupials, but its formation coincided with the formation of the unilaminar blastocyst. The diameter of the corpus luteum remained constant throughout diapause. The histology of the reproductive tract was generally similar to other marsupials. There were no sperm storage crypts in the female reproductive tract.

The length of pouch-life in the honey possum was 55-65 days, and the interval between litters of the same size varied between 65 and 100 days. Embryonic diapause may reduce the time between production of successive litters in the honey possum, but lifetime reproductive potential is reasonably low. Females had up to four litters over the period that they were captured. Thus, each litter represents a substantial proportion (25%) of a female’s lifetime reproductive output. Reproductive amortization occurred, with 61% loss overall, due to overproduction of ova, loss of conceptuses and reduction of the litter during lactation.

The behavioural dominance of females suggests that multiple mating is an active strategy, and this presumably allows the genetic quality of their offspring to be maximized. Males that succeed in sperm competition may be of better intrinsic quality. Overproduction of conceptuses by females presents the opportunity for them to select those fertilized by intrinsically viable males or genetically compatible males. Sexually active males are present all year round. Females were not synchronous in their sexual receptivity, and this would lead to a skewed operational sex ratio, with more reproductive males than oestrous females. Since adult males are significantly
smaller than adult females and possess no ornaments or armaments, it is unlikely that males overtly fight for access to females. Rather, males appear to monitor the reproductive status of females through smell, and probably compete in their ability to locate oestrous females. The risk and intensity of sperm competition is high, sexual selection for a large investment in spermatogenesis is evident and competition after copulation is probably an important factor in the mating system. It is likely that males, as well as females mate multiply, and the mating system is promiscuous.
Table of Contents

Chapter 1: Introduction
1.1 The honey possum Tarsipes rostratus ................................. 1
   1.1.1 Phylogeny and similarity to other small possums .......... 1
   1.1.2 Adaptation to a diet of nectar and pollen .................. 3
   1.1.3 Distribution of the honey possum in relation to habitat ... 6
1.2 Theoretical predictions and scope of the thesis .................. 9
1.3 Thesis overview ....................................................... 15

Chapter 2: Site Description and Field Methods
2.1 Fitzgerald River National Park ........................................ 16
2.2 Sampling of the honey possum in the FRNP ....................... 18
   2.2.1 Description of trapping sites within the FRNP .......... 22
   2.2.2 Trapping regime .................................................. 24
   2.2.3 Sampling of honey possums and mark-recapture .......... 25
   2.2.4 Tissue sampling .................................................. 28

Chapter 3: Life-history of the Honey Possum
3.1 Introduction ............................................................. 30
3.2 Longevity ............................................................... 30
3.3 Size and maturity ...................................................... 33
3.4 Litter size and brood reduction ..................................... 36
3.5 Growth, pouch-life and weaning .................................... 38
3.6 Dietary constraints on reproduction ................................ 42
3.7 Breeding cycle throughout the year ............................... 45
   3.7.1 Population structure and breeding ......................... 45
   3.7.2 Food resources and condition indices ..................... 48
   3.7.3 Annual cycle of breeding ..................................... 48
3.8 Behaviour and sociality ............................................. 51
3.9 Conclusion ............................................................. 53

Chapter 4: Population Density and Structure Over Time
4.1 Introduction ............................................................. 54
4.2 Methods and results .................................................. 54
   4.2.1 Population density ............................................... 55
   4.2.2 Population structure ............................................. 56
      Females with pouch young ........................................ 64
      Sex ratios .................................................................. 68
      Sexing of pouch young using a molecular marker .......... 71
   4.2.3 Recaptures between sessions and dispersal ............... 73
      Recaptures ........................................................... 73
      Detection of sex biased dispersal using genetic data ....... 76
4.3 Discussion ............................................................... 78
4.4 Conclusion .............................................................. 86
Chapter 5: Microsatellite Analysis of Multiple Paternity

5.1 Introduction ................................................................. 88
5.2 Methods .................................................................... 90
   5.2.1 Samples genotyped at microsatellite loci ....................... 90
   5.2.2 Extraction of DNA .................................................. 91
   5.2.3 Genotyping of microsatellite loci ................................ 93
   5.2.4 Analysis of microsatellite data .................................... 101
5.3 Results ...................................................................... 103
   5.3.1 Genetic diversity ..................................................... 103
   5.3.2 Multiple paternity .................................................... 107
5.4 Discussion ................................................................. 116
   5.4.1 Genetic diversity ..................................................... 116
   5.4.2 Multiple paternity .................................................... 117
   5.4.3 Microsatellites in the honey possum and recommendations for future study ..................................................... 122
5.5 Conclusion ................................................................. 126

Chapter 6: Reproduction in the Female Honey Possum

6.1 Introduction ................................................................. 127
   6.1.1 Reproductive anatomy and physiology of marsupials .......... 128
       Ovary ..................................................................... 129
       Fertilization, cleavage and conceptus development ................... 131
   6.1.2 Patterns of reproduction and embryonic diapause ............... 134
       Embryonic diapause .................................................... 135
       Role of the corpus luteum in diapause .................................. 138
   6.1.3 This study of female reproduction .................................. 140
6.2 Methods .................................................................... 142
   6.2.1 Collection of samples and histology ................................ 142
   6.2.2 Behavioural observations .......................................... 145
   6.2.3 Individual female histories ......................................... 146
6.3 Results ...................................................................... 147
   6.3.1 Overview of the reproductive stages of the females ........... 147
   6.3.2 Size of conceptuses in relation to pouch-young ................ 150
   6.3.3 Conceptus descriptions ............................................ 152
   6.3.4 Degenerating conceptuses and reproductive amortization .... 158
   6.3.5 Ovarian structures ................................................... 161
       Corpus luteum ............................................................. 161
       Degenerate structures and interstitial tissue ......................... 164
   6.3.6 Histological characteristics of the tract at different stages of reproduction ............................................................. 167
   6.3.7 Morphology of the reproductive tract ............................. 172
   6.3.8 Absence of sperm storage crypts .................................. 174
   6.3.9 Behavioral observations ............................................ 178
   6.3.10 Individual female histories ........................................ 179
   6.3.11 Asynchrony of female cycles ..................................... 182
6.4 Discussion ................................................................. 185
   6.4.1 Reproductive histology and anatomy .............................. 185
   6.4.2 Mating behaviour ................................................... 188
   6.4.3 Embryonic diapause and reproduction in the female honey possum 189
       Post-partum oestrus and the nature of embryonic diapause ........ 189
       Cycle length, reactivation and embryonic diapause ................ 193
Chapter 7: General Discussion – The Mating System of the Honey Possum

7.1 The mating system of the honey possum and sexual selection .................. 205
7.2 The male perspective ........................................................................ 205
  7.2.1 The female tract as an ‘arena’ for sperm competition ...................... 206
  7.2.2 Adaptations to sperm competition ............................................. 209
  7.2.3 Competition before and after copulation amongst male honey possums ................................................................. 215
7.3 The female perspective ..................................................................... 222
  7.3.1 Benefits of multiple mating – the hypotheses ............................. 222
    Benefits of mate choice ................................................................. 223
    Benefits of mechanisms that bias post-copulatory paternity .......... 224
    Genetic diversity and bet-hedging ................................................. 229
  7.3.2 Possible benefits of multiple mating for female honey possums .... 230
7.4 Genetic diversity and multiple paternity in the honey possum ............ 236
7.5 Synthesis of the mating system ....................................................... 237

References .................................................................................. 239
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Chapter 1: Introduction

1.1 The honey possum *Tarsipes rostratus*

South-western Australia is home to an unusual marsupial, the honey possum *Tarsipes rostratus*. It is only distantly related to other marsupials (Kirsch *et al.* 1997) and is endemic to this floristically rich region of Western Australia. It is one of the smallest marsupials, with adult females (8-12g) being larger than adult males (6-9g). It is also short-lived, with most individuals living less than two years (Russell and Renfree 1989; Wooller *et al.* 2000; this study). The honey possum feeds exclusively on nectar and pollen (Wooller *et al.* 1984), and has a number of adaptations associated with this flower-feeding (Richardson *et al.* 1986). This chapter provides an introduction to the honey possum, its feeding ecology and its habitat. This background is essential to the later interpretation of its mating, reproduction and life-history. In addition, this chapter highlights the intriguing features of honey possum reproduction that prompted a study into its mating system.

1.1.1 Phylogeny and similarities to other small possums

The honey possum is a diprotodont marsupial that was once placed in its own superfamily, Tarsipedoidea (Kirsch 1977). However, more recent evidence suggests that it is less distantly related to other marsupials and should be placed into its own family, Tarsipedidae (Kirsch *et al.* 1997). Studies based on morphological, immunological and DNA analysis show that Tarsipedidae, Petauridae, Acrobatidae and Pseudocheiridae are best viewed as one superfamily (Petauroidea), with the first three families most closely related and forming an unresolved clade within this group (Baverstock 1984; Aplin and Archer 1987; Baverstock *et al.* 1987; Edwards and Westerman 1995; Kirsch *et al.* 1997). Molecular studies suggest that there is a relationship between the Petauridae and Tarsipedidae (Baverstock 1984; Edwards and Westerman 1995; Kirsch *et al.* 1997), although others have suggested that
Tarsipes may have its closest relationship with the Acrobatidae (Aplin and Archer 1987; Baverstock et al. 1987; Harman et al. 1990).

The honey possum and Acrobatidae, consisting of the feathertail glider Acrobates pygmaeus and the feathertail possum Distoechurus pennatus, have a range of morphological, reproductive and ecological characteristics in common. The feathertail glider (11-18 g: Ward 1990a) has a diet consisting of a large proportion of nectar and pollen, but also some seeds and insects (Turner 1984a; Huang et al. 1987). The feathertail glider has a small litter size, slow-growing young and embryonic diapause, all features it shares with the honey possum (see Chapters 3 and 6; Renfree 1980; Ward and Renfree 1988a; Ward 1990a; Wooller et al. 1999). Most other taxa of possums consist of species with a larger body size. The family Burramyidae, a group of five species of pygmy-possums, all with small body size, was once thought to be related to the Acrobatidae, but is now recognized as distinct, and is placed in the superfamily Phalangeroidea. The family Burramyidae includes the mountain pygmy possum Burramys parvus, the western pygmy possum Cercartetus concinnus, the eastern pygmy possum C. nanus, the long-tailed pygmy possum C. caudatus, and little pygmy possum C. lepidus. The Burramyidae are mostly insectivorous, but some species also eat nectar and pollen, and even fruits and seeds (Strahan 1995). In addition to their broadly similar diets and small body size (<60g as adults), the Tarsipedidae, Burramyidae and Acrobatidae share similarities in life-history (see Chapter 3) and reproduction (see Chapter 6). The small possums are all polypoestrous and polytocous, and at least one species from each of the three families exhibits embryonic diapause (Renfree 1980; Tyndale-Biscoe and Renfree 1987; Ward and Renfree 1988a; Ward 1990c).

Fossils of possum and glider families, other than Tarsipes, suggest that they diverged between 22 and 55 million years ago (Edwards and Westerman 1995). The earliest fossils of honey possums are dated approximately 33 thousand years ago (Russell and Renfree 1989). Preliminary divergence times based on molecular data indicate that the Burramyidae diverged from a common ancestor about 47
million years ago, Acrobatidae from the others about 46 million years ago, and the Petauridae from *Tarsipes* about 39 million years ago (Edwards and Westerman 1995). Although these dates are acknowledged as speculative, this divergence coincides with gradual floristic changes that resulted in a mosaic of habitats in the early to late Eocene, when Australia separated from Antarctica (Edwards and Westerman 1995).

1.1.2 Adaptation to a diet of nectar and pollen

The honey possum feeds mostly on flowers from the Proteaceae family and also some from the Myrtaceae family (Wooller *et al.* 1984; Saffer 1998; Everaardt 2004). It appears to be the only non-flying mammal in the world that relies solely on nectar and pollen as a food source (Wooller and Wooller 2003). The honey possum is specialized for a life of nectar and pollen feeding, and these morphological and physiological adaptations are more pronounced than those of other nectar-feeders that supplement their diet with insects.

The small size and agility of the honey possum presumably enables them to manoeuvre amongst thin-stemmed, terminal flowers where they feed with ease (Renfree *et al.* 1984; Richardson *et al.* 1986). They tend to move between adjacent plants by coming down and running along the ground (Garavanta 1997). The honey possum’s eyes have a large binocular overlap, giving good depth perception for its semi-arboreal lifestyle (Harman *et al.* 1990). They have grasping hands and feet with opposable digits, the first of which is clawless and the others have reduced nails, presumably to aid in gripping branches when feeding. An almost prehensile tail is used for balance. Russell (1986) summarized these adaptations in her description of captive animals: “[*Tarsipes*] were able to move over the surface of the inflorescences and cling to individual flowers, even being suspended upside down while they probed, gripping with hind-feet and tail while they used forefeet to manipulate the flowers”.

3
The honey possum has a dorsoventrally flattened head, with an elongated snout (Richardson et al. 1986; Rosenberg and Richardson 1995). When collecting nectar it buries its head deep into the floral inflorescence (Renfree et al. 1984; Russell 1986), and its head shape is thought to be related to the inflorescence morphology of banksias and dryandras that are its most frequently visited food-plants (Richardson et al. 1986). When feeding on flowers such as *Lambertia inermis*, with singular floral tubes it either pushes its snout deep into the tube, or pierces the base of the tube with its lower incisors, in order to collect the nectar (personal observation). It licks pollen grains from stamens of the flowers, which are often held in the forefeet while “being licked like an ice-cream” (Russell 1986). The honey possum carries large numbers of pollen grains on its snout and head (Wooller et al. 1983), and it has been shown to play a role in the pollination of several species of *Banksia* (Wooller and Wooller 2001, 2002; Wooller and Wooller 2003).

The long protrusible tongue is stiffened by a keratinized keel and has a brush tip and upper surface (Richardson et al. 1986). Nectar is collected by capillary action with the long papillae on the tip of the tongue when probing deep into flowers. Pollen grains adhere to the short papillae on the upper surface of the tongue when licking stamens. They are then scraped from the tongue by a series of combs on the roof of the mouth as the tongue contracts (Richardson et al. 1986).

The jaw bones and dentition in the honey possum are greatly reduced, with all teeth only small pegs, except for the characteristic diprotodont lower incisors (Richardson et al. 1986; Rosenberg and Richardson 1995). The bones of the skull are thin and translucent, the mandible and zygomatic arch are slender and flexible, and the jaw muscles are much reduced (Rosenberg and Richardson 1995). As a result the honey possum has greatly reduced masticatory power compared to small carnivores, such as the dasyurids. Moreover, it also has significantly less jaw strength than other nectarivores, such as the western pygmy possum and the feathertail glider, that supplement their diet with insects, seeds and fruit. This is reinforcing evidence of the total reliance of the honey possum on nectar and pollen as a food source.
The high fluid intake from nectar is resolved by a kidney that has an unusually large cortex relative to its medulla (Slaven and Richardson 1988). This allows the honey possum to excrete more than its own body weight in water each day. A diverticulum off the stomach may be used for storing nectar (Richardson et al. 1986). Although nectar is rich in energy, it contains very few other nutrients, and so nutrients must come solely from the pollen grains (Wooller et al. 1999). The contents of the pollen grains are digested, not in the stomach, but in the simple, elongate large intestine over about 6 hours (Richardson et al. 1986; Wooller et al. 1999). The digestive process is unresolved (Yamada et al. 1989), but probably occurs by enzymic digestion of the contents of the pollen grain through pores in the exine coat (Richardson et al. 1986).

The honey possum has a higher body temperature and higher basal metabolic rate than expected for a marsupial of its body mass (Withers et al. 1990). However, the field metabolic rate (FMR) is lower than expected for insectivorous marsupials of its body mass (Nagy et al. 1995; Bradshaw and Bradshaw 1999). Bird and bat species that feed at least partially on nectar have field metabolic rates similar to the honey possum, and it has been suggested that foraging for nectar may be less costly energetically than an insectivorous diet for small vertebrates provided food is abundant (Nagy et al. 1995; Bradshaw and Bradshaw 1999). Another reason why the FMR is lower than expected in the honey possum is due to their ability to enter a deep, short-term torpor, in which the body temperature drops to 5°C for about 10 hours (Collins et al. 1987; Withers et al. 1990; Nagy et al. 1995). Entry into torpor is stimulated by low temperatures and low food availability. The Queensland blossom bat Syconycteris australis, also a nectar and pollen feeder, utilizes regular short-term torpor (<12h), as a means of conserving energy (Geiser et al. 1996). This pattern of torpor is different to the shallow, short-term torpor shown by dasyurids, and the deep, multi-day torpor shown by pygmy possums (Withers et al. 1990). Unlike pygmy possums, honey possums do not store fat reserves, either in the field or in captivity. Deep short-term torpor probably provides maximum energy savings without
compromising their tiny body size, which may allow feeding on terminal flowers (Withers et al. 1990). Torpor may provide energy savings to offset spurts of costly feeding activity when food is sparsely located.

Estimates indicate that a 9g honey possum requires 6-7ml nectar and 1g of pollen per day to maintain mass balance (Bradshaw and Bradshaw 1999). There is variation in the estimate of daily nitrogen requirements (Wooller et al. 1999; Bradshaw and Bradshaw 2001), but honey possums may have a much lower nitrogen requirement than is predicted by their body weight for other marsupials (Bradshaw and Bradshaw 2001). This lower estimate is similar to that of other nectar and pollen feeders, such as the sugar glider and the eastern pygmy possum, and has been suggested as an adaptation to low protein diets in nectar and pollen feeders (Bradshaw and Bradshaw 2001).

The honey possum is crepuscular and nocturnal in its activity (Arrese and Runham 2002) and structural components of the eyes suggest that its vision is adapted to low light conditions rather than the dark (Arrese et al. 2002). It possesses colour vision capabilities beyond strictly nocturnal species that may facilitate feeding at dawn and dusk (Arrese et al. 2002). However, the structure of the nasal cavity and its open connection to the oral cavity, suggests that olfactory adaptations associated with searching for food are also important (Kratzing, 1982). Sniffing is also important in the little social interaction that occurs (Russell 1986).

1.1.3 Distribution of the honey possum in relation to habitat

Given this suite of specialist adaptations to its diet of nectar and pollen, it is not surprising to find that the distribution of the honey possum follows floristic patterns in south-western Australia (Figure 1.1). This region is renowned for its floristic richness (Lamont et al. 1984; Lamont and Connell 1996). There are two nodes of high species richness for the Proteaceae family, corresponding to the sandplains on the western and southern coasts of this corner of Western Australia. These nodes of
flowering plant species richness are reflected in other kwongan plant families (Lamont et al. 1984). The greatest abundance of honey possums occurs essentially within these two areas (Figure 1.1). Their distribution was probably once continuous, as was the vegetation (Beard 1984). However now, most populations of the honey possum are found within these two areas, after extensive vegetation clearing between 1940 and 1982 (Saunders and Ingram 1995), with a few small relictual populations in-between.

The honey possum has no known ecological counterpart in other mediterranean areas or any other parts of the world (Wooller et al. 1984). Flowering periods in south-western Australia are not only staggered, but often overlap, ensuring food availability throughout the year. Other mediterranean areas, such as Chile and southern California, have peaks of flowering in spring and little thereafter (Bell and Stevens 1984). Only southern Africa and south-eastern Australia have a relatively even distribution of flowering activity throughout all months of the year (Bell and Stevens 1984). Yet in comparison to south-eastern Australia, the south-west has a greater proportion of species in flower each month, and a less marked annual peak. This may explain why eastern Australian marsupials, such as the feathertail glider and the little pygmy possum, include substantial proportions of nectar and pollen in their diet, but do not rely solely upon it.

No other place in the world has a greater diversity of species from the Proteaceae and Myrtaceae families than the south-western sandplain heathlands. The Proteaceae in particular contains many species with large compound inflorescences that produce substantial amounts of nectar, and this is the group upon which the honey possum primarily feeds (Wooller et al. 1984). South-western Australia is perhaps the only place where sufficient quantities of nectar are available all year round to support a specialized feeder such as the honey possum (Wooller et al. 1984).
Figure 1.1: Distribution of *Tarsipes rostratus*. Each dot represents a specimen in the Western Australian Museum and the highest rate of capture in field studies of the honey possum also corresponds to these areas. The lines are isoflors joining points of equal plant species richness in the family Proteaceae. Numbers of plant species for each area within the isoflors is shown (after Wooller 1984).
Although the honey possum is relatively common in the heathlands of south-western Australia, it has a restricted distribution overall and is vulnerable to habitat destruction. Apart from direct destruction through clearing, its habitat is vulnerable to fire and plant disease, for example, the dieback fungus *Phytophthora cinnamomi* and canker fungi *Botryosphaeria*, to which members of the Proteaceae and Myrtaceae are particularly susceptible. On a local scale, high intensity fires remove large tracts of habitat, and although populations recover slowly, burn intervals of less than 10 years may not be sufficient for their foodplants to recover (Everaardt 2004).

### 1.2 The mating system and reproduction of the honey possum: the limited current knowledge and the scope of this study

Perhaps one of the most intriguing features of the honey possum is related to its reproduction. The testes of the honey possum are very large and represent 4.2% of body weight (Renfree *et al.* 1984). This is much larger than all other marsupials; most have a relative testes mass of less than 1% (Taggart *et al.* 1998; Taggart *et al.* 2003). It is also amongst the largest recorded for eutherian mammal species (Kenagy and Trombulak 1986; Breed and Taylor 2000). Honey possum sperm are the longest recorded for any mammal, at 356µm (Cummins and Woodall 1985).

There is a positive relationship between body mass and testes mass in both eutherian and marsupial mammals (Harcourt *et al.* 1981; Harvey and Harcourt 1984; Kenagy and Trombulak 1986; Rose *et al.* 1997; Taggart *et al.* 1998). Once this allometric relationship is taken into account there is still much variation in relative testes mass between species, and this variation is related to the mating system. Mammal species in which the female mates with more than one male in a single oestrus (referred to throughout as multiple mating), have a larger relative testes mass than species in which the female mates with a single male (Harcourt *et al.* 1981; Harvey and Harcourt 1984; Kenagy and Trombulak 1986; Hosken 1997; Rose *et al.* 1997; Gomendio *et al.* 1998; Hosken 1998; Taggart *et al.* 1998). Having larger testes shows a larger investment in spermatogenesis (Harvey and Harcourt 1984;
Møller 1988, 1989). This is an adaptation to the sperm competition which occurs in multiple mating species, where the gametes of two or more males have to compete to fertilize a given set of ova (Birkhead and Møller 1998; Parker 1998; Anderson and Dixon 2002).

Such an association between testes mass and mating system has also been found in birds (Møller and Briskie 1995), fish (Stockley et al. 1997), frogs (Jennions and Passmore 1993) and butterflies (Gage 1994). A relationship between mating system and sperm length is not as clear amongst all taxa as with testes mass (see Gomendio and Roldan 1991; Parker 1993; Stockley et al. 1997; Gomendio et al. 1998; Parker 1998). Long sperm have been associated with sperm competition in birds (Briskie et al. 1997; Johnson and Briskie 1999), butterflies (Gage 1994) and cichlid fishes (Balshine et al. 2001), but varying results have been found for mammals (Gomendio and Roldan 1991; Gomendio et al. 1998; Gage and Freckleton 2003).

Testes mass in a species is therefore thought to be a good predictor of the mating system (Gomendio et al. 1998). The testes mass of the honey possum is much greater than expected for its body size (Taggart et al. 1998), and this, together with the extremely long sperm, suggests that strong selection operates on characteristics of the ejaculate, possibly due to female multiple mating. However, caution should be applied in such predictions since ejaculate characteristics may be selected in co-evolution with female reproductive tract biology (Gomendio and Roldan 1993a). Observations of honey possums in captivity have indicated that no pair-bonds or social groups exist (Russell 1986). Studies of movement patterns, and overlap between male and female honey possums in the wild, have indicated that they lead essentially solitary lives, consistent with a promiscuous mating system (Garavanta 1997). However, no study has previously been undertaken to examine the mating system of the honey possum or document female multiple mating.
This study focused on the mating system and reproduction of the honey possum in natural populations in the Fitzgerald River National Park. It was the intention to collect information that reflects natural conditions rather than the artificial conditions in captivity. Moreover, the sensitive nature of the honey possum makes it unsuitable for the constant handling needed for high-resolution studies in captivity. Indeed, pouch-young born in a captive situation, where animals could be readily observed, have died (Russell 1986).

Behavioral observations are very difficult on small, nocturnal animals that live in dense habitat with poor visibility (Gomendio et al. 1998). Previous attempts to study the behaviour of the honey possum in the wild have led to little success (Garavanta 1997), due to their cryptic nature, dense habitat and sensitivity to noise. Moreover, behavioral observations of social and mating systems may, on their own, be misleading (Gomendio et al. 1998), as has been extensively demonstrated in birds (Moller and Birkhead 1993; Hughes 1998). Therefore, the addition of molecular techniques to yield information about the mating system of animals is now well established and recommended. Such techniques allow documentation of how frequently multiple mating results in broods that are multiply sired. Given the strong suggestions that female multiple mating occurs in the honey possum, microsatellite markers were used in this study to assess patterns of female mating and the frequency of multiple paternity.

Multiple paternity has been documented using genetic techniques in a wide variety of animals (Watson 1991; Birkhead 1998; Alderson et al. 1999; Baker et al. 1999; Zane et al. 1999; Kraaijeveld-Smit et al. 2002). Determining the frequency of multiple paternity in a population provides valuable insight into the mating system and reproduction, which may be used in conservation management. The frequency of multiple paternity is also important for hypotheses concerning the adaptive significance of polyandry. Females may adopt multiple mating as a strategy to increase their reproductive success, including by increasing the genetic quality of their offspring (see Jennions and Petrie 2000; Hosken and Stockley 2003). Multiple
paternity can also have an influence on population-level phenomena. Multiply sired broods increase the effective population size and preserve greater genetic diversity, which may be important in populations that experience environmental change (Sugg and Chesser 1994; Chesser and Baker 1996). A study of multiple paternity in wild populations of the honey possum would provide valuable insight into the mating system and how it may interact with population-level processes.

Multiple paternity in the honey possum would also provide evidence that sperm competition exists. Sperm competition is defined by Parker (1998) as the “competition between the sperm of two or more males for the fertilization of a given set of ova”. The ‘competition’ arises from the fact that in multiple mating species, gametes from two or more males are trying to fertilize the same set of ova (see Birkhead and Møller 1998; Anderson and Dixon 2002). Thus, it is well established that multiple mating results in sperm competition, and multiple mating is frequently used as a gage of sperm competition when studying its relationship with relative testis size (eg. Harcourt et al. 1981; Harvey and Harcourt 1984; Gomendio and Roldan 1991; Anderson and Dixon 2002). In mammals, all ova are produced simultaneously, and remain fertile for about 24 hours, and spermatozoa are also short-lived, usually surviving for a few days (Gomendio et al. 1998). For sperm of different males to be in competition to fertilize a set of ova, all copulations must occur near the time of ovulation, and sperm from different males must coexist within the tract (Gomendio et al. 1998). Sperm must survive until ovulation, with enough time to allow transport to the oviduct (site of fertilization) and maturation of spermatozoa (Gomendio et al. 1998). Due to the methodological difficulty of observing sperm within the female reproductive tract of mammals, evidence for sperm competition has focused on other methods that show that sperm from different males are trying to fertilize the one set of ova (Gomendio et al. 1998). Multiple paternity is the most direct evidence and is “unquestionable proof of sperm competition” (Gomendio et al. 1998). The ability of sperm from different males to achieve fertilization within one ovulation event, perforce means they are simultaneously surviving within the female tract and therefore competing to fertilize the ova.
Whilst multiple paternity of litters is direct evidence of sperm competition, the occurrence of females with single-sired litters does not preclude sperm competition (Gomendio et al. 1998). However, direct observation of sperm from different males within the female tract involves serious methodological problems for mammals generally (Gomendio et al. 1998), but also specifically for a study of the honey possum in the wild. Even if the female was sacrificed and the sperm recovered from the tract, the female would need to be sampled after mating and before fertilization. It would require detailed knowledge of the timing and length of oestrus for each female to be sampled, yet this information is not yet known for the honey possum. In addition, detection of sperm from different males within the female reproductive tract, through genetic identification, would not necessarily confirm that the sperm were alive and viable. Successful fertilization of embryos by different males giving multiply sired pouch-young indicates that sperm from different males are simultaneously surviving, and therefore in competition to fertilize one set of ova. Levels of multiple paternity were assessed in the honey possum for the intrinsic value of this information in describing the mating system, but also its potential to provide evidence of sperm competition in a non-invasive manner.

One of the reasons that observations of mating behaviour and information on the mating system of the honey possum has been difficult to collect, is that there has been little detailed information on female reproduction. Whilst there is much information on the breeding and life-history of the honey possum in the wild (Wooller et al. 1981; Renfree et al. 1984; Russell and Renfree 1989; Wooller and Richardson 1992; Wooller et al. 1999; Wooller et al. 2000, and see Chapter 3), there has been no detailed description of female reproduction. In particular, the honey possum has previously been found to exhibit embryonic diapause (Renfree 1980). However, no investigation of this has been undertaken since. This study used wild-caught females at various stages of breeding, to investigate embryonic diapause and describe female reproduction.
The aim of this thesis was to determine female mating patterns and the frequency of multiple paternity and therefore sperm competition, and to describe embryonic diapause and female reproduction in natural populations of the honey possum in the Fitzgerald River National Park (FRNP). In addition, long-term trapping data from these populations since 1984 have revealed that population density changes over time in relation to the available resources, and such fluctuations were observed during the three years of this study. The long-term data was, therefore, utilized to document these changes in population density and determine if the structure of the population changed in a way that affected reproduction and mating. This provided an integrated approach to the understanding of reproduction and the mating system of the honey possum. The objective was to then interpret these results within the framework of existing knowledge on the life-history and ecology of this species to formulate a provisional synthesis of the mating system.

Consequently, the specific aims of this study were:

(1) To document changes in the population density and structure of the honey possum populations in the FRNP, that may have implications for the mating system and reproduction.
(2) To examine whether females mate with more than one male, whether there is multiple paternity within litters, and therefore if there is evidence for sperm competition.
(3) To determine how wide-spread multiple paternity is in the population.
(4) To determine if the incidence of multiple paternity is consistent during the changes in population density and available food resources experienced in the years 2000-2002.
(5) To describe embryonic diapause, conceptus development and the changes in the female reproductive tract throughout the reproductive cycle.
(6) To document the reproductive status of all females captured in the population, and utilize this information to investigate the temporal spread of reproductive females in the population. Further, to document the reproductive histories of individually marked females in the population and thereby aid our understanding of reproduction and breeding during a female’s lifetime.

(7) To interpret the findings of this study in the light of the life-history and ecology of the honey possum.

1.3 Thesis overview

This thesis is organized into seven chapters. Chapter 2 describes the Fitzgerald River National Park and the study sites, together with trapping and general sampling protocols. Chapter 3 reviews the existing knowledge of the life-history and breeding of the honey possum. Chapter 4 presents the results of the analysis of the long-term dataset with respect to population densities and population structure over time. Chapter 5 describes the genetic analyses and assessment of multiple paternity using microsatellite markers. Chapter 6 begins with an overview of functional marsupial reproduction and embryonic diapause against which the histological descriptions of the honey possum female reproductive tract and conceptus development may be interpreted. This chapter investigates embryonic diapause and reproductive processes in the female. It also presents new information on the reproductive cycle which is discussed in the light of those previous findings presented in Chapter 3. Finally, Chapter 7 draws together these aspects of the life-history, ecology, reproduction and mating patterns in order to discuss the mating system of the honey possum.
Chapter 2: Site Description and Field Methods

2.1 Fitzgerald River National Park

The Fitzgerald River National Park (FRNP) is a 328 026 hectare reserve situated on the south coast of Western Australia, approximately 500km south-east of Perth, between the coastal towns of Bremer Bay and Hopetoun (Figure 2.1). The Fitzgerald is an International Biosphere Reserve, recognised by the United Nations Educational Scientific and Cultural Organization (UNESCO). It is renowned for its highly diverse flora and fauna, including many rare species, and also for its spectacular coastal scenery, gorges, inlets, extensive plains and Barren Ranges (Moore et al. 2001). Much of the park is free of any human interference and therefore represents a very large area of ‘natural’ ecosystem.

Five major and two minor landforms are recognized within the Park (Moir and Newbey 1995; Newbey and Chapman 1995; Moore et al. 2001). In the north of the park there are ‘uplands’ and ‘greenstone’ areas which are characterized by a gently undulating plain and V-shaped river valleys, with underlying rock of granite and gneiss, and loamy sand soils with exposed granite. These northern areas represent 34% of the area of the FRNP. In the remaining south of the park there is a ‘marine plain’ (44% area) of sandy and clay soils with exposures of the spongolite siltstone bedrock and numerous swamps, extensive ‘spongolite gorges’ (10% area) down the Fitzgerald and Hamersley Rivers, and the rugged ‘Barren Ranges’ (10% area) which are mainly in the coastal areas and consist of quartzite and phyllitic schist. The two minor landforms are coastal dunes and inlets. There are five rivers in the FRNP which are all saline and all end in large inlets. There is also much laterite over the different rock types throughout the park (Moore et al. 2001).
The FRNP has a dry-mediterranean climate with cool damp winters, warm to hot summers and erratic rainfall (Moir and Newbey 1995). Cold fronts, strong on-shore winds and moist mid-level cloud are common in both winter and summer. Rainfall occurs in all months of the year, but is greatest over winter with only occasional heavy rain during summer (Moir and Newbey 1995; Wooller et al. 1998). Monthly rainfall averages 60-75mm in winter and 20-30mm in summer (Wooller et al. 1998). The average number of raindays is 103 per year. Rainfall decreases markedly inland from the coast, but temperature is less variable (Moir and Newbey 1995). Average summer maximum temperatures are 25-29°C and average winter minimum temperatures are 5-8°C (Moir and Newbey 1995). Wind is an important factor in the Fitzgerald climate and days without wind are uncommon.

The south-west of Western Australia is recognized for its diverse flora, and the FRNP is within one of two nodes of high species richness (see Figure 1.1) and is therefore an important conservation reserve. It contains 1748 identified plant species, including 75 endemic species and 250 rare or geographically restricted species (Moore et al. 2001). Generally the vegetation consists of very open mallee, mallee and shrubland, with heath more common in coastal areas and woodlands mainly along rivers or in swamps (Newbey 1995; Moore et al. 2001). The flora is dominated by plants from the Proteaceae and Myrtaceae families. The peak flowering season is between August and November (Moir and Newbey 1995), although there are many species in flower at all times of year.

The Fitzgerald area is also one of the most important faunal conservation reserves in Western Australia. It contains more species of vertebrates than any other conservation reserve in south-western Australia, and many of these species are remnants of a once widespread and rich fauna present before European settlement (Chapman 1995). The decline in mammal species (42% of species lost, Kitchener et al. 1980) in south-western Australia has been attributed to a number of factors including clearing, feral cats, foxes, grazing and changes in fire regimes. The large size of the FRNP and its lack of widespread habitat degradation make it an important
reserve for fauna, as well as forming part of a corridor of uncleared vegetation from the coast to inland areas (Chapman 1995; Moore et al. 2001). At present in the FRNP, there are 22 species of native mammals (seven of which are declared rare), 41 species of reptiles, 12 species of frogs, 184 species of birds (3 of which are declared rare) and 4 species of inland fish (Chapman 1995; Moore et al. 2001). The honey possum is the most abundant and widespread small mammal in the FRNP and is found throughout the park (Chapman 1995).

2.2 Sampling of the honey possum in the FRNP

Trapping of honey possums in the western end of FRNP has been the primary focus of a long-term study, started in 1984, by Murdoch University researchers led by Associate Professor R. D. Wooller (eg. Wooller and Richardson 1992; Wooller et al. 1993; Wooller et al. 1998; Wooller et al. 1999; Wooller et al. 2000). Together with the trapping undertaken during the present study (2000-2002), this resulted in a total of 19 years of data available from the trapping sites described below. These long-term data have been utilized in later chapters (3 and 4). The trapping for the present study was conducted in order to monitor the populations and to study the mating system and reproduction of the honey possum. This involved a continuation not only of the mark-recapture study, but also collection of tissue samples for the study of multiple paternity by genotyping, as well as collection of samples for exploration of the female reproductive tract. The following sections describe the general field methods for trapping honey possums, with more specific methodologies described in greater detail in the appropriate chapter.
Figure 2.1: Location of Fitzgerald River National Park and the study sites A, B, C, D and E in relation to Gairdner Road. Refer to Figures 2.2 and 2.3 for enlargement of the area within the dashed line.
Figure 2.2: Location of the two trapping grids at site A, in long unburnt mature vegetation.
Figure 2.3: Location of the two trapping grids at site B, three trapping grids at site C, and one trapping grid at site D, collectively referred to as the 'hill area'.
2.2.1 Description of trapping sites within the FRNP

Honey possums were caught at the western end of the FRNP (34°12’S, 119°22’E), off Gairdner Road, in heathland and shrubland typical of the marine plain, the most extensive vegetation complex within the park. The area contains about 70-80 species of flowering plants, mainly from the families Proteaceae, Myrtaceae and Leguminosae (Wooller et al. 1993). There were five trapping sites (sites A, B, C, D and E). These differed in their vegetation structure and time since fire. Site A was located in long-unburnt mature vegetation (Figure 2.2) and sites B, C and D were all located within the same hill area with various burn regimes (Figure 2.3). The hill area was approximately 3.5km from the long unburnt area (site A), and site E was a further 3km from site A (6.5km between the hill and site E). For studies of the mating system and reproduction, trapping was concentrated in sites A and C, because they were stable populations that had not suffered from fire recently.

The trapping sites each had square grids of pitfall traps consisting of 10 x 10 pitfall traps, each 5m apart. Earlier studies had shown this to be the optimal trap spacing for sampling all individuals within an area (Wooller et al. 1981; Wooller and Richardson 1992; Garavanta et al. 2000). The pitfall traps consisted of lengths of PVC pipe (40cm deep and 15cm in diameter) sunk vertically into the ground, so that the rim was flush with the surface. The traps were open at the bottom and lined with mesh to allow drainage, and thus safeguard against flooding, yet not permit animals to dig. The traps were left in situ and fitted with a secure lid when not in use.

Site A consisted of two grids approximately 350m apart, each of 100 traps. They were located in high, dense scrub vegetation which had remained unburnt for 50 years or more. It contained mature vegetation with an upper canopy up to 3m high consisting mainly of Banksia baxteri, Hakea victoria and B. coccinea. It had a dense understorey ranging from 0.5–1m tall with a median projective foliage cover of 75% (Everaardt 2004). Honey possum foodplants characteristic of the understorey at site A included Banksia nutans, their key summer-flowering foodplant, as well as B.
Bauera and Dryandra species, which provide their staple diet over winter (Wooller et al. 2000).

Site C consisted of three grids in a line and approximately 100m apart, each with 100 traps. For studies of the mating system and reproduction, trapping was concentrated in grids 2 and 3 at this site. The grids were located in low open mallee heath last burnt in December 1989. This site consisted mainly of a low (0.5-1.0m) stratum of regenerating vegetation with a median projective foliage cover of 35% (Everaardt 2004). It also contained about 70-80 species of flowering plants. The sparse upper canopy consisted mainly of Eucalyptus tetragona, E. buprestium and Lambertia inermis. Honey possum foodplants characteristic of site C included Banksia baueri, Dryandra spp., and E. buprestium.

Long-term trapping studies have also been undertaken at sites B and E and, although not the focus of studies on the mating system and reproduction, these sites provided ancillary data. In addition, animals commonly moved between sites B and C (see Section 4.2.3) and therefore tissue samples were taken from males at site B to maximize the genetic sampling of potential sires. Site B contained two grids, each of 100 traps. It had been burnt in May 1998 and consisted mostly of seedlings and regenerating plants, less than 0.5m high, with sparsely occurring shrubs of Adenanthes cuneatus and Calothamnus gracilis, as well as Eucalyptus buprestium of greater height. The median projective foliage cover rose to only 15% during the study (Everaardt 2004) and there was very little in flower at this site. Site E consisted of one grid of 100 traps in Banksia baxteri shrubland and was located 3km from site A.

Site D was located approximately 450m from site C, and trapping at this site focused primarily upon the collection of honey possums for study of the female reproductive tract (Chapter 6). This distant site was used in order to minimize the impact that removal of animals might have had on the population monitoring sites. Therefore, analyses of population density (Chapter 4) do not include data from this site.
However, information from this grid was included for detection of movements of individuals between grids (Section 4.2.3) and for genetic studies (Chapter 5). This area had been last burnt in December 1989 and the vegetation was similar to site C.

Plants common to all sites, that are commonly utilized by honey possums, include; *Calothamnus gracilis, Eucalyptus buprestium, Banksia nutans, B. baueri, Dryandra plumosa, D. cuneata, D. nivea, D. falcata, Lambertia inermis, Adenanthes cuneatus, B. gardneri, B. repens, B. coccinea, B. attenuata* and *Beaufortia spp.* (Saffer 1998; Everaardt 2004).

2.2.2 Trapping regime

At each site honey possums were caught in pitfall traps over periods of three consecutive nights, during which the traps remained open. Previous studies have shown that three nights was the optimal duration of trapping in order to catch most individuals present in an area, whilst minimizing the deleterious effects of repeated capture upon individuals (Wooller et al. 1981; Wooller and Richardson 1992; Garavanta 1997). All traps were checked daily during each trapping session, shortly after sunrise, to minimize the length of time that animals remained in the traps.

Table 2.1 shows the timing of trapping sessions from 1984 onwards. For studies of the mating system and reproduction (2000-2002) there were three objectives when deciding the spacing of trapping periods. The first objective was population monitoring using mark-recapture. The second was to collect tissue samples from litters and adult animals for the study of multiple paternity by genotyping. The third objective was to collect samples for exploration of the female reproductive tract. The spacing of trapping periods was therefore determined by the need to target periods particularly informative to mating and reproduction from June 2000 to July 2002. A limited amount of trapping was also undertaken in 2003 to collect reproductive tract samples and note information on the movement of recaptured individuals marked in 2002 (see Section 4.2.3). Since the incidence of females with pouch-young was
highest in winter (see Section 3.7), trapping periods were concentrated over the winter months in 2000, 2001 and 2002 at sites A and C (Table 2.1). Trapping at other times provided mark-recapture data and information on the prevalence of pouch-young, which formed the basis of decisions as to when the next appropriate time of sampling for mating system studies would be. Trapping periods conducted in the months prior to winter enabled sampling of potential fathers of those offspring subsequently sampled in winter (see Section 5.2.1). In general, however, no more than 2 months elapsed between trapping sessions, giving regular collection of tissue samples and therefore as high a resolution as practicable.

2.2.3 Sampling of honey possums and mark-recapture

Each honey possum captured was sexed, marked with an individual number, measured and then released near the trap in which it had been caught. Individuals were marked by making small notches in the margins of the ears using a 1 mm ear punch. Notches were placed in set positions, from anterior to posterior along the ear margin, which gave the possibility of numbers 1, 2, 4, and 8 in the left ear and 16, 32, 64, and 128 in the right ear. This gave a possible 255 combinations forming individual numbers, although no more than 3 notches were made in each ear due to welfare considerations, so that some combinations remained unused. The ear tissue from each individually marked animal was preserved for DNA analysis. During some trapping periods, animals of a particular class, for example juveniles or non-reproductive females, were marked with the same number to serve as a date stamp and indicator of life-history stage. This was necessary to conserve numbers. Otherwise, the large number of individuals caught would have exhausted all possible combinations. Individual numbers were duplicated on males and females. Sites B, C and D on the hill area were subject to a single set of number combinations because of their proximity and the possibility of movement by individuals between the sites. Again, to save number combinations, the same set of numbers were, however, duplicated at the distant site A. Previous studies have shown that the home range of *Tarsipes* is 1277m$^2$ for males and 701m$^2$ for females with the greatest distance
travelled for most individuals as 163m (Garavanta 1997; Garavanta et al. 2000). Thus, it was considered that the possibility of migration between sites A and C would be very low.

The live weight of animals was measured in the bag to ± 0.1g with electronic scales. Using vernier callipers, to an accuracy of ± 0.1mm, head length was measured from the back of the skull to the tip of the snout, the width of the tail at its base was taken as an indicator of condition and, for males, maximal scrotal length and scrotal width were measured, including the epididymes.

Females were examined for the presence and number of pouch-young by gently opening the pouch with a pair of blunt forceps. Pouch-young were not measured directly as this would have involved their removal from the pouch, which is impossible while they are permanently attached to the teat during the first third of pouch life (Russell 1982); after that time there is a risk of rejection by the mother (Wooller and Richardson 1992). Instead, the crown to rump size of the curled pouch-young was estimated by eye. Tissue samples for DNA analysis were also taken from pouch-young (see below for details). If a female had no pouch-young, but had enlarged teats and mammary glands, this indicated she was still lactational. If only the teats were elongated then she was described as being post-lactational.

Those female honey possums caught at site D which were at the particular reproductive stages needed for exploration of the reproductive tract, were sacrificed by barbiturate overdose and preserved as described in Section 6.2. Any animals that accidentally died during the mark-recapture study were also preserved. A limited number of animals were also sacrificed from areas A and B in order to minimize any impact on those sites used for population monitoring, but to supplement the collection of females at very specific reproductive stages.
Table 2.1: Timing of trapping sessions over the year for long term studies in the FRNP (1984-2002). In addition, limited trapping sessions in 2003 provided data on recaptures of individually marked animals (section 4.2.3) and samples for reproductive tract examination only. Studies of the mating system and reproduction from 2000-2002 were primarily at sites A and C (grids 2 and 3). Most often only one session was conducted per month. A dashed horizontal line indicates the establishment of a grid.

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2.2.4 Tissue sampling

During all trapping periods, ear biopsies from all individually marked animals of both sexes captured from all sites were preserved for DNA analysis. In order to eliminate cross-contamination of DNA, the instruments used for tissue sampling (ear punch, scissors and forceps) were washed with 100% ethanol and wiped with a lint free tissue before each sample was taken. Samples were preserved in approximately 1 ml of 20% DMSO solution saturated with NaCl. The samples were kept at 4–8°C until extraction of the DNA.

Tissue samples were also taken from mothers and litters of pouch-young to analyze litters for multiple paternity. Different techniques were tested to obtain tissue samples from young, because of the difficulty of the task. Given that honey possums do not use permanent nests, the only way to gain access to the young was to sample them in the pouch. The tiny pouch-young, ranging in crown-rump size from 3 to 25mm, are present deep within the pouch which is tightly closed by a sphincter muscle. In addition, honey possums show brood reduction (Section 3.4). Thus, as the young become larger and easier to sample, there are fewer of them, thereby reducing the power of detecting multiple paternity. Taking hair samples was not feasible because the extraction of DNA from hair was unsuccessful, and furring young were only present well after brood reduction had already occurred. Removal of tissue from the body, ears or limbs was too difficult and deemed unethical. In addition, female honey possums can become stressed if inspection of the pouch is too invasive. There is then a consequent risk that young will be expelled from the pouch, and often expulsion or removal of young from the pouch results in permanent rejection of that young by the mother. The only successful sampling strategy was therefore to take a tiny piece of tissue (≤ 1mm) from the tip of the tail without removing the young from the pouch. This was performed only on young of crown rump size 8 to 10mm or larger, and only when the female was not unduly stressed. Where females were active, they were placed in a calico bag, on freezer bricks in an insulated container for several hours, in order to induce a natural torpor, or at least
sleepiness, in which they were unaware of the sampling. This technique allowed sampling of litters still containing three to four young, whilst minimizing the stress on the animals. Many of the litters sampled in this way were subsequently recaptured and appeared unaffected by the sampling. Tissue samples were also taken from any litters that had been sacrificed for reproductive tract studies. Anaesthetic was considered as an option, but administering the correct dose to such a tiny animal proved extremely hazardous. In general, only pouch-young from the recently unburnt areas A, C and D were sampled.
Chapter 3: Life-history of the Honey Possum

3.1 Introduction

The purpose of this chapter is to present some of the existing knowledge of the life-history of the honey possum, including breeding cycles throughout the year. The long-term data set (1984-2002), including that from the present study, is utilized to provide updated data for illustration of existing knowledge. Subsequent findings are then presented later in the results sections of Chapters 4, 5 and 6.

3.2 Longevity

The lifespan of the honey possum has been estimated to be between 1 and 2 years (Russell and Renfree 1989; Wooller et al. 2000). Previous studies of the honey possum in the Fitzgerald River National Park (FRNP) from 1984 to 1996 have shown that most individuals were recaptured between 1 and 6 months after their original capture, and none more than 18 months after their original capture (Garavanta 1997). The estimated annual mortality rate from this data was 86% (Wooller et al. 2000). Data collected during the last three years (2000-2002) for studies of the mating system and reproduction, revealed that most individuals were recaptured between 1 and 4 months after their original capture, with a small number of individuals caught up to 24 months after their original capture (Figure 3.1). A maximum lifespan of 2 years has been observed in captive honey possums, where no adults lived for more than 2 years, even when fed *ad libitum* (Wooller et al. 2000). Honey possums are clearly shorter-lived than larger possums and gliders (10-20 years: Smith and Lee 1984), and other species of small possum (Table 3.1). However, a lifespan of 1-2 years is common amongst the dasyurids (McAllan 2003) and survival to little more than 1 year, or one or two breeding seasons, is common amongst small eutherian mammals, such as rodents and shrews (Macdonald 2001).
Figure 3.1: Interval between first and last capture of (a) males and (b) females caught in at least two different trapping sessions. Data are for individuals first captured in March 2000 through to October 2001, and subsequently recaptured from March 2000 to September 2002, when trapping sessions were 1-3 months apart. Data have been pooled from all trapping areas.
Table 3.1: Comparison of life-history attributes of very small mammals. Litter size is given as a mean where data was available. Diet: N=nectar, P=pollen, S=seeds, F=fruit, I=insects

<table>
<thead>
<tr>
<th>Species</th>
<th>Adult body size</th>
<th>Lifespan</th>
<th>Age at maturity</th>
<th>Litter size</th>
<th>Diet</th>
<th>Breeding</th>
<th>Sociality</th>
<th>References</th>
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<tr>
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<td></td>
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<tr>
<td>Tarsipes rostratus</td>
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<td>8-12</td>
<td>1-2</td>
<td>4-6</td>
<td>N, P</td>
<td>All months</td>
<td>Solitary</td>
<td>see text</td>
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<td>12-18</td>
<td>12-16</td>
<td>&gt;3</td>
<td>7-18</td>
<td>N, P, S, I</td>
<td>July-Jan</td>
<td>Family groups in nest boxes</td>
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<tr>
<td>Cercartetus nanus</td>
<td>23-40</td>
<td>3-5</td>
<td>4.5-10</td>
<td>4.5-10</td>
<td>N, P, S, I</td>
<td>Nov-March</td>
<td>Solitary</td>
<td>5, 6</td>
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<td>-</td>
<td>N, P, I</td>
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<td>-</td>
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<td>N, P, I</td>
<td>most months?</td>
<td>-</td>
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<td>2-11</td>
<td>12</td>
<td>12</td>
<td>I, S, F</td>
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<td>9.5-16</td>
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<td>1-4</td>
<td>6.5</td>
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<td>Aug-Dec</td>
<td>-</td>
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<td>1-2?</td>
<td>8</td>
<td>-</td>
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<td>I</td>
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<td>1-2</td>
<td>-</td>
<td>4</td>
<td>7-10</td>
<td>I</td>
<td>July-Feb</td>
<td>Solitary</td>
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<td>Common shrew</td>
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<td>1-2?</td>
<td>12</td>
<td>9-14</td>
<td>6-10</td>
<td>I</td>
<td>April-Oct</td>
<td>Solitary</td>
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<td>Mus musculus domestica</td>
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<td>2</td>
<td>1-2</td>
<td>4-8</td>
<td>S, I</td>
<td>Oct-April</td>
<td>Family groups</td>
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</tbody>
</table>

3.3 Size and maturity

Detailed data on the size and sexual dimorphism of honey possums caught during long-term studies in the FRNP have not been presented previously. Figure 3.2 depicts frequency histograms for weight and head length of all free-living honey possums captured at all trapping areas in the FRNP from 1984 to 2003. Honey possum young leave the pouch at about 2 to 2.5g (Renfree et al. 1984) and free-living young were captured in pitfall traps from this stage onwards (Figure 3.2). The sexes are readily distinguishable in large pouch-young and onwards. Adult females (8-12g) are markedly larger than adult males (6-9g) (Wooller et al. 1981; Russell and Renfree 1989). Both sexes reach maturity at 6g and 24mm head length (see below). Therefore animals with body measurements equal to, or greater than, these were included in the following analysis of sexual dimorphism. Adult female mean weight (10±3.2g, n=1898, SE=0.07) and head length (27.1±2.0mm, n=2112, SE=0.04) were significantly greater than adult male mean weight (7.8±1.5g, n=2352, SE=0.03) and head length (26.3±1.5mm, n=2644, SE=0.03) (weight: $t_{(2601)}=-26.4$, $p=0.000$; head length: $t_{(3891)}=-14.7$, $p=0.000$). The t-tests did not assume equal variances. Growth rates of the head lengths of males and females are similar, but females continue to get heavier (Wooller et al. 1981).

Both males and females become sexually mature at around 4-6 months of age. A recent study investigated sexual maturity in males using 25 individuals collected from the FRNP, ranging in size from 2.4g to 10.3g (Rutter 2001). Nine males of 5.2g and less showed no beginning of spermatogenesis in the testes and all had small prostate glands of less than 1.0mm diameter. Of two males examined, both weighing 5.5g, one showed only the initial stages of spermatogenesis, but no mature sperm; the other displayed active spermatogenesis, and his testes and epididymis contained mature sperm. These males had prostate glands of 1.0mm and 3.1mm diameter respectively. All males 6g and above displayed active spermatogenesis and had mature sperm in the testes and epididymis, indicating their ability to sire offspring. A 6g honey possum has a head length of about 24mm, a size reached
after approximately 6 months. In these 25 males the prostate diameter and scrotal volume increased sharply at a body weight of approximately 5.2g. The increase in scrotal volume after 5g was also consistently found in measurements of males during long-term trapping data (1984-1996) (Rutter 2001). Interestingly, a slight decline in scrotal volume, also apparent in long-term trapping records, and in prostate diameter was detected in males greater than 9g. However, the small number of individuals examined still had active spermatogenesis and mature sperm in the testes and epididymis (Rutter 2001). Thus, the onset to maturity in males appears to occur rapidly between 5 and 6g, around six months after birth.

Generally, females first breed when about 6g and 24mm in head length, but a small number of females were found to have pouch-young when smaller in size (Figure 3.2 e and f). The oestrous cycle and ovulation begins, in at least some individuals, at a size of about 4g and 22mm head length (see Chapter 6).

Honey possums therefore become sexually mature whilst they are still growing, and females have the potential to breed at less than half their final adult size. This is a characteristic shared by many macropods (Jarman 1983). Age at sexual maturity is at the low end of values reported for other small possums (Table 3.1), and is much less than the 9-12 months to maturity for most of the short-lived dasyurids (McAllan 2003). However, when compared to the tiniest dasyurids weighing less than 20g, such as the fat-tailed dunnart, *Sminthopsis crassicaudata*, Giles’ planigale, *Planigale gilesi* and the narrow-nosed planigale, *Planigale tenuirostrius*, time to maturity is similar (see Table 3.1 and McAllan 2003). Time to maturity is certainly shorter than other small eutherian mammals, such as shrews (Table 3.1), and is not related to territory establishment, as seen in voles (Ratkiewicz and Borkowska 2000).
Figure 3.2: Frequency histograms of weight and head length for all males (a, b) and females (c, d), and for reproductive females only (either with pouch young or lactational/post lactational: e, f) first caught between 1984 and 2003. Sample sizes as follows: (a) 2985, (b) 3322, (c) 2447, (d) 2693, (e) 1081, (f) 1275.
3.4 Litter size and brood reduction

Female honey possums have four teats; two anterior and two posterior to the pouch opening. As marsupial young remain attached to the same teat for the first few weeks of life, the number of teats determines the maximal litter size (Russell 1982). Honey possums have between 2 and 4 young that are enclosed completely within the pouch throughout most of lactation (Renfree et al. 1984). Incidences of females carrying only one young have been recorded, but are relatively rare. A study of populations at the FRNP showed that the litter is reduced throughout pouch life (Wooller and Richardson 1992). As the size of the young increases, the number of young decreases. Mean litter size soon after birth (approximately 2mm crown-rump length) was 3.6, but this decreased to 2.4 around the time of first pouch exit (approximately 24mm crown-rump length). Most litters of 4 pouch-young consisted of small young (mean crown-rump length 6.7±0.4mm), whereas litters of 3 (10.2±0.3mm) and particularly 2 (14.0±0.5mm) contained proportionately more large pouch-young (Wooller and Richardson 1992). Of 599 females in this study, only 2% had one young, 28% had two young, 47% had three young and 23% had four young.

There was a significant positive correlation between the head length of females and the size of their pouch-young (eg. r=0.927 for 3 young, and r=0.805 for two young) (Wooller and Richardson 1992). This may have been because smaller mothers raise smaller young or lose their young more often. Alternatively, this may reflect the normal growth of females, which begin breeding whilst still small, during the two months of carrying young in the pouch. For particular sizes of young, larger females carried a larger litter, and this difference was significant for litters of 2 and 3 young (Table 3.2: Wooller and Richardson 1992). Weight showed a similar trend, but may be influenced by the additional weight that larger young add to a female’s overall weight. For females with 2 or 3 young, an increased size in pouch-young correlated with increased condition index of the mother. This was not the case for females with four young, in which condition was lowest in those females with large young (Table 3.2). Only the largest, presumably oldest, females are probably able to rear four
young to independence, but their relatively poor body condition may indicate the stress that this places on them (Wooller and Richardson 1992). As a short-lived animal, each litter of pouch young may represent a substantial proportion of lifetime reproductive potential, and reduction in litter size to less than four after birth, rather than before, may be expected (Russell 1982; Wooller and Richardson 1992). Chapter 6 explores amortization over the entire reproductive process in the honey possum. Smaller females, or those in relatively poor condition, may gauge their reproductive investment on the basis of its sustainability (Wooller and Richardson 1992). Older females with larger body size and experience, might be better able to access rich food sources, provide more nutritious milk (Green and Merchant 1988, and see Sections 3.6 and 3.8 below) and thereby be more successful at rearing young. Their expectation of further life is also less than younger females, and therefore their reproductive output may not be limited by future survival (sensu Charnov and Krebs 1974). The honey possum is unusual among small marsupials in the relatively protracted time for which it carries young in the pouch (Section 3.5 below) and larger females may also be more capable of carrying a larger load of young (Russell 1986).

Litter size in the honey possum is relatively small compared to other small possums (Table 3.1). Amongst these small possums, litter size is higher for the Burramys-Cercartetus group and smallest for the honey possum and the feathertail glider, Acrobates pygmaeus (Ward 1998). The honey possum certainly has a much smaller litter than dasyurid marsupials and small eutherians of similar body size (Table 3.1). In this way the honey possum deviates from the typical pattern of large litter size in species with a small body size and limited longevity (Smith and Lee 1984). Smaller litter size is a condition noted for all possums and gliders (phalangeroids), compared to dasyurids, if body size is taken into account (Smith and Lee 1984).
Table 3.2: Mean (±SE) head length, weight and condition index of female honey possums with small (crown-rump length 1-8mm), medium (9-16mm) and large (17-24mm) pouch-young, shown separately for those with two (n=165), three (n=280) and four (n=141) young. Table after Wooller and Richardson (1992).

<table>
<thead>
<tr>
<th>Crown-rump length of pouch young (mm)</th>
<th>Two pouch-young</th>
<th>Three pouch-young</th>
<th>Four pouch-young</th>
</tr>
</thead>
<tbody>
<tr>
<td>Head length (mm)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1-8</td>
<td>26.8 (±0.2)</td>
<td>27.4 (±0.2)</td>
<td>28.0 (±0.2)</td>
</tr>
<tr>
<td>9-16</td>
<td>27.2 (±0.2)</td>
<td>28.1 (±0.2)</td>
<td>27.8 (±0.5)</td>
</tr>
<tr>
<td>17-24</td>
<td>28.0 (±0.2)</td>
<td>29.2 (±0.2)</td>
<td>31.0 (±0.6)</td>
</tr>
<tr>
<td>Weight (g)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1-8</td>
<td>9.9 (±0.4)</td>
<td>10.6 (±0.4)</td>
<td>11.5 (±0.3)</td>
</tr>
<tr>
<td>9-16</td>
<td>11.2 (±0.4)</td>
<td>12.7 (±0.4)</td>
<td>12.7 (±0.6)</td>
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<tr>
<td>17-24</td>
<td>14.4 (±0.4)</td>
<td>15.2 (±0.8)</td>
<td>18.8 (±0.6)</td>
</tr>
<tr>
<td>Condition index</td>
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<tr>
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<td>13.1 (±0.4)</td>
<td>12.8 (±0.3)</td>
<td>13.2 (±0.3)</td>
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<tr>
<td>9-16</td>
<td>13.0 (±0.5)</td>
<td>13.8 (±0.4)</td>
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<td>17-24</td>
<td>14.1 (±0.5)</td>
<td>13.6 (±0.6)</td>
<td>12.2 (±0.7)</td>
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</tbody>
</table>

3.5 Growth, pouch-life and weaning

Female honey possums have a post-partum oestrus and exhibit embryonic diapause throughout lactation (Renfree 1980; Chapter 6). This has made the active gestation length difficult to determine (Renfree et al. 1984). Newborn honey possums are the smallest mammalian young at birth (Renfree et al. 1984). In one litter, the three neonates weighed 3, 4 and 6mg (Renfree 1980). In the present study, one litter had one neonate weighing 3.9mg and another weighing 4.7mg (see Table 6.1). Head lengths of neonates are approximately 1-2mm (Wooller et al. 1999; this study, Chapter 6).
The pouch is deep and well developed, completely enclosing the young, and it is closed by a sphincter muscle (Renfree et al. 1984). As Russell (1982) notes; “as the young grow it becomes deep and bilocular, extending on each side almost onto the flanks on the animal”. The mammary glands are well developed in lactating honey possums and the teats become extremely elongated (Renfree et al. 1984). The honey possum is distinct from the dasyurids in these features.

As with other mammals, much of the variation in life-history parameters of marsupials such as length of pouch life and time to weaning, can be explained by differences in body size (Russell 1982; Smith and Lee 1984). Compared to marsupials of similar size, the growth of honey possum young is relatively slow (Wooller et al. 1999). Wooller et al. (1999) compared the growth curves of the honey possum with other similar sized small marsupials (Figure 3.3). Three small insectivorous dasyurids are shown on the left of Figure 3.3, and these are Giles’ planigale, Planigale gilesi (data from Whitford et al. 1982), the southern ningaui, Ningaui yvonneae (data from Fanning 1982), and the stripe-faced dunnart, Sminthopsis macroura (data from Frigo and Woolley 1997). The feathertail glider, Acrobates pygmaeus (growth data from Ward 1990a) and the eastern pygmy possum, Cercartetus nanus (growth data from Ward 1990b) both include insects, nectar, pollen and seeds in their diet. Faecal samples from the feathertail glider show predominantly pollen (Turner 1984a) and, in general, fewer insects and more pollen than the eastern pygmy possum, where the two species are sympatric (Huang et al. 1987). Data from one captive western pygmy possum, Cercartetus concinnus, fit the growth curve of the eastern pygmy possum (Wooller et al. 1999). Approximate time of weaning is indicated by an arrow in Figure 3.3. The rate of development in the eastern pygmy possum is similar to other fast growing insectivorous marsupials in terms of first appearance of morphological features (Russell 1982) and growth rate (Figure 3.3). However, both the honey possum and the feathertail glider, the two species with the largest components of nectar and pollen in the diet, lack the period of rapid growth early in pouch life that the other more insectivorous species exhibit, and their time to weaning is longer (Wooller et al. 1999; Figure 3.3). Smith and Lee (1984) noted that,
compared to other possums and gliders, the honey possum has a lower offspring production rate (litter size x weight of young/time to weaning) than predicted for its body size. Russell (1986) also noted a low parental investment (weight of litter/female body weight) in the honey possum and feathertail glider.

Figure 3.3: Growth curves showing the more rapid growth in size and earlier onset of weaning (indicated by an arrow) in three small marsupials with purely insectivorous diets (*Planigale gilesi*, *Ningaui yvonneae* and *Sminthopsis macroura*) and one with a partially insectivorous diet (*Cercartetus nanus*), compared with two small marsupials (*Tarsipes rostratus* and *Acrobates pygmaeus*) in which nectar and pollen form large components of their diets. After Wooller et al. (1999).
The pouch life of the honey possum lasts between 56 and 65 days (Renfree et al. 1984; Wooller et al. 2000). It is much longer than other small possums, such as the 33-37 days of the eastern pygmy-possum (Ward 1990b), the 25 days of the western pygmy-possum (Ward 1990c), and the 30 days of the mountain pygmy-possum (Mansberg and Broome 1994), but similar to the feathertail glider (65 days, Ward 1990a). In the last few weeks of pouch-life, the female honey possum is obviously encumbered by her litter (Russell 1986). First pouch-exit is followed by a few days when the young still return to the pouch, and the transition to permanent exit is rapid (Russell 1982). The young are between 2 and 2.5g when they first leave the pouch. They are fully furred, including the dorsal stripes, and have their eyes open (Renfree et al. 1984; Russell and Renfree 1989). At first, their mother leaves them at a safe location whilst she forages. Honey possums do not appear to construct a nest, but rather use existing structures such as abandoned bird’s nests, branches or skirts of grass trees (Russell and Renfree 1989). Within one week the young are active and able to run and climb (Russell 1986). They may cling to the mother’s belly or ride on her back, but she appears to discourage them from doing so. In captivity, the young continued to suckle, but ate some honey one week after first pouch exit. In captivity, weaning occurred at around 90 days (Russell and Renfree 1989; Wooller et al. 1999), but in the wild lactation may be shortened. Eight females captured with pouch-young and later recaptured with another litter of the same size, indicated a length for this entire cycle of 65-67 days (Wooller et al. 2000). Additional data on the cycle length of the honey possum will be presented in Chapter 6. There is a period after weaning during which the young travel and associate with their mother. This has been observed both in captivity (Russell 1986) and by various field-workers during mark-recapture studies. Where a female was caught with a juvenile in a pitfall trap, upon release the juvenile would, on some occasions, climb on the female’s back or assume close association with her. It is possible that this association may last after the birth of the next litter, although it is not likely to be prolonged, as Garavanta (1997) noted no association between adult female and juvenile home ranges and concluded that young disperse soon after weaning.
The advanced stage to which the honey possum carries its young clearly differs from the typical dasyurid pattern of leaving the young in the nest half way through lactation, whilst they are still blind and naked (Russell 1982; Renfree et al. 1984). It is also different from the pygmy possums and the feathertail glider that leave their young in the nest before they are fully developed and before their eyes are open (Russell 1986). Not only do honey possums have slower growth and development, they spend a longer period of their development in the pouch. This places a great burden on the mother (Russell 1986).

### 3.6 Dietary constraints on reproduction

The slow growth of honey possum young and their comparatively small litter size has been attributed to the limitations of a diet of nectar and pollen (Wooller et al. 1999). In early lactation, whilst the young are permanently attached to the teat, the milk of marsupials is dilute, but milk production and concentration increase throughout lactation, to meet the increasing demands of the young (Green and Merchant 1988). Also, both the protein and lipid content of the milk increase significantly throughout lactation. Certain amino acids in the milk predominate at different stages of lactation, and these changes are thought to be related to the different amino acids required during development at different stages (Renfree 1981, cited in Green and Merchant 1988). Similarly, changes in vitamins and mineral content of the milk throughout lactation are thought to be related to the changing requirements of the young (Green and Merchant 1988).

The energetic burden on lactating mothers appears to be high and their own energy requirements increase substantially during lactation (Green et al. 1997). Transfer of nutrients through the milk of the mother is responsible for differences in the growth rate of young among marsupial species (Merchant and Sharman 1966). Greater milk production by larger female tammar wallabies *Macropus eugenii* has been implicated in the faster growth shown by young of larger mothers (Green and Merchant 1988). The slow growth and long lactation of a single young in large macropod species has
been attributed to their herbivorous diet and grazing in habitats with low water, nutrient and energy resources (Green et al. 1997). The low protein, low energy diet of large leaf-eating marsupials, and the time it takes for the microbial digestion of cellulose, have been implicated in the dilute milk they have compared to other marsupials, resulting in small litter sizes, long lactational periods and slow growth of young relative to other marsupials of similar size (Smith and Lee 1984; Munks et al. 1991). This may help to spread the burden of lactation over a longer period (Green et al. 1997). In medium-sized phalangeroids, a low proportion of protein intake (from insects and pollen) relative to energy intake (from nectar, saps, gums and honey dew) has been suggested to limit reproduction because of the high proportion of protein in milk and in offspring tissue (Smith and Lee 1984).

Similarly, the nutrient constraints of a diet of nectar and pollen have been suggested as the reason for slow growth and small litter size in the honey possum (Russell 1986; Wooller et al. 2000). Nectar is essentially a sugar solution and is energy rich, but contains very few other nutrients, whereas pollen is rich in carbohydrates, protein, lipids and trace nutrients (Simpson and Neff 1983). Pollen is thus the sole source of protein and nitrogen in the honey possum diet (Wooller et al. 1999; Bradshaw and Bradshaw 2001). Whilst the honey possum has many adaptations to feeding on nectar and pollen (see Section 1.1.2), they are only slightly more efficient or faster at digesting the contents of pollen grains than other mammals (Wooller et al. 1999). The estimated nitrogen intake of honey possums feeding in the field show that their intake exceeds their everyday requirements (Bradshaw and Bradshaw 2001). However, the nitrogen requirements for reproduction are additional to their maintenance nitrogen requirements (Bradshaw and Bradshaw 2001) and have not yet been investigated. Honey possums may also be limited by the profile of amino acids and other nutrients available in pollen, restricting the growth rate of the pouch-young (Wooller et al. 1999).

There are likely to be time limitations in the collection of energy and nutrients by the honey possum (Wooller et al. 1999), similar to those for large folivores (Smith and
Nectar is 50-90% water and, although the honey possum is able to excrete more than its own body weight in water each day (Slaven 1988), there is likely to be a maximum volume per day that can be ingested (Wooller et al. 1999). Captive honey possums fed on a nitrogen-deficient food mixture, did not attempt to increase their nitrogen intake by ingesting more of the liquid mixture than those animals that were fed on a higher nitrogen mixture. Wooller et al. (1999) suggested a parallel with hummingbirds, which must wait for their digestive tract to empty before ingesting more nectar (Diamond et al. 1986). A similar restriction may place time limits on energy acquisition by honey possums. Foraging in the wild requires visiting many Banksia and Dryandra inflorescences that have large numbers of flowers that open progressively over a period of 1-4 weeks. Once these inflorescences are located they would provide food over a period of time, but this may have to be shared with other honey possums and birds. Spool-and-line tracking of individuals showed that they visit a series of inflorescences once or more each night, but that 3-4 different individuals may visit the same inflorescence each night, and honeyeater birds also visit these inflorescences during the day (Garavanta 1997). Amongst honey possums, large females with young have priority access to food sources, at least in captivity, due to their larger size and dominance (see Section 3.8 below and Russell 1986), even if only when the paths of animals cross. Wooller et al. (1999) suggest that obtaining the essential nutrients from pollen needed for lactation, and the time needed to collect and digest these plant products, are the most likely explanation of the slow growth of honey possum young. Another advantage of a prolonged association between mother and young may be that the young can learn foraging techniques (Munks et al. 1991), and this may be all the more important to the honey possum given its short lifespan.

Smith and Lee (1984) suggested that there may be a physical restriction on the number of young that a honey possum can rear, given that it carries them in the pouch for so long. However, as Russell (1986) points out, if nutrient limitation is not a factor, then even if this long pouch life imposes litter size restrictions, the growth rate of young would be greater. It is not known why female honey possums carry
their young for such a long period, although it may be beneficial to carry the young with them during the time consuming process of foraging, rather than repeatedly returning to a nest (Russell 1986).

3.7 Breeding cycle throughout the year

The availability of food also appears to influence the annual pattern of breeding in the honey possum. Wooller et al. (2000) illustrated the annual patterns of breeding in the FRNP populations. The data presented in this section includes that from Wooller et al. (2000), supplemented by data collected during the present study, resulting in long-term trapping data collected over 19 years (1984-2002; see Section 2.2). Data relating to population structure are from trapping sites A and C using only data from long unburnt vegetation (ie. before fire in Dec 1989 at site C; see Section 2.2.1).

3.7.1 Population structure and breeding

In both areas, the number of individuals captured per trapping session (100 traps open for 3 days) was greatest in winter and lowest in spring (Figure 3.4). Further details on population densities are presented in Chapter 4 and the data provided here depicts only the overall seasonal trends in population numbers. Pouch-young were present in all months and in most trapping sessions (91%). Of the trapping sessions when no females with pouch-young were encountered, the majority were in March/April (6), with one such session in February and another in August. Thus, any break in the sequence of births tended to occur in early autumn, but was not recorded in all years. The percentage of adult females with pouch-young was greatest in winter (68-74%), falling slowly through to summer (57%), before a more sudden decrease in autumn (19-29%) (Figure 3.4). In contrast, the percentage of free-living young among individuals captured was smallest in winter, and increased from spring through to autumn. Thus, the large number of pouch-young carried in winter through to early summer, appear to become the free-living young captured in summer and autumn, before they themselves start to breed by the next winter.
Figure 3.4: Seasonality in the number of individuals caught per 3 day/100 trap session (mean ±SE) and in the percentages of individuals consisting of young, adult females (with and without pouch-young) and adult males. Site A and C refer to different areas of long-unburnt vegetation. Data from Wooller et al. (2000), supplemented with data from the current study. Adults weigh >6g or >24mm head length. Sample sizes (n) equals number of trapping sessions.
Table 3.3: The flowering phenologies and food rewards of the plants visited most often by honey possums. A small circle indicates that a species flowers in that month; peak flowering periods are indicated by large circles (After Wooller et al. 2000).

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Mean ± SE percentage carrying pollen</th>
<th>Species in flower</th>
<th>Mean ± SE energy (J) in nectar standing crop per inflorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SE</td>
<td>Winter</td>
<td>Spring</td>
</tr>
<tr>
<td></td>
<td>percentage carrying pollen</td>
<td>J</td>
<td>J</td>
</tr>
<tr>
<td>Banksia baueri</td>
<td>22.1 ± 7.8</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>Banksia gardneri</td>
<td>7.8 ± 4.1</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>Banksia repens</td>
<td>3.6 ± 2.1</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>Banksia coccinea</td>
<td>3.0 ± 1.2</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>Dryandra nivea</td>
<td>10.6 ± 4.3</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>Dryandra cuneata</td>
<td>30.4 ± 8.6</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>Dryandra plumosa</td>
<td>86.0 ± 3.1</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>Banksia nutans</td>
<td>35.8 ± 9.7</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>Banksia baxteri</td>
<td>2.9 ± 1.7</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>Banksia attenuata</td>
<td>6.5 ± 2.8</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>Lambertia inermis</td>
<td>23.6 ± 4.4</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>Adenanthos cuneatus</td>
<td>21.1 ± 6.5</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>Calothamnus gracilis</td>
<td>24.6 ± 6.1</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>Eucalyptus buprestium</td>
<td>88.8 ± 3.4</td>
<td>○</td>
<td>○</td>
</tr>
</tbody>
</table>
3.7.2 Food resources and condition indices

Table 3.3 from Wooller et al. (2000) shows the flowering phenologies of the major foodplants visited by the honey possum in FRNP. Overlapping flowering phenologies of these species ensure that nectar and pollen are available throughout the year. However, banksias provide much more energy than dryandras (Table 3.3). Food resources appear greatest over winter, when Banksia baueri and several Dryandra species flower, and also over summer, when Banksia nutans and Lambertia inermis flower (Wooller et al. 2000). Food appears most scarce in autumn and, rather surprisingly, spring (Wooller et al. 2000), particularly since Lambertia inermis and Adenanthis cuneatus are harvested extensively by honeyeater birds (Saffer 1998). Condition indices of the honey possum in different seasons are congruent with this pattern of flowering phenologies, being highest in winter and low over summer and autumn (Figure 3.5; Wooller et al. 2000).

3.7.3 Annual cycle of breeding

In the FRNP, as well as at a site 100km west (Manypeaks) where earlier studies of the honey possum were undertaken, breeding is continuous throughout the year (Wooller et al. 1981; Wooller et al. 2000). Adult males are clearly present in the population all year round, and they have active spermatogenesis and are capable of siring offspring in all months (Scarlett and Woolley 1980; Rutter 2001). There is also no seasonality evident in either scrotal index or testes volume. Rutter (2001) found a slight decline in scrotal index (scrotal volume after compensation for body size), scrotal length and, to a lesser extent, scrotal width in January, February and March, although this was not linked to any reduction in siring capability. It is possible that this indicates a slight decrease in the quantity of sperm produced by males at this time, which may be linked to a reduction in reproductive effort due to poor body condition at this time of year (Figure 3.5).
Annual capture rates are highest in years following higher than average rainfall (Wooller et al. 1998), presumably because there is more nectar available at these times (Wooller et al. 1993). It is, therefore, not surprising that the incidence of lactation is highest over winter when rainfall is greatest and food availability is highest. Honey possums appear to breed whenever food conditions are sufficient for lactation, regardless of the later consequences for their offspring (Wooller et al. 2000). Although breeding occurs throughout the year, there are times in the FRNP (Wooller et al. 2000) and at Manypeaks (Wooller et al. 1981) when there are few or no females carrying pouch-young. There appears to be a link between the annual cycle of reproduction, population structure and condition indices of the honey possum, and the flowering phenologies of local foodplants (Wooller et al. 2000). This prompted Wooller et al. (2000) to propose a tentative annual breeding cycle for the honey possum, and it is this model that is described below.

Marked synchrony in breeding is not evident, but the greatest proportion of females with pouch-young occur over winter when food is most abundant and body condition indices are highest. Spring is a time of much lower food availability, when mortality of adults and young is probably greatest, resulting in a smaller number of individuals being captured and only moderate percentages of females with pouch-young. This is followed by a period over summer when Banksia nutans provides a good nectar supply and young of the survivors emerge from the pouch, leading to more captures of adults and of young. These young mature over autumn and breed in the winter. There is a marked drop in the percentage of females breeding in autumn, presumably linked to the low food availability at this time, since substantial numbers of adult males are present and capable of siring offspring all year round (Scarlett and Woolley 1980; Rutter 2001). The proportion of free-living young in the population is lowest in winter following this low point in breeding in autumn.

The low point in breeding over March and April in the FRNP, differs from that at Manypeaks, where the only two records of trapping sessions with no pouch young were both in December (Wooller et al. 1981). The two study areas have similar
climates, but differ in the suite of foodplants used by the honey possum (Wooller et al. 1984; Wooller et al. 2000). *Banksia nutans* is utilized at both sites, but the subspecies at Fitzgerald (*B. n. nutans*) that flowers from November to January, is different to *B. n. cernuella* at Manypeaks that flowers from January to April (Wooller et al. 2000). This difference would result in a dearth of nectar in autumn at FRNP, but over summer at Manypeaks. Both these periods coincide with the low incidence of breeding at each site. This may indicate that food supply constrains the virtually continuous annual breeding cycle (Wooller et al. 2000). If conditions allow, females are capable of producing more than one litter in a year. One female, who was recaptured sequentially, had four litters in one year (Wooller et al. 2000).

![Condition index graph](image)

Figure 3.5: Mean (±SE) condition indices for adult male and female honey possums in each season. After Wooller et al. (2000). Condition indices were calculated from basal tail diameters and head lengths measured in all years from 1984-1999, using the methods of Krebs and Singleton (1993).
3.8 Behaviour and sociality

The behaviour and sociality of small, nocturnal mammals is difficult to determine in the wild. A study of social interactions in the field has been attempted, but few observations were made (Garavanta 1997). However, the behaviour of the honey possum has been studied in captivity (Russell 1986). In many respects its behaviour is typical of other marsupials, with the exception of specialized behaviours for feeding (Russell 1986).

 Unless occupied in grooming or feeding, the honey possum runs fast and is very active, with exploratory behaviour involving sniffing other objects, the air or other individuals (Russell 1986). Sniffing is important in social interactions, usually by nose-to-nose sniffing, which may be followed by sniffing of the head, pouch, scrotum or cloaca, in encounters between the same and the opposite sex.

In captivity, much of the honey possum’s non-active time was spent huddling with other animals (Russell 1986), and this has also been observed when more than one animal has been caught down a pitfall trap. Thus, many individuals appeared tolerant of each other. Females huddled with lactating young after pouch exit, but non-related animals of the same and opposite sex also formed part of huddling groups (Russell 1986). Individuals that had had agonistic encounters were found in the huddling groups, but not close together. There is, however, no evidence of protracted pair bonds or socially organized groups. Of animals caught in pitfall traps together with another animal, these associations did not differ from that expected by chance with respect to either sex or size (Rutter 2001). The home ranges of individuals in the wild, both male and female, overlap, and there is no evidence of territoriality or a social unit (Wooller et al. 1981; Russell 1986; Garavanta 1997). Interaction between animals in the wild is most likely to be driven by day-to-day encounters, and they appear to lead essentially solitary lives.
Although in captivity interactions between animals may be somewhat influenced by the artificial environment, consistent patterns emerged (Russell 1986). In the honey possum, ‘dominance’ was related to the outcome of competitive encounters, rather than a form of social organization as such (Russell 1986). Size appeared important and the largest female was dominant and supplanted other individuals at food, without obvious aggression. Females generally lived together amicably, but, being larger than males, were always dominant to males. Females, particularly those with young, aggressively threatened males and supplanted them from food. This was also observed when animals were kept in captivity overnight during this study (Chapter 6). In this way, breeding females in the wild may have priority access to rich food resources (Wooller et al. 1981; Wooller et al. 2000). Males interacted less with other individuals, but Russell (1986) noted that any aggression was usually directed from the largest male to the smaller ones.

Agonistic interactions are typified by an initially slow approach towards another individual, with a fixed stare and tail held straight out behind (Russell 1986). This may be followed by a variety of aggressive acts increasing in intensity, from a push-away with the forelimbs, to lunging or jumping at the opponent, who leaps away and flees, and if caught may be involved in a brief wrestling fight. Honey possums do not have any armaments, their teeth are vestigial and the reduced nails are only present on the hindlimbs, so that aggressive encounters are basic and unritualized.
3.9 Conclusion

In some respects, the honey possum follows a fast life-history pattern typical of mammals with a small body size (sensu Read and Harvey 1989). It is short-lived and matures early. However, it has a small litter, which is reduced during lactation, and the young have a slow rate of growth. Reproduction is probably constrained by the nutritional limitations of an unusual diet consisting exclusively of nectar and pollen. Although breeding occurs continuously throughout the year, its pattern appears to be affected by the different overlapping flowering phenologies of foodplants throughout the year, particularly in autumn when food availability is at its lowest. Breeding appears to be largely opportunistic, occurring whenever conditions will allow. Larger females appear to wean a larger litter and the behavioural dominance of these larger females may allow them priority access to rich food sources, enabling this greater investment in reproduction.
Chapter 4: Population Density and Structure Over Time

4.1 Introduction

Natural cycles of peaks and troughs in population density have previously been noted in the honey possum (Wooller et al. 1998), and were experienced during the present study of the mating system and reproduction. The long term data set (1984-2002) is analyzed in this chapter in a manner not previously undertaken, to highlight these natural fluctuations. It will be determined if these fluctuations are associated with changes in the structure of the population that may have implications for the breeding and the mating system. In addition, this chapter presents data on recaptures and movement patterns of individually marked animals, in order to establish basic patterns of movement for males and for females. Sex-biased dispersal is common in mammals and birds, and is often related to whether the mating system is monogamous or polygamous (Greenwood 1980; Dobson 1982). Genotype data at microsatellite loci were employed to test for sex-biased dispersal. The trapping methods have been described earlier in Chapter 2. Results are presented here together with details on the methods of analysis.

4.2 Methods and results

Sites A and C, described in Chapter 2, were the focus of this study. A concurrent study of honey possums in the Fitzgerald River National Park (FRNP), found significant associations between the densities of honey possums and the burn history of an area, and also with the area per se (Everaardt 2004). Whilst the focus of the present study was not centred on vegetation and burn history, density data from the two areas have been presented separately and their different fire histories have been accounted for where an area had been burnt. In general, Everaardt (2004) found that the structure of the honey possum populations was not affected by the time since an area had been last burnt, nor by the trapping area per se. However, the sole exception was in juvenile sex ratios; in this case data from long unburnt areas only were used in the present study (see Section 4.2.2). Trapping data were
available for site A, in long unburnt, mature vegetation (50+ years since fire) from 1984 to 2002. At the second site, C, the ‘hill area’, data were available for one of these grids from 1984 to 2002 and for the other two grids from 1991/1992 to 2002 (see Table 2.1). Site C had been last burnt in December 1989. In addition, where analyses of covariance (ANCOVA) were used to investigate the relationship of several factors and covariates with population variables, sites B and E were included (see Section 2.2). Statistical tests were performed using SPSS version 11.5. The four seasons were based on differences in climate and are defined by the Bureau of Meteorology as: summer (1 December to 28 February), autumn (1 March to 31 May), winter (1 June to 31 August), and spring (1 September to 30 November). Summer comprised the three months with lowest monthly rainfall, winter those with the heaviest rainfall and the other two seasons were intermediate. Methods and results are presented in three sections, namely population density, population structure and recapture data.

4.2.1 Population density

Over the 19 years, almost all trapping sessions were conducted over three consecutive nights using grids of 100 pitfall traps, but occasionally there were minor variations to this protocol. Corrections were therefore applied in order to allow valid comparisons, and these were calculated separately for each grid. Where trapping sessions lasted two rather than three days (<9% all cases), the number of individuals captured over three days was estimated from the strong regression of captures over three days with captures over two days (Pearson product moment correlation coefficients ranged from $r=+0.943$ to $+0.991$). Sometimes a trap was left closed, for example due to ant infestation, to protect the well-being of the animals. In these cases the number of captures was increased pro rata (eg. 1% for one trap left closed in a grid of 100 traps). If individuals were marked identically across several trapping sessions (<5% all cases), then the number of different individuals was estimated from the strong regression with total captures (Pearson product moment correlation coefficients ranged from $r=+0.966$ to $+0.987$). In this way a value was calculated for
each trapping session of the number of different individuals caught in 100 pitfalls over three consecutive nights (data corrections performed by Everaardt 2004). This was an index of capture and can be used as a minimal estimate of density.

To highlight fluctuations in the capture index over the 19 years, the mean number of individuals was calculated for each trapping session, for all grids within an area during a season. Fluctuations in the capture index occurred from season to season within years, and also from year to year at both trapping areas (Figure 4.1). Similar fluctuations in the capture index were reflected across the two areas. Aside from the local effect of fire at site C, if numbers were high in one area, they were also high in the other area. Alternately, if numbers were low in one area, they were also low in the other area. The three years of studies of the mating system and reproduction (2000-2002) had fluctuations as great as any seen in the entire 19 years. In 2000, numbers were high relative to other years, reaching as high as 38 individuals per average trapping session at site C and 23 per session at site A (Figure 4.1). This is equivalent to 76 individuals per hectare and 46 individuals per hectare at sites C and A respectively. However, this was a year of exceptionally low rainfall (Figure 4.2), and the following year had much lower numbers of honey possums. The number of individuals reached as low as 5 per average trapping session at site C (10/hectare), and down to 2 individuals (4/hectare) at site A. Indeed, spring 2001 had amongst the lowest densities seen in all the 19 years. However, higher than average rainfall occurred in 2001 and the densities in 2002 were markedly greater (Figure 4.1). In winter 2002, Site C had 36 individuals per trapping session (72/hectare) and site A had 34 individuals per trapping session (68/hectare). This provided a natural experiment that allowed a comparison of multiple paternity across three years with very different densities. This comparison is detailed in Chapter 5.
Figure 4.1: Mean number of individuals in 3 day/100 trap session in each season over grids at (a) long unburnt area, site A and (b) the hill area, site C. Number of trapping sessions per season: (a) 1-6 and (b) 1-7. Years or seasons without data indicate that no trapping occurred.
Previously, annual capture rates have shown a significant, positive correlation with rainfall in the previous year, rather than rainfall in the present year (Wooller et al. 1998). The capture index, as an estimate of density, for each trapping session from 1984 to 2002 was used in an analysis of covariance (ANCOVA) to examine the extent to which a number of factors and covariates, including rainfall, could predict density. Rainfall data were collected near the study sites (Figure 4.2). After inspection, the capture index data was subjected to a log_{10} (capture index +1) transformation prior to the ANCOVA. Levene’s test was subsequently non-significant and therefore variances were assumed homogenous. Clearly, density is significantly affected by annual rainfall in the previous year, as well as by time since fire, and to a lesser extent by season and site-specific factors (Table 4.1).

Table 4.1: ANCOVA results on the log transformed capture index of honey possums (number of different individuals per 3 day/100 trap session) with rainfall and time since fire as covariates and season, trapping area and season-by-trapping area interaction as factors.

<table>
<thead>
<tr>
<th>Factor</th>
<th>hypothesis df</th>
<th>F value</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annual rainfall in previous year</td>
<td>1</td>
<td>69.608</td>
<td>0.000</td>
</tr>
<tr>
<td>Years since last burnt</td>
<td>1</td>
<td>100.069</td>
<td>0.000</td>
</tr>
<tr>
<td>Season when trapped</td>
<td>3</td>
<td>13.016</td>
<td>0.000</td>
</tr>
<tr>
<td>Area where trapped</td>
<td>7</td>
<td>20.900</td>
<td>0.000</td>
</tr>
<tr>
<td>Season x area</td>
<td>21</td>
<td>0.818</td>
<td>0.699</td>
</tr>
</tbody>
</table>

Nectar availability is related to rainfall (Wooller et al. 1993). Although the effect of rainfall on the population was not the focus of this study, rainfall was a significant determinant of population fluctuations and thereby provided a means by which to look at years of high and low resources and population density, as well as the intrinsic impacts these changes have on reproduction and sex ratios. Rainfall thus provided a framework within which the fluctuations seen in the three years of the genetical study might be interpreted. For this purpose, years were categorized by the annual (January to December) rainfall in the previous year. The long-term mean
annual rainfall was 552mm (Figure 4.2). If rainfall in the previous year was 1-10% higher or lower than this long-term mean, the year was classed as high (+) or low (-). If rainfall in the previous year was >10% higher or lower than the long-term mean, the year was classed as very high (++) or very low (- -). Only one year had rainfall within 1% of the mean (0). In analyses of densities and population structure, season was separated out as a factor for its intrinsic interest as a significant covariate of population density. Similarly, areas were analyzed separately and burn history accounted for.

Absolute densities were calculated using the minimal number of individuals known to be present per trapping session per hectare. The mean capture index was calculated for all trapping sessions during a season, over all grids at an area, within each category of rainfall-year. To account for the capture of individuals whose home range may have been only partly on the trapping grid, a boundary strip (see Krebs 1999) of 12.75m (half the average distance moved by both sexes between captures within a trapping session, see Garavanta et al. 2000) was added to the grid margin. This adjusted the 0.25ha grid area to an effective trapping area of 0.497ha, and the mean capture index was calculated per hectare accordingly. As site A was unaffected by fire, the entire data set from that site was included. To avoid the complication of fire at site C, only data from the one grid present before the fire in 1989 was included, together with data from the last three years for all grids at this site. Long term data indicates that the capture index of honey possums stabilizes approximately 10 years following a fire (Figure 4.3; Everaardt 2004). Thus, by the start of the current study into the mating system and reproduction in 2000, site C would have been experiencing little, if any, effects from the fire in 1989. Due to small sample sizes, high (+), average (0) and low (-) years at site C were pooled, to produce three, rather than four categories.
Figure 4.2: Annual rainfall recorded near the study area in FRNP. Yearly rainfall was categorized as >10% higher (++) or lower (- -), or 1-10% higher (+) or lower (-) than the long term mean (broken line). 1985 was within 1% of the long term mean (0). Note that the categories here relate to rainfall in the current year, whereas density and population structure data are categorized according to the rainfall in the previous year. Data provided by Department of Conservation and Land Management, Western Australia, from their ranger station only 5-12 km from the trapping sites.
Figure 4.3: \( \log_{10} \) of the average annual capture index (mean number of different honey possums caught per 3 day/100 trap session in one year) in relation to the years since a site was last burnt. Data are from site E (■) after it was burnt in 1980 (one grid), site C (×) after it was burnt in 1989 (3 grids), and site B (▲) after it was burnt in 1998 (2 grids). A logarithmic trendline was fitted \( [y=0.362\ln(x) + 0.435] \) to the longest series of data from site E. The capture index has been corrected for differences in seasonality of capture effort and annual differences in rainfall. After Everaardt (2004).
Population densities were highest following years of very high rainfall, and lowest following years of very low rainfall (Figure 4.4). For each season and at both sites, densities declined with rainfall, with two exceptions in summer and spring in the categories closest to mean rainfall. These intermediate rainfall categories showed some overlap with years following very high rainfall, but years following very low rainfall had substantially lower densities. Overall, densities were highest in winter and lowest in spring. In general, densities were higher at site C (hill area) than site A (long unburnt area). In years following very high rainfall, densities in winter peaked at 88 individuals per hectare for site C and 61 individuals per hectare at site A. The lowest seasonal densities in these years after heavy rainfall were still as high as 32 individuals per hectare for site C and 23 individuals per hectare for A. In years following very low rainfall, the lowest densities recorded were in spring with 10 individuals per hectare for site C and 8 individuals per hectare for site A.

4.2.2 Population structure

Given the fluctuations in population densities, the data were analyzed to determine if the changes in years following high and low rainfall were reflected in the structure of the population, in a way that might affect the mating system. For analyses of population structure, the total number of different individuals caught (capture index) for each trapping session was divided into five categories: adult males, adult females (with pouch-young and without pouch-young), juvenile males and juvenile females. Individuals were classified as adults or juveniles according to size at sexual maturity. For both males (Rutter 2001) and females, adult status, as defined by sexual maturity, corresponded to a head length >24mm or a weight of >6g (see Section 3.3). A very small number of females had pouch-young when slightly less than adult size and these females were classified as adults.
Figure 4.4: Mean number of individuals (+SE) per hectare. (a) Site A, the long unburnt site with mature vegetation (b) site C (hill area). As this site C was burnt in 1989, data from one grid from before this date was combined with data from the 3 years of this study (2000-2002) to avoid the effects of fire (see text). Number of trapping sessions is indicated above each column.
Generally, fire did not affect the proportions of particular classes of animals (Everaardt 2004) and all years of data were included in the analysis. The one exception, juvenile sex ratios, is outlined below and, in this case, only data from years prior to the fire were used. The data have been presented with years classified according to the rainfall in the previous year, as for reasons noted earlier. Due to small sample sizes in the very high and very low categories, categories were pooled to give one high and one low category for visual presentation, but all categories (+++, +, 0, -, - -) were retained for statistical tests on this data. Season has been presented separately due to its intrinsic interest, and the two main trapping areas have also been presented separately.

**Females with pouch-young**

Fewer females with pouch-young were caught in years following low rainfall than after high rainfall, in all seasons and at both trapping areas (Table 4.2). A general log-linear analysis performed on this categorized data revealed a significant three-way interaction between the proportion of females with pouch-young, rainfall in the previous year and season at site A ($\chi^2_{(df=12)} = 43.12$, $p=0.000$) and site C ($\chi^2_{(df=12)} = 26.43$, $p=0.01$). Season was then included, along with rainfall, as covariates in a logistic regression, in order to isolate the effects of each variable separately on the proportion of females with pouch-young. Both season and rainfall significantly affected the proportion of females with pouch-young at both site A (Season: Wald’s $\chi^2_{(df=3)} = 98.64$, $p=0.000$; Rainfall: Wald’s $\chi^2_{(df=4)} = 26.62$, $p=0.000$) and site C (Season: Wald’s $\chi^2_{(df=3)} = 88.68$, $p=0.000$; Rainfall: Wald’s $\chi^2_{(df=4)} = 10.18$, $p=0.038$). The proportion of females that had pouch-young was highest in winter and then declined through spring and summer to the lowest proportions in autumn (Table 4.2). This highly significant seasonal trend was consistent over years of high and low rainfall. The low point in breeding in autumn, also noted in Chapter 3, was more prominent in years following low rainfall.
Table 4.2: Percentage of adult females caught that had pouch-young in years following high or low rainfall. Total number of adult females caught in parentheses.

<table>
<thead>
<tr>
<th></th>
<th>Site A: Mature Vegetation</th>
<th>Site C: Hill area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rainfall in previous year</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Very high/high</td>
<td>Low/very low</td>
</tr>
<tr>
<td></td>
<td>++ / +</td>
<td>- / -</td>
</tr>
<tr>
<td>Winter</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>79% (238)</td>
<td>63% (123)</td>
</tr>
<tr>
<td>Spring</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>65% (60)</td>
<td>61% (57)</td>
</tr>
<tr>
<td>Summer</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>58% (144)</td>
<td>49% (61)</td>
</tr>
<tr>
<td>Autumn</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>42% (109)</td>
<td>15% (65)</td>
</tr>
</tbody>
</table>

Three elements may be involved in the relationship between rainfall and the proportion of females breeding. Firstly, following dry years, females that are without young may not be in a condition to breed. Secondly, following dry years, a greater proportion of females may have just moved beyond the juvenile stage (>24mm head length), but not yet had the opportunity to mate. Thirdly, following dry years, females may not have pouch-young because they have not yet encountered a mate. Mating patterns of females were analyzed in relation to the different densities in the three years of the genetical study, and the incidence of multiple paternity was independent of density effects (see Chapter 5). This suggests that the availability of males was not a limiting factor. Indeed, when densities were at their lowest, minimal estimates still showed 8 individuals present per hectare.

The possibility that the lower proportion of females with pouch-young occurred due to a greater number of late juvenile/pre-reproductive females at such times was investigated. A significant negative regression was detected between the proportion
of females with pouch-young and the proportion of females that were juvenile
\((F_{1,438}=6.663, r^2=0.015, p=0.01, \text{ slope}= -0.351)\). An ANCOVA was performed on the
proportion of females with pouch-young for each trapping session and at all areas
from 1984 to 2002 (Table 4.3). This included the proportion of females that were
juvenile, together with rainfall in the previous year and burn history as covariates, and
season, trapping area and season-by-area interaction as factors. The results
showed that there was no longer a significant relationship between the females with
pouch-young and the proportion of females that were juveniles (Table 4.3). The only
significant factor was season. Therefore, the observed relationship between the two
variables was simply determined by seasonal variation and indicated that the number
of post-juvenile adult females was not an important source of variation in the
proportion of females with pouch-young. The first explanation therefore, appears
most probable, and suggests that in years following low rainfall, there were a greater
number of females whose condition precluded them from breeding.

Table 4.3: ANCOVA results for the proportion of adult females with pouch-young. The
variables rainfall, time since fire, and the ratio of female juveniles to adults are included as
covariates, and the season, trapping area and area-by-season interaction are included as
factors. Season shows the only significant interaction.

<table>
<thead>
<tr>
<th>Factor</th>
<th>hypothesis df</th>
<th>F value</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annual rainfall in previous year</td>
<td>1</td>
<td>0.926</td>
<td>0.337</td>
</tr>
<tr>
<td>Years since last burnt</td>
<td>1</td>
<td>1.272</td>
<td>0.260</td>
</tr>
<tr>
<td>Season when trapped</td>
<td>3</td>
<td>26.521</td>
<td>0.000</td>
</tr>
<tr>
<td>Ratio of female juveniles to adults</td>
<td>1</td>
<td>2.420</td>
<td>0.121</td>
</tr>
<tr>
<td>Area where trapped</td>
<td>7</td>
<td>0.491</td>
<td>0.834</td>
</tr>
<tr>
<td>Area x season</td>
<td>21</td>
<td>0.751</td>
<td>0.779</td>
</tr>
</tbody>
</table>

Although the ANCOVA showed that the proportion of females with pouch young was
not significantly related to rainfall (Table 4.3), a significantly lower proportion of
females bred in years following low rainfall, with all these years combined, compared
to all years combined that followed high rainfall (outlined above). This was probably
due to a greater ability to detect differences without the noise created by small captures in some sessions included in the ANCOVA data.

Litter size was analyzed over the years 2000, 2001 and 2002 to determine whether females that bred in 2001, when population densities were low and food was sparse, had a smaller litter. The size and condition of the female and the size of the pouch-young were considered possible factors, after the findings of Wooller and Richardson (1992) (see Section 3.4). Measurements of adults were taken by three measurers. Parallel measurements of the same honey possum taken by myself and R.D. Wooller, and by myself and A.E. Everaardt were significantly correlated (r=0.84 and 0.87 respectively). The slope of the regression lines did not significantly differ from 1.0 (p>0.05), indicating that there were no systematic differences in measurements between measurers. Univariate ANOVAs showed that the measurer and the trapping area were not significant factors in predicting adult female head length and body weight. The condition index of the females were calculated from tail-base diameter and head length using the methods described by Krebs and Singleton (1993). An ANOVA was performed with the condition index as the dependent variable, and showed that head length was a significant factor in determining condition, but there was no significant relationship between the number of pouch-young and the condition index of the female (Table 4.4). Condition was therefore excluded as a factor in litter-size analysis.

<table>
<thead>
<tr>
<th>Factor</th>
<th>df</th>
<th>F value</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female head length</td>
<td>1</td>
<td>4.689</td>
<td>0.032</td>
</tr>
<tr>
<td>Number of pouch-young</td>
<td>3</td>
<td>0.680</td>
<td>0.566</td>
</tr>
<tr>
<td>Number of pouch-young x head length</td>
<td>3</td>
<td>0.696</td>
<td>0.556</td>
</tr>
</tbody>
</table>
Likelihood ratio tests were performed to test for an association between the number of pouch-young and the size of the pouch-young, the year and the size of the female (Table 4.5). There was a significant association between pouch-young size and the number of pouch-young, as expected given brood reduction (Wooller and Richardson 1992). However, neither the head length of the mother, nor the year, were significantly associated with litter size.

Table 4.5: Results of the likelihood ratio test for association between the number of pouch-young and the size of the young, the head length of the female and the year. The years analyzed included 2000, 2001 and 2002, when population densities went from high to low to high again, shadowing a similar change in rainfall. Size of the pouch-young was an estimate of crown-rump length (see Section 2.2.3).

<table>
<thead>
<tr>
<th>Effect</th>
<th>-2 Log likelihood of reduced model</th>
<th>$\chi^2$</th>
<th>df</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size of pouch-young</td>
<td>366.074</td>
<td>17.866</td>
<td>9</td>
<td>0.037</td>
</tr>
<tr>
<td>Year</td>
<td>361.468</td>
<td>13.259</td>
<td>9</td>
<td>0.151</td>
</tr>
<tr>
<td>Female head length</td>
<td>359.993</td>
<td>11.784</td>
<td>9</td>
<td>0.226</td>
</tr>
</tbody>
</table>

**Sex ratios**

Sex ratios were calculated over all sessions for all areas between 1984 and 2002. Sex ratios were significantly biased toward males, which comprised 56.2±1.8% of juveniles ($t_{367}=3.417$, $p=0.001$), and 58±1.0% of adults ($t_{469}=8.356$, $p=0.000$). This small, yet significant, bias was consistently evident in both high and low years (Tables 4.6 and 4.7).

There was no relationship between rainfall and adult sex ratios (Table 4.6). A general log-linear analysis showed no significant three-way interaction between adult sex ratios, rainfall and season at either site A ($\chi^2_{(df=12)}=10.13$, $p=0.604$) or site C ($\chi^2_{(df=12)}=20.36$, $p=0.060$). When the effects of these two covariates on adult sex ratios were considered separately in a logistic regression, slight differences appeared
between the two areas. At site A, rainfall did not significantly affect adult sex ratios (Wald’s $\chi^2_{(df=4)} = 5.87$, $p=0.209$), but season was a significant determinant (Wald’s $\chi^2_{(df=3)} = 10.42$, $p=0.015$). The proportion of males at site A was slightly lower in autumn and winter, increased in spring and was highest in summer (Table 4.6). At site C, neither season (Wald’s $\chi^2_{(df=3)} = 3.04$, $p=0.385$) nor rainfall (Wald’s $\chi^2_{(df=4)} = 9.309$, $p=0.054$), significantly affected adult sex ratios.

The fire history of an area significantly affected the juvenile sex ratio, with proportionately fewer males at intermediate times 20-40 years after fire and proportionately more juvenile males in both recently burnt (1-3 years after fire) and long unburnt areas (Everaardt 2004). In order to exclude this bias, data from site C (burnt in 1989) were only included from the one grid that was present before the fire (Table 4.7). A general log-linear analysis showed no significant three-way interaction between juvenile sex ratios, rainfall and season at either site A ($\chi^2_{(df=12)} = 15.81$, $p=0.200$) or site C ($\chi^2_{(df=9)} = 6.23$, $p=0.717$). The effects of these two covariates on juvenile sex ratios were considered separately in a logistic regression. At site A, neither season (Wald’s $\chi^2_{(df=3)} = 2.68$, $p=0.444$) nor rainfall (Wald’s $\chi^2_{(df=4)} = 4.74$, $p=0.314$) significantly affected juvenile sex ratios. Similarly, at site C neither season (Wald’s $\chi^2_{(df=3)} = 0.68$, $p=0.878$) nor rainfall (Wald’s $\chi^2_{(df=3)} = 0.40$, $p=0.274$) significantly affected juvenile sex ratios. This was despite the proportion of juveniles that were male appearing lower in years following low rainfall at site C (Table 4.7). This trend may have been due to the small sample sizes when years after fire were excluded. However, the trend for fewer males after dry years was significant when all years were included in the analysis but the proportion of males was then biased by the fire history of the area.
Table 4.6: Percentage of adults caught that were male in years following high or low rainfall. Total number of adults caught is shown in parentheses.

<table>
<thead>
<tr>
<th></th>
<th>Site A: Mature Vegetation</th>
<th>Site C: Hill area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rainfall in previous year</td>
<td>Rainfall in previous year</td>
</tr>
<tr>
<td></td>
<td>Very high/high ++ / +</td>
<td>Very high/high ++ / +</td>
</tr>
<tr>
<td></td>
<td>Low/very low - / - -</td>
<td>Low/very low - / - -</td>
</tr>
<tr>
<td>Summer</td>
<td>60% (361)</td>
<td>56% (165)</td>
</tr>
<tr>
<td></td>
<td>61% (157)</td>
<td>50% (109)</td>
</tr>
<tr>
<td>Autumn</td>
<td>53% (230)</td>
<td>55% (155)</td>
</tr>
<tr>
<td></td>
<td>53% (137)</td>
<td>54% (158)</td>
</tr>
<tr>
<td>Winter</td>
<td>52% (492)</td>
<td>59% (426)</td>
</tr>
<tr>
<td></td>
<td>51% (253)</td>
<td>53% (326)</td>
</tr>
<tr>
<td>Spring</td>
<td>56% (136)</td>
<td>52% (139)</td>
</tr>
<tr>
<td></td>
<td>59% (138)</td>
<td>65% (231)</td>
</tr>
</tbody>
</table>

Table 4.7: Percentage of juveniles caught that were male in years following high or low rainfall. Total number of juveniles caught is shown in parentheses.

<table>
<thead>
<tr>
<th></th>
<th>Site A: Mature Vegetation</th>
<th>Site C: Hill area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rainfall in previous year</td>
<td>Rainfall in previous year</td>
</tr>
<tr>
<td></td>
<td>Very high/high ++ / +</td>
<td>Very high/high ++ / +</td>
</tr>
<tr>
<td></td>
<td>Low/very low - / - -</td>
<td>Low/very low - / - -</td>
</tr>
<tr>
<td>Summer</td>
<td>56% (71)</td>
<td>56% (16)</td>
</tr>
<tr>
<td></td>
<td>56% (64)</td>
<td>47% (34)</td>
</tr>
<tr>
<td>Autumn</td>
<td>47% (76)</td>
<td>50% (16)</td>
</tr>
<tr>
<td></td>
<td>53% (32)</td>
<td>43% (14)</td>
</tr>
<tr>
<td>Winter</td>
<td>54% (85)</td>
<td>58% (24)</td>
</tr>
<tr>
<td></td>
<td>44% (27)</td>
<td>40% (5)</td>
</tr>
<tr>
<td>Spring</td>
<td>47% (15)</td>
<td>60% (15)</td>
</tr>
<tr>
<td></td>
<td>67% (18)</td>
<td>25% (12)</td>
</tr>
</tbody>
</table>
Sexing of pouch-young using a molecular marker

Female honey possums carry their young, rather than leave them in a nest, and the tiny young are held within a deep pouch with a small, muscular opening. This meant that it was not possible to sex young visibly. Removal of young from the pouch would have been inimical to the welfare of the animals (see Section 2.2). Tissue samples for DNA analysis of pouch-young were obtained whilst the young remained in the pouch (see Section 2.2), and in addition to analysis for multiple paternity (Chapter 5) these young were sexed using a molecular marker. Tissue samples were also taken from young preserved when the mother was sacrificed for reproductive tract studies (Chapter 6) and most were analyzed for multiple paternity in litters. The small size of some of the young preserved made it difficult to sex them on external characteristics. All these preserved young were also sexed using a molecular marker. Samples for sexing were therefore limited to those collected for genetical and reproductive studies.

The sex determining region on the Y chromosome for marsupials, the SRY region (Foster 1992), was amplified using SRY primers (F:ATCATATGGTCACGGAGTCAGCGG, R:GTGTTTAGCGCAAACGTTTGGC) designed for the bandicoot Isoodon macrourus (Watson et al. 1998). These were used in a multiplex PCR reaction with primer set 1, at a final concentration of 0.4µM, as described in section 5.2.3. This ensured that a positive PCR reaction could be confirmed in the absence of an SRY band (indicating females). The 5’ end of the forward SRY primer was labelled with fluorescent dye (HEX: APPLIED BIOSYSTEMS). The 160 bp SRY product was resolved along with primer 1, as described in section 5.2.3.

The SRY primers were multiplexed in the PCR with primer set 1 for all genotyped samples. This included 115 adult males and 40 adult females, the sex of which were known definitively through anatomical observation. The genetic sexing method correctly sexed these animals in all cases. In addition, 22 of the pouch-young preserved during reproductive studies (Chapter 6) were large enough to sex using
external characteristics and were also genetically sexed. The genetic sexing method correctly sexed these pouch-young in all cases. Thus this genetic sexing method produced accurate results.

The number of male and female pouch-young in the population was equal when data were combined for all years (Table 4.8). As noted previously, during this period densities changed from high in 2000, to low in 2001, and then back to high in 2002. Although sample sizes were small, no difference was detected between numbers of males and females among pouch-young when the data were separated into the three separate years. Sex ratios did not appear to differ according to the size of the young, although again, sample sizes were small.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 8mm</td>
<td>11</td>
<td>8</td>
<td>3</td>
<td>7</td>
<td>6</td>
<td>9</td>
<td>20</td>
<td>24</td>
</tr>
<tr>
<td>8.1 – 14.9mm</td>
<td>11</td>
<td>12</td>
<td>6</td>
<td>5</td>
<td>16</td>
<td>13</td>
<td>33</td>
<td>30</td>
</tr>
<tr>
<td>≥ 15mm</td>
<td>5</td>
<td>6</td>
<td>5</td>
<td>3</td>
<td>5</td>
<td>5</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td><strong>Totals</strong></td>
<td>27</td>
<td>26</td>
<td>14</td>
<td>15</td>
<td>27</td>
<td>27</td>
<td>68</td>
<td>68</td>
</tr>
</tbody>
</table>

4.2.3 Recaptures between sessions and dispersal

Recaptures

The movement patterns of honey possums in the FRNP have been considered in detail elsewhere (Garavanta et al. 2000). Data on recaptures of animals are
presented here in order to provide some basic parameters on the recapture of males and females. The proportion of individuals caught that were previously unmarked were calculated only from trapping sessions 1-4 months apart from 1984 to 2002, during which animals were individually marked and the trapping session lasted three days. The proportion of unmarked individuals was consistently greater than 70% at both sites in all seasons (Table 4.9).

Table 4.9: The percentage of unmarked individuals in an average trapping session from 1984 to 2002. Trapping sessions were 1-4 months apart.

<table>
<thead>
<tr>
<th>Trapping site</th>
<th>Summer</th>
<th>Autumn</th>
<th>Winter</th>
<th>Spring</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>86</td>
<td>72</td>
<td>81</td>
<td>72</td>
</tr>
<tr>
<td>C</td>
<td>89</td>
<td>81</td>
<td>73</td>
<td>77</td>
</tr>
</tbody>
</table>

Despite these high turnover rates, the recapture of individually marked honey possums between sessions was considered in more detail. These sessions were at least one month apart in the period March 2000 to June 2003. Table 4.10 depicts the movements of individual males and females that were marked in one session, and then later captured in one or more subsequent sessions. These data have been presented for all grids within all trapping sites A to E, in order to allow the detection of movements of animals between grids, and between areas, in comparison to those that stayed within the same grid. There were 206 movements made by 131 males, and 136 movements made by 101 females. The proportion of individuals that moved to a different grid was significantly greater ($\chi^2_{(df=1)}=6.36, p=0.012$) for males (43.7%) than females (30.15%). Also, the number of males (7) moving between the hill area (sites B and C) and sites A and E, some 3.5 and 6.5km distant respectively, was greater than for females (0). However, the proportions changing areas (of those that moved between different grids) were non-significant ($\chi^2_{(df=1)}=3.37, p=0.066$).
Incidences of animals changing areas were only included where the individual mark was either not duplicated in the other area or the identity of the animal could be confirmed by other means (eg. size or simultaneous capture of the other individual with that number).

The proportions of males and females changing grids were also analysed separately for the two main trapping sites, A and C (Table 4.11). The proportion of females moving to a different grid did not differ between the two areas \( \chi^2_{(df=1)} = 2.45, p=0.117 \). However, the proportion of males moving away was significantly greater for area C than for area A \( \chi^2_{(df=1)} = 4.68, p=0.031 \).

For individuals marked from March 2000 to July 2002, across all sites, 29% of 499 males were recaptured, and 26% of 406 females were recaptured. Of these recaptured animals, over 70% for both males and females were captured in only 2 sessions (Figure 4.5). These distributions did not differ for males and females (Mann-Whitney U \( z=1.47, p=0.141 \)).
Table 4.10: Movements within and between grids of male and female honey possums recaptured between sessions at least one month apart (March 2000 to June 2003). Movements within a trapping area are surrounded by a double line and movements within a grid are shown in bold. Sites B and C are closest together with 5 grids over about 500m, site D is about 450m distant to these sites (Figure 2.2). Site A is 3.5km away from this hill area, and site E a further 3km distant.

(a) Males

<table>
<thead>
<tr>
<th>From</th>
<th>E</th>
<th>A1</th>
<th>A2</th>
<th>B1</th>
<th>B2</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>1</td>
<td>12</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td>5</td>
<td></td>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>1</td>
<td></td>
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(b) Females

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<th>B1</th>
<th>B2</th>
<th>C1</th>
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Table 4.11: The number of male and female honey possums caught within sites A or C that moved to a different grid, either within the same area or to the surrounding area.

<table>
<thead>
<tr>
<th></th>
<th>Same grid</th>
<th>Different grid</th>
<th>% moving</th>
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<tbody>
<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Site A</td>
<td>26</td>
<td>10</td>
<td>28%</td>
</tr>
<tr>
<td>Site C</td>
<td>66</td>
<td>61</td>
<td>48%</td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Site A</td>
<td>29</td>
<td>7</td>
<td>19%</td>
</tr>
<tr>
<td>Site C</td>
<td>53</td>
<td>27</td>
<td>34%</td>
</tr>
</tbody>
</table>

_Detection of sex biased dispersal using genetic data_

Genotyping of 155 individuals (115 males and 40 females) at four highly polymorphic microsatellite loci was undertaken for assessing multiple paternity (Chapter 5). Characteristics of the genotype data are outlined in section 5.3.1. This genotyping data was used to test for sex-biased dispersal. Genetic data are a valuable way to test for sex-biased dispersal because of difficulties with estimating dispersal from field data (Goudet _et al._ 2002). Marking of honey possum pouch-young is not possible and detection of dispersing individuals would rely on free-living young being marked before dispersal. If there is sex-biased dispersal, genetic relatedness between individuals of one sex would be greater than the relatedness between individuals of the other sex. The program _FSTAT_ (version 2.9.3.2 Goudet 1995) was used to test for sex-biased dispersal using relatedness calculated in the manner described by Queller and Goodnight (1989). Relatedness is calculated in the program over all individuals for each sex, and then a randomisation approach is used to generate the null distribution for significance testing. The approaches used and the power of the test are described in Goudet _et al._ (2002). Given that relatedness between full siblings is 0.5, average relatedness calculated between all individuals was low, at −0.0044. Nevertheless relatedness between females (0.0277) was significantly greater (p=0.0298) than relatedness between males (-0.0031), providing evidence of male-biased dispersal.
Figure 4.5: The number of different sessions in which male (a) and female (b) honey possums were recaptured. This data is collated from all trapping sites A to E, and from trapping sessions 1-3 months apart from March 2000 to September 2002. Sample sizes are not equal.
4.3 Discussion

Marked density fluctuations occurred in the honey possum population in the Fitzgerald River National Park, both seasonally and also from year to year, significantly related to rainfall in the previous year. The proportions of females carrying young were also significantly affected by season and rainfall, but the sex ratios, in general, were not.

Total annual captures of honey possums have previously been found to be significantly positively related to rainfall in the previous, rather than the current year (Wooller et al. 1998). In the present study, the number of different individuals present in the population throughout the year showed a similar relationship to rainfall in the previous year, with years following very low rainfall showing markedly lower densities than other years. This relationship is most likely to be mediated by the impact of rainfall on the flowering of the honey possum’s foodplants in the following year (Wooller et al. 1998). The lag effect shown probably occurs because plants from the Proteaceae family have deep tap roots that reach the water table, and therefore respond to the accumulation of water over time (Wooller et al. 1998). Indeed, the capture rates of the honey possum in the Fitzgerald have previously been found to have a close relationship with the nectar production of their proteaceous foodplants (Wooller et al. 1993). Years following lower than average rainfall were, therefore, times of low resource availability and were followed by troughs in the honey possum population. In contrast, years following higher than average rainfall were times of higher resource availability, and were followed by higher densities of honey possums.

The honey possum is a small, non-flying mammal, specialized for feeding on nectar and pollen, and is unable to change to other foods when nectar is low. Although there are site-specific differences in the vegetation (see Section 2.2.1), in the generally homogenous heathland of FRNP the effect of water availability on flowering would presumably be similar in different areas, following dry years. Honey possums
are unlikely to be able to escape starvation by moving elsewhere. Indeed fluctuations due to rainfall were mirrored across the two main sites considered in this study. Short-term torpor by honey possums (Collins et al. 1987; Withers et al. 1990) only represents a temporary solution to food shortage. The lower numbers of honey possums and the significantly lower proportion of females carrying young that followed dry years, suggest that the lower population densities result from greater mortality due to starvation and a lower rate of recruitment. The impact of dry years generally did not appear to last longer than one year. The significantly greater number of females initiating offspring and the continuous breeding throughout the year, coupled with higher survival would allow this rapid recovery. Such a succession of high to low to high densities was seen during the three years of trapping for study of the mating system and reproduction (2000-2002) and this shadowed a similar succession of changes in rainfall. This provided an opportunity to assess the incidence of multiple paternity during changes in the availability of resources and population densities (see Chapter 5).

In the Stirling Range National Park, 110 km west of the Fitzgerald River National Park, Rose (1995) found that the captures of honey possums more than doubled following a doubling of rainfall after drought conditions broke in 1988. There was a lag effect with numbers peaking about six months after the drought broke. The squirrel glider *Petaurus norfolcensis*, a species in which nectar and pollen make up a large portion of the diet, also showed a response to changes in rainfall at the same time as the honey possums in the FRNP (Sharpe 2001). On the eastern coast of Australia, in New South Wales, numbers of squirrel gliders in the year 2000 reached a five-year high of 30 per 38 hectare trapping grid. This corresponded to greater than usual flowering of key foodplants. Dry conditions during this year, similar to the FRNP, saw a lack of flowering in spring, then a fall to seven individual squirrel gliders at the same site by November 2000. These changes mirror those for the honey possum in the FRNP where numbers fell dramatically at site C from 76 individuals per hectare in spring 2000 to 12 individuals per hectare in summer 2001 (Figure 4.1), with a similar yet less dramatic change at site A. The body condition of many honey
possums in spring 2000 was low, and all carried mites. Changes in rainfall, and in turn, the availability of flowers, therefore, appear to be an important factor in the population dynamics of nectar and pollen feeders.

Significant seasonal variation in the densities of honey possums showed that numbers were generally highest in winter and lowest in spring in both areas, and this agrees with previous findings for capture rates (Wooller et al. 2000). The flowering phenologies of key foodplants indicate that winter is a time of year when the most food is available (see Section 3.7). Autumn and, surprisingly, spring, are times of lowest food availability, during which natural attrition is probably greatest (Wooller et al. 2000). The condition indices of honey possums are consistent with these seasonal changes, being highest in winter, and lowest in summer and autumn, presumably for those that survive through the spring to summer (see Figure 3.5; Wooller et al. 2000).

Honey possums are essentially continuous breeders and seasonal changes in population densities are probably mostly affected by differential survival, rather than by cycles of breeding. The seasonal changes in the proportions of females breeding, related to the availability of food, were outlined in Chapter 3 and have here been shown to be significant. This, together with the significant seasonal changes in densities, provides support for the annual cycle outlined in Chapter 3. The greatest number of females with pouch-young occur in winter, when food resources are greatest, with more moderate proportions through spring and summer, and the lowest levels in autumn, corresponding to a dearth in food. This trend is consistent in years following high or low rainfall. However, the composite effect of low rainfall and the autumn dearth of food, is dramatic; only 7% of females at site C and 15% of females at site A had pouch-young during such times (Table 4.2).

The significantly lower proportion of females with pouch-young after lower rainfall in the previous year indicates that the availability of resources affected the proportion of females in a condition to breed. It is interesting to note, however, that even in low
years, the proportion of females with pouch young was still around 50%, if not greater (Table 4.2). The exception to this, of course, is in autumn. If females that were noted with an empty pouch, but with mammary glands swollen, clearly indicating they were lactational, were included, these proportions would increase. It is perhaps not surprising that many females would attempt to reproduce in a bad year, given their short lifespan of one to two years. Of those females that did breed, there was no evidence that the litter size was smaller in 2001, a time of low resources, compared to 2000 and 2002. This suggests that if females are in a condition to produce young, they do so unreservedly. Perhaps females in a lower condition delay carrying pouch-young altogether, but they may hold conceptuses in diapause (see Section 6.4.3).

The head length of a female was not significantly related to litter size in the three years analyzed (2000-2002). This is contrary to the findings of Wooller and Richardson (Wooller and Richardson 1992) for data from 1984 to 1989 (see Section 3.4). That study showed that females carrying three young were significantly larger, in head length and weight than females carrying two young. Females with four young were even larger, but this difference was not significant (Wooller and Richardson 1992). It was clear from their data that only the largest females carried four young to a size where they left the pouch. The discrepancy between the two findings may be due to smaller sample sizes in the present study or that the trend was weak. Further investigation is needed to resolve this issue.

The large proportion of unmarked individuals trapped at every trapping session (over 70%) indicates that turnover rates between trapping sessions are high. An annual mortality rate of 86% for honey possums has been estimated, based on the first and last captures of individually marked animals (Wooller et al. 2000). Even given this high mortality rate, death of individuals and their replacement by newborn young are unlikely to fully account for the high turnover rate between sessions even over intervals of several months. This indicates that the mobility of honey possums is probably greater than previously thought (Garavanta et al. 2000) and that some
individuals probably remain alive, but move to adjacent areas where they are not trapped.

The honey possum in the FRNP has previously been reported as relatively sedentary (Garavanta et al. 2000). Using mark-recapture data, the average distance of movements between trapping sessions of 1-3 months apart was 54±99m (n=344) for males and 56±107m (n=222) for females, with most individuals moving less than 30m (Garavanta et al. 2000). Home ranges could only be calculated from individuals recaptured at least 6 times, and therefore used a relatively small number of individuals. The average home range of 30 males was 1277m$^2$ (0.13 ha) (range 150-3494 m$^2$) and was significantly larger than the average of 20 females 701 m$^2$ (0.07 ha)(range 112-2212 m$^2$). Several methods to detect dispersal beyond the grids in this study showed little dispersal. The home ranges are probably reasonable indicators of the limited movements of a proportion of honey possums and even in the current study 54-70% of individuals recaptured had not moved from the grid where they were marked. However, as over half of all individuals marked were never recaptured, there may be many individuals that disappeared, not because they died, but rather because they moved further than the grid areas. Greater movements may be necessary at times, in order to search for new food sources.

Studies using radiotracking to estimate the areas utilized by honey possums (ie movements over a short time period of 5-10 days) (Arrese and Runham 2002; Bradshaw and Bradshaw 2002) also have limitations. The small transmitters have a limited range in dense vegetation. Honey possums are very sensitive to sound and movement, and may be disturbed by tracking. Nevertheless, Bradshaw and Bradshaw (2002) found that although female movements did not differ when estimated using either trapping or radiotracking, the areas utilized by males were significantly greater (0.79ha) when assessed using radiotracking than by mark-recapture (0.03ha). One male utilized an area of 2.2 ha, although this was in recently burnt vegetation.
Given the limitations of estimating movements of honey possums, and the high turnover rates revealed in this study, movement patterns were not analyzed in detail from the mark-recapture data. However, movements of recaptured animals between grids were presented in order to give some idea of the differences between males and females. Significantly more males than females changed grids, and males made more long distance movements (3-5km) than females, although this difference was not significant. This is consistent with the greater movement patterns shown by males in the two other studies (Garavanta et al. 2000; Bradshaw and Bradshaw 2002). There was no appreciable difference in the recapture rate of males and females in this study. However, Garavanta et al. (2000) found that more males (41%) were recaptured than females (29%). This is probably a consequence of their greater movement enhancing their trappability.

Female honey possums are larger than males and are behaviourally dominant (see Section 3.8; Russell 1986). In captivity, they exclude males from flowers and in the wild, males may be forced to range more widely for food. Females, burdened by carrying young may thus monopolize concentrated food resources. Garavanta (1997) found greater movement of adult and juvenile males in nectar sparse years, but no greater movement of females. However, although females often remain sedentary, their ability to carry their young in the pouch, rather than leave them in a nest, gives them the ability to travel further afield in search of new food sources if necessary. More extensive movement patterns by males may also reflect their search for females in oestrus (see Chapter 7), and males probably have ‘mobile’ home ranges (Garavanta 1997). Movements by individually marked males and females in this study often overlapped, and a number of males and females were trapped in close proximity. Garavanta (1997) found no territoriality and found that male and female home ranges overlapped in a way that would allow access by each sex to several members of the opposite sex. The areas utilized by male and female honey possums, as assessed by Bradshaw and Bradshaw (2002) also overlapped.
The significantly greater genetic relatedness of females in the population, compared to males, provides evidence of male-biased dispersal. Such male-biased dispersal is common in mammals, and is often associated with promiscuous and polygynous mating systems, where local mate competition drives male dispersal (Greenwood 1980; Dobson 1982). Sex-biased dispersal can also facilitate inbreeding avoidance (Pusey 1987).

Overall, the sex ratio was biased towards males in both adults (58%) and juveniles (56%), and a slight bias towards males was consistent following years of both high and low rainfall. There was, however, no bias detected in the sex ratios of pouch-young at the population level, even following brood reduction. The pouch-young samples, collected over all years, consisted of 68 males and 68 females. Wooller and Richardson (1992) also sexed litters of pouch-young that were sacrificed for other purposes and found that the sex ratio did not differ from parity, with 25 females and 27 males. Although both these studies have modest data sets, taken together, they provide no evidence to suggest that females routinely bias sex ratios during reproduction in order to favour production of male offspring. This does not preclude adaptive adjustment of sex ratios by females during reproduction, but suggests that if it occurs, the direction of the bias is individually-adaptive and differs amongst females in such a way that the overall population sex ratio of young remains at parity. For example, sex ratio manipulation by brood reduction occurs in the agile antechinus *Antechinus agilis* according to the age of the mother, but the population sex ratio of young before and after brood reduction remains at parity (Cockburn 1994). Adaptive adjustment of sex ratios in marsupials can also occur at conception (Davison and Ward 1998; Johnson and Ritchie 2002). Significant departures from parity in offspring sex ratios at the population level are common in other marsupials (Cockburn 1990), but is not evident from the available data in honey possums.

Therefore if in the honey possum a bias towards males among both juveniles and adults exists, it must be established at the juvenile, free-living stage, perhaps through differential survival of the sexes. Survivorship of adult males and females does not
differ (Wooller et al. 2000). As there is evidence of male-biased dispersal, this may point to a possible source of differential survival of male and female young. However, movement to an unknown area may decrease male survival, or conversely, increase it because of greater food availability in other areas. Provisioning of more resources to male offspring during lactation and weaning is unlikely given that honey possum juveniles do not differ in size (Wooller et al. 1981). The growth rate of young males and females are similar, but females continue to grow larger than males (Wooller et al. 1981). Perhaps the most likely explanation for the slight male bias in sex ratios is that males are not in greater abundance, but rather are more trappable, because they traverse greater distances in their normal activities. Therefore, the effective census area for males is greater than for females per grid and may then include more males in its sample. The greater recapture rate of males in previous studies supports the idea that they are more trappable, and that this is the source of the bias (Wooller et al. 2000).

Generally, sex ratios did not differ in the years that followed high or low rainfall, and again this suggests that females do not favour production of a particular sex due to specific environmental or resource conditions. There was a trend toward a lower proportion of juvenile males in years following low rainfall, but only at site C. Differences between populations in sex ratios in response to local conditions do occur in other marsupials, for example the brush tail possum (Johnson et al. 2001) and kangaroos (Johnson and Jarman 1983). However, sex ratios of honey possum young across changing resources and population densities in 2000, 2001 and 2002 were at parity, albeit based upon low sample sizes. Since the difference in juvenile sex ratio only occurred at one site, it probably represents a site-specific difference related to available food resources following drier years. This may lead to greater juvenile male dispersal away from this area or greater mortality of juvenile males than females at this area. Another anomaly was the significant interaction with season and adult sex ratios at site A. Again, this may also be due to a site-specific difference. Site A was in mature vegetation where the understorey had a dense stand of Banksia nutans, the key summer food source for honey possums (see
Section 3.7). As males travel further than females, the higher proportion of males at this site in summer may be due to an influx of males seeking the rich nectar and pollen source localized in this area. This trend did not occur among juveniles at this site and therefore is unlikely to represent adaptive sex allocation by females.

### 4.4 Conclusion

The population densities of honey possums fluctuate from season to season with changes in flowering food resources available. There are also year-to-year differences in the intensity of those fluctuations produced by rainfall and probably mediated by changes in flowering intensity of their foodplants. The impact of changing nectar and pollen levels was also evident in female breeding, both at a seasonal level and from year to year, again in relation to rainfall. Years following low rainfall had a lower proportion of females in a condition to breed. The population sex-ratio of pouch-young was at parity, but with a bias towards males among both juveniles and adults. This was probably due to greater movements shown by males. Sex ratios were not affected by high and low rainfall years. Dispersal appears male-biased and the movement patterns of males show that they move greater distances than females do during their normal activity.

Despite a high turnover rate, the densities of honey possums presented here represent the number of individuals present at the locality of each area trapping area during each session, and are a minimal estimate of density per hectare. Although there were peaks and troughs in the estimated densities of honey possums, numbers per hectare still remained reasonably high, except following very dry years. The highest mean densities were recorded in winter in years after very high rainfall. At these times, estimates of honey possums reached 88 individuals per hectare at site C and 61 individuals at site A. In other seasons of these years, numbers only went down to 32 individuals per hectare at site C and 23 individuals per hectare at site A. In years following low, near average and high rainfall, mean densities ranged from 17 to 54 individuals per hectare. The lowest mean densities were recorded in the spring.
after dry years, with 10 individuals per hectare at site C and 8 individuals per hectare at site A. The home ranges of honey possums estimated using mark-recapture in the FRNP (Garavanta et al. 2000), were 701 m$^2$ for females and 1277 m$^2$ for males, and are likely to be a minimal estimate. Given this, in the extreme, only 14 females per hectare or 8 males per hectare would be needed for home ranges to meet at the margins. With mean densities ranging from 17 to 88 individuals per hectare for all but those times following very low rainfall, home range overlap and the potential for encounters between the opposite sex may very often be substantial. At times, some males may move several kilometres (Bradshaw and Bradshaw 2002). Therefore even at the lowest mean density of 8 individuals per hectare following a dry year, interaction between one individual and several of the opposite sex may still occur, particularly if these interactions are actively sought.

Honey possums are the most abundant and widespread mammal in the FRNP. At the trapping sites described here, on average 88% of all small mammals captured in pitfall or box traps from 1984 to 2002 were honey possums (Wooller and Wooller 2003; Everaardt 2004). Only small numbers of house mice *Mus musculus*, ash-grey mice *Pseudomys albocinereus*, southern bush-rats *Rattus fuscipes*, grey-bellied dunnarts *Sminthopsis griseoventer*, and occasionally the western pygmy possum *Cercartetus concinnus*, were caught in the area (Everaardt 2004). Chapman (1995) reported a similar result for an extensive survey across the entire FRNP.
Chapter 5: Microsatellite Analysis of Multiple Paternity

5.1 Introduction

As stated in Chapter 1, the large testes and long sperm of the honey possum suggest that sperm competition due to female multiple mating may be an important part of the mating system. However, no previous study has assessed multiple mating or multiple paternity in the honey possum. Microsatellite markers were used in this study to determine the frequency of multiple paternity and thus provide integral information on the mating system of the honey possum. In animals, when a female mates with several males, and the sperm from different males are concurrently viable within the reproductive tract, those sperm from different males must compete to fertilize the set of ova (Gomendio and Roldan 1993b; Gomendio et al. 1998; Parker 1998). In mammals, all ova are released and fertilized simultaneously and therefore multiple paternity is direct evidence of multiple mating and the occurrence of sperm competition (Gomendio and Roldan 1993b; Gomendio et al. 1998).

Highly polymorphic microsatellite loci were the tools of choice for analysing multiple paternity in this study and many successful studies have been undertaken in mammals using this approach. They are a nuclear, codominant marker, inherited in a Mendelian fashion, and composite genotypes over a number of single-locus markers provide very precise, repeatable data for analyses at the individual level, such as identity and parentage analysis (Beaumont and Bruford 1999; Scribner and Pearce 2000; Sunnucks 2000). This makes them superior to dominant, multi-locus markers, such as AFLPs (amplified fragment length polymorphisms) where DNA fragments can only be scored as present or absent. Whilst AFLPs are highly repeatable, they are really only comparable to other data within the same study (Sunnucks 2000). Microsatellites on the other hand, provide allele frequencies as well as genotype data, and thus offer a more flexible approach to population genetics.
Microsatellites consist of non-coding regions of the DNA, the majority are assumed to be selectively neutral, and they are widely dispersed in the eukaryotic genome (Bruford and Wayne 1993; Jarne and Lagoda 1996; Hancock 1999; Scribner and Pearce 2000). They are subject to a high mutation rate ($10^{-5}-10^{-2}$ per generation), through slippage during DNA replication, causing length variation among alleles. In comparison, rates of $10^{-9}-10^{-10}$ have been recorded for point mutations (Hancock 1999). Recent advances in analysis of molecular data and the availability of computer packages allows accurate provision of information that was previously unattainable (Luikart and England 1999; Sunnucks 2000).

Microsatellites often incur a cost in time and research funds, because loci must be identified separately for each species, or at least for each family of species. Specific primers need to be designed to bind to the flanking regions and thereby allow amplification of each locus (Jarne and Lagoda 1996; Scribner and Pearce 2000). Furthermore, the presence of a large number of short, simple repeat sequences that define the microsatellite locus (Bruford and Wayne 1993; Jarne and Lagoda 1996), can create problems with slippage during amplification. ‘Stutter bands’ behind the target product or non-specific product can form (Scribner and Pearce 2000). This, together with the specific nature of the individual species’ DNA, means that the conditions of the PCR amplification need to be optimised for each new primer set. Rigorous approaches are needed to overcome any problems and these can be time consuming and expensive (Beaumont and Bruford 1999).

Despite these costs, microsatellite markers proved successful in determining the extent of multiple paternity in populations of the honey possum in the Fitzgerald River National Park (FRNP). In addition, provisional studies whereby paternity was assigned to offspring, have resulted in recommendations for further study. The genetic analyses included a determination of the amount of genetic diversity in the populations, since this is pertinent to any analysis of paternity. Throughout this chapter, genetic diversity has been separated from the more specific topic of multiple paternity, because of its own intrinsic interest and significance.
5.2 Methods

5.2.1 Samples genotyped at microsatellite loci

In order to analyze litters for multiple paternity, samples from females and the pouch-young they carried were taken over the period from June 2000 to July 2002 and genotyped at four microsatellite loci. Most of the litters were sampled at sites A and C in the June to October period of each year, a time when the incidence of females with pouch-young was highest and trapping was therefore concentrated (see Table 2.1).

In order to sample potential sires, samples were taken, where practicable, in the months prior to litter sampling, to allow for the lag period between conception and sampling of the young, due to embryonic diapause. There are several reasons that a specific time period in which potential sires could be caught could not be directly pinpointed. Firstly, unlike other small mammals which have a defined breeding season, the honey possum is an opportunistic breeder which has pouch-young almost all year round (see Chapter 3 and Wooller et al. 2000). Secondly, embryonic diapause (see Chapter 6) and the short life-span of the honey possum (1-2 years), mean that potential fathers of medium-sized pouch-young (approximately one month old) sampled at any given time may have been any adult male present in the population up to four months earlier. This is only an estimate; given a pouch life of 56-63 days (Wooller et al. 2000), a post-partum oestrus, a possibly variable length of time that embryos spend in diapause, and the fact that the length of gestation after exiting diapause is also unknown. Conversely, the short life-span and high annual mortality rate (86%; Wooller et al. 2000), means that a large proportion of the females with young may be reproducing for the first time, when presumably they do not undertake a diapause strategy. This implies that the father of an offspring might have been a male present in the population only two months prior to litter sampling, a considerable simplification with regards to sampling. The sampling for sires therefore required anticipation of litter sampling by two to four months. The increase in breeding after the February to April low-point proved difficult to predict, particularly
following the population decline after October 2000. However, given that most litters were sampled in July of all three years (see Table 5.6) sampling in the months prior to this was successfully achieved (see Table 2.1 for trapping sessions).

In order to determine the success of paternity assignment, a sub-sample was taken, which included all litters sampled from May to October 2001, and the potential fathers for those litters were analysed. This period was chosen because its greater concentration of trapping sessions prior to the sampling of litters gave the greatest chance of successfully assigning paternity (see Table 2.1). In addition, more litters were sampled in 2001 than in 2000 or 2002, although it was subsequently decided not to analyze the 2-young litters, due to limited time and resources and the limited success of paternity assignment. The potential sires for 2001 litters were made up of all individually marked males caught in trapping sessions in December 2000, and February, April, May, June, July, August, September and October of 2001. Male samples were included from December 2000 because no trapping occurred in January 2001, as the regularity of field trips was offset by very limited capture rates at this time. Even so, trapping effort for the sires of 2001 offspring was intense. Sampling of litters was concentrated in the main trapping sites (A and C). Male samples were available for both areas in all of the aforementioned months, except in June when no trapping was undertaken at site A (see Table 2.1). For the purposes of paternity assignment, male samples from sites B and D were included, because of movement of individuals between sites B, C and D in the hill area. Male samples were available from most months at these sites also (Table 2.1). In total, 115 males were analysed.

5.2.2 Extraction of DNA

Genomic DNA (gDNA) was extracted from samples with limited tissue, such as pouch-young tail tip samples, using an EPICENTRE Masterpure™ DNA Purification Kit which uses a salting out process similar in principle to that of Miller et al. (1988) (Table 5.1). All other samples (adult ear tissue, body tissue from preserved pouch-
young) were extracted using a similar salting out process, using 4M ammonium acetate for precipitation of proteins (Table 5.1). There were some variations to the kit protocol (Iovannisci 2000), which were also included in the ammonium acetate protocol, and were designed to maximize the purity and quantity of the DNA extract (steps 3, 6, 7, 10). All rounds of extraction included a negative control which contained no tissue, but was treated identically. The DNA extraction yield was estimated by fluorometry (Sambrook and Russell 2001) using a HÖFER DyNA Quant 200. The concentration of DNA in the extracts after suspension in 35-50µl TE buffer were between 1 and 300 ng/µl. Pouch-young tail tip samples frequently yielded 20-50ng/µl DNA, despite the sample containing a small amount of tissue. Only 8 out of 128 pouch-young, and 15 out of 155 adult samples, yielded less than 10ng/µl DNA and none less than 1ng/µl. Typically nanogram quantities of DNA are used in PCR (Taberlet et al. 1996). Therefore, ample DNA was available when 2µl was used as a PCR template.

<table>
<thead>
<tr>
<th>Table 5.1: DNA extraction protocol used with EPICENTRE Masterpure™ DNA Purification Kit (KIT) and with ammonium acetate (AA). Protocol same for both except where indicated. Modifications to kit protocol based on (Iovannisci 2000).</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Take tissue from DMSO and rinse with ultra pure ddH₂O.</td>
</tr>
<tr>
<td>2. KIT: Add 300µl Tissue and Cell Lysis Solution + 1µl 50µg/µl Proteinase K to sample.</td>
</tr>
<tr>
<td>AA: Add 250µl Digestion Solution (20mM EDTA, 50mM Tris, 120mM NaCl, 1% SDS) + 2-5µl 20µg/µl Proteinase K to sample.</td>
</tr>
<tr>
<td>3. Vortex and incubate overnight at 55°C with continuous rotation.</td>
</tr>
<tr>
<td>4. KIT ONLY: Cool to 37°C, add 1µl 5µg/µl RNAase, vortex and incubate at 37°C for 30 min.</td>
</tr>
<tr>
<td>5. KIT: Place samples on ice for 5 min, then add 150µl MPC Protein Precipitation.</td>
</tr>
<tr>
<td>AA: Add 250µl 4M ammonium acetate.</td>
</tr>
<tr>
<td>6. Vortex vigorously for 10 sec and chill on ice for 30 min.</td>
</tr>
<tr>
<td>7. Pellet the debris by centrifugation at 4°C for 15-30 min.</td>
</tr>
<tr>
<td>8. Transfer supernatent to clean tube and discard the pellet.</td>
</tr>
<tr>
<td>9. KIT: Add 500µl isopropanol to the recovered supernatent.</td>
</tr>
<tr>
<td>AA: Add 2 volumes of 100% EtOH to the recovered supernatent.</td>
</tr>
<tr>
<td>10. Invert tube 40 times. Place at -20°C overnight.</td>
</tr>
<tr>
<td>11. Pellet the DNA by centrifugation at 4°C for 30 min.</td>
</tr>
<tr>
<td>12. Remove isopropanol/ethanol without disrupting pellet and rinse twice with 70% ethanol.</td>
</tr>
<tr>
<td>13. Dry pellet and resuspend in 35-50µl TE buffer (10mM Tris-Cl, 0.1mM EDTA).</td>
</tr>
</tbody>
</table>
5.2.3 Genotyping of microsatellite loci

Identification of microsatellite loci had been carried out previously and primers were designed for 20 loci (Spencer and Bryant 2000). Fifteen of these appeared to be monomorphic, with five being highly polymorphic. In this study, the primers for these five polymorphic loci were optimized for use with fluorescent labelling and resolution on an ABI PRISM® 377 DNA Sequencer. A subset of samples were used for optimization which appeared successful. Unfortunately, when a further 17 litters were genotyped, the results revealed a serious allele dropout problem. Depending on the primer, between 5 and 32% of young did not appear to have either of the maternal alleles, and this was always associated with either a homozygous female or homozygous young. Faint alleles were also common. In some individuals, one allele was amplified weakly than the other, but in other individuals one allele did not amplify, such that heterozygotes were appearing as homozygotes. This occurred at all five microsatellite loci for a range of different individuals and DNA sample types, including DNA from adult ear tissue, pouch-young tail tips, and pouch-young body tissue.

This problem has highly significant consequences for population studies that use microsatellite markers. Although at each locus between 5 and 32 % of young were mismatching their mother, the extent of the allele dropout problem could have been much greater, because no homozygote could be confirmed. Thus, without genotyping a substantial number of family groups with which to test primer sets, allele dropout such as this may not be detected in a population genetic study, compromising the accuracy of the results.

The problem was not caused by low DNA concentration, as is usually the case concerning allele dropout (Taberlet et al. 1996; Gagneux et al. 1997; Taberlet et al. 1999). Experimental trials have shown that allele dropout becomes a problem when 0.05ng of DNA or less are present in the PCR (Gagneux et al. 1997). The amount of DNA used in the PCRs of honey possum samples had not been this low (see Section 5.2.2), and increasing the amount of DNA did not remedy the allele dropout. A few
samples were identified that had a high concentration (25ng/µl) of DNA in the extract, and also produced good results and these were used as a benchmark for testing. Since 2µl of template was used, these samples had 50ng of template DNA in the PCR. This is well above the level at which allele dropout has been documented, and 10 of the 17 litters were retested using 50ng of template DNA. The incidence of allele dropout was not reduced. Furthermore, varying the amount of template DNA in the PCR from <1ng to >100ng, also did not alter the incidence of allele dropout or change the genotype results of litters that were showing mismatches.

The allele dropout could be distinguished from a null allele problem, in which mutation in the flanking regions of a locus interferes with primer binding and particular alleles do not amplify in a population (Pemberton et al. 1995). In this study, the specific alleles involved were amplifying in some individuals, but weakly, or not at all in others. The other source of non- or weakly amplifying alleles in microsatellite analysis is short allele dominance (Wattier et al. 1998). In this case, short allele dominance was not the problem, as the non or weakly amplified allele in a heterozygote was not always the larger sized allele.

Direct trials of different DNA quantities, as outlined above, revealed that DNA quantity was not a contributing factor. The quality of the DNA was also investigated, since contaminants in the DNA extract may inhibit PCR. A range of samples were extracted using both the EPICENTRE kit and the ammonium acetate protocols outlined in section 5.2.2, as well as with a QIAGEN QIAamp Tissue Kit for ultra-pure DNA extraction. A spectrophotometric analysis at wavelengths from 220 to 350nm revealed no difference between samples extracted using the different methods. There were low background absorbance readings. Readings were close to zero at 230nm and ≥300nm which indicated no significant contamination from organic compounds or particulate matter respectively (see Sambrook and Russell 2001). There were clear peaks in absorbance at 260nm corresponding to nucleic acid. A range of DNA extracts from all extraction methods were run on a 2% agarose gel. Again, no difference was detected between samples extracted using the different
methods. All extracts had a crisp band of high molecular weight DNA, with no degradation. The unstained gel was visualized under UV light to check for the turquoise-coloured fluorescence of substances that may inhibit PCR (see Sambrook and Russell 2001), and none were present.

Previously, BSA (Bovine-serum albumin) had been included in the PCR at a concentration of 0.1 µg/µl to bind inhibitors that may be present and prevent them from inhibiting the reaction. Trial PCRs were performed in which the BSA was increased to 0.5µg/µl or in which no BSA was added. This did not affect the level of mismatch between mother and pouch-young alleles. All of this evidence therefore suggested that the faulty amplifications were not due to poor DNA quality or the presence of inhibitory compounds.

Exhaustive experimentation with elements of the PCR ingredients and the PCR amplification reaction were performed as controlled experiments and the genotypes and peak profiles compared between the treatments. These changes to the PCR were tested primarily with 13 litters and the microsatellite clone, which was a female that had 4 pouch-young. This female and her young had previously been genotyped using the same primer sets with radioactive labelling, such that comparison of genotypes could be made. It should be noted that using this radioactive method, smearing of DNA bands had been a problem. In addition, large numbers of litters were not genotyped as in the present study, and thus allele dropout may have been occurring but escaped detection. Therefore, returning to radioactive labelling could offer no greater guarantee of success.

All of the trials of different PCR conditions failed to solve the allele dropout problem and mismatches between mothers and pouch-young remained. These experiments will be briefly recounted below, however it is not the intention to recount specific nuances in banding patterns for each trial, since none of the trials improved the overall allelic dropout outcome. The basic PCR around which variations were made consisted of a 10µl reaction using 0.5U SIGMA® Taq DNA polymerase, 2µl template
DNA and final concentrations of 200µM dNTPs, 1.5mM MgCl, 1X Buffer (10mM Tris-Cl, 50mM KCl), and 0.5-0.7µM primer. The PCR included an initial denaturation step of 94°C for 5 min, followed by 30-40 cycles of 94°C for 30 sec, 45 sec at the annealing temperature for each primer plus a 72°C extension for 45 sec. A final 72°C extension for 30-45 min was included.

New stock solutions of dNTPs, BSA, Taq DNA polymerase and its buffer and MgCl solution, and fresh working solutions of the primers were made up from stock. For one primer set, new primer solutions were ordered. These new solutions did not correct the allele dropout. SIGMA® Taq DNA polymerase was increased to 0.75U and PCRs were also performed with APPLIED BIOSYSTEMS AmpliTaq Gold® DNA polymerase, but this did not increase the accuracy of the PCR. The MgCl concentration in the PCR reaction had been previously optimized at 1.5mM. This was increased to 2.5mM, but whilst this gave crisper peak profiles it did not increase the amplification of faint alleles or remedy the allele dropout. For one primer set the concentration of the forward and reverse primer were tested independently of each other, in order to compensate for possible interference by the fluorescent label with primer binding for some alleles. The forward primer concentrations of 0.2, 0.5, 0.7 and 1.0µM were tested against each of those same concentrations of the reverse primer. The clarity of the DNA bands varied with these different concentrations, but the genotypes of the two problematic samples tested remained the same. The annealing temperature for all primers was lowered by 3 and 5°C in the standard PCR to aid primer binding to different alleles. Touchdown PCR was also tested extensively. Again, these conditions did not decrease the incidence of mismatch between mothers and pouch-young. Comparison of results between PCRs for all the above conditions indicated that the same set of alleles for each individual were being detected. However, for some individuals, including the microsatellite clone, faint alleles would drop in and out of the results, but this was not consistently associated with any particular PCR condition. It was therefore concluded that the efficiency of
primer binding and amplification of certain alleles was not related to specific PCR conditions.

Consequently, new primers were designed according to recommendations from Applied Biosystems and were placed well away from the microsatellite region. The primers were manufactured with a 7 base extension on the reverse primer (pig-tailing) to ensure the A-nucleotide addition on all products (Brownstein et al. 1996), therefore standardizing size comparisons. One of these five new primer sets still showed similar problems and was discarded, so that eventually four primer sets were analyzed (Table 5.2).

Table 5.2; Sequences of microsatellite primers designed and used for *Tarsipes rostratus*. Repeat motif based on the sequenced clone. Note these are new primers for previously described loci as follows: previously pTr3A11, now Tr16; previously pTr6a3, now Tr18; previously pTr6F9, now Tr1; previously pTr5H3, now Tr14.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer set name</th>
<th>Repeat motif</th>
<th>Primer sequence (5’ – 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tr16</td>
<td>16</td>
<td>(GT)$<em>{19}$GC(GT)$</em>{23}$</td>
<td>F:CAAAATAATCTGAAACATTTGCAAGAAGTCTCCTTATCCTTTA TGGTCCTGGTCTCTGCTCTTTAA</td>
</tr>
<tr>
<td>Tr18</td>
<td>18</td>
<td>(GT)$_{33}$</td>
<td>F:GCCAAAGTCTCCCTCCTACAGT R:TTTTGGTTTTGCACTCATGGAC</td>
</tr>
<tr>
<td>Tr1</td>
<td>1</td>
<td>(CA)$_{26}$</td>
<td>F:TTTCTAGTCTAAACCCGAGATGT R:CCTTCTGGCACTCTGCAC</td>
</tr>
<tr>
<td>Tr14</td>
<td>14</td>
<td>(GT)$<em>{29}$N$</em>{21}$(TAGA)$<em>{14}$TA(TAGA)$</em>{5}$</td>
<td>F:CCACTAAACCTTTGTCTATTATAGAGACCAGT R:GTCCTCCCTCTGACACTTACCTATT</td>
</tr>
</tbody>
</table>

Strict adherence to an optimised set of protocols for each of the new primers was necessary to ensure complete amplification of both alleles. Under these conditions, outlined below, alleles not previously detected were amplified, such that litters which had previously had mismatches between mothers and offspring were now showing
Mendelian inheritance of alleles. No mismatches were detected in any of the 128 pouch-young genotyped with the new primers under the conditions stipulated below. PCRs for at least 30 of the mother/offspring samples were performed at least two times for each primer set, and for each, identical results were achieved. The most important factor in the protocol was the use of the EPPENDORF Triplemaster® PCR system for primer sets 1, 14 and 16. Alleles that were amplifying weakly were more strongly amplified using this system. Triplemaster comprises an enzyme mixture with a blend of DNA polymerases, a proofreading enzyme and a processivity-enhancing factor. It also contains a buffer designed for high fidelity amplifications. DMSO was added as a GC-destabilizing co-solvent and PCRs were prepared as two separate mixes, so that a pre-amplification denaturation step at 98°C could be performed with the separate primer/template mix, without damaging the enzyme mix which is added later. These Triplemaster protocols enhance separation of the DNA strands in difficult templates, allowing primer binding. The difficulties experienced with amplification of the honey possum loci and their remedy through using re-designed primers with a PCR system for difficult templates, suggests that there may be a particular secondary or tertiary biological structure to the honey possum DNA, which poses difficulty with denaturation and primer-binding in some samples.

Including 20ng of DNA as the template in the PCR was determined as giving the clearest banding patterns. Therefore, working solutions of all extracts were made by diluting to approximately 10ng/µl and 2µl was used as the template DNA in PCR. For those few samples that contained less than 10ng/µl in the neat extract, 3µl of this undiluted extract was used and the number of PCR cycles was increased. No allelic dropout was detected in these samples.

The 5’ end of the forward primers were labelled with fluorescent dye markers (ABI PRISM® 6FAM, NED, HEX: APPLIED BIOSYSTEMS). For primer sets 1, 14 and 16, the EPPENDORF Triplemaster® PCR system for difficult templates was used. DMSO was added as a GC-destabilizing co-solvent and PCRs were prepared as two separate mixes of 5µl to give a final volume of 10µl, with a pre-amplification denaturation step
performed with the separate primer/template mix. The final volumes for all mixes were adjusted with the addition of PCR-grade sterile water. Mix 1 consisted of 1X High Fidelity Buffer® (containing 2.5mM Mg$^{2+}$), 0.4µM each primer, 2µl template DNA, and DMSO concentrations of 3%, 2% and 4% for primer sets 1,14 and 16 respectively. Note, the primer set 1 mix also contained 0.4µM SRY primers (see Section 4.2.3). Mix 1 was denatured at 98°C for 30 sec, and placed immediately on ice before the addition of mix 2. Mix 2 consisted of 1X High Fidelity Buffer® (containing 2.5mM Mg$^{2+}$), 0.5U Triplemaster® Enzyme Mix and dNTP concentrations of 300µM, 100µM and 400µM for primer sets 1,14 and 16 respectively. Combined PCR mixes were then placed into a preheated thermocycler (GeneAmp® PCR System 9700 APPLIED BIOSYSTEMS). For primer set 1 an initial denaturation step of 96°C for 10 min was followed by 40 cycles of 96°C for 30 sec plus 60°C for 60 sec. For primer set 14 an initial denaturation step of 96°C for 10 min was followed by 32 cycles of 96°C for 30 sec plus 64°C for 40 sec. For primer set 16 an initial denaturation step of 96°C for 10 min was followed by 40 cycles of 96°C for 30 sec plus 60°C for 90 sec.

For primer set 18, PCRs were prepared in a single step in a final volume of 10µl, using 0.5U SIGMA® Taq DNA polymerase, 2µl template DNA, and final concentrations of 200µM dNTPs, 1.5mM MgCl, 1X Buffer (10mM Tris-Cl, 50mM KCl), 0.5µM each primer, and 2% DMSO. The PCR included an initial denaturation step of 96°C for 10 min, followed by 40 cycles of 96°C for 30 sec plus 60°C for 60 sec.

For a small number of samples that were difficult to amplify, BSA was included in the PCR at a final concentration of 0.5µg/µl, in order to bind any inhibitors and exclude them from the reaction. All rounds of PCR included a negative control, which contained as its template the negative extraction control.

PCR products for all primers were diluted 1:2 with PCR-grade water before combining PCRs from primer set 16 with 18 (1.4 + 0.6µl respectively + 1.0µl PCR-
grade water), and 1 with 14 (1.8 + 1.0µl respectively) for lane multiplexing. These combined products had 2µl of loading buffer, as follows; 1.0µl formamide, 0.6µl loading dye (50mg/ml blue dextran, 25mM EDTA: APPLIED BIOSYSTEMS) and 0.4µl Genescan® 400 ROX labelled internal size standard (APPLIED BIOSYSTEMS), added to them. They were denatured at 95°C for 10 min, and snap cooled on ice immediately prior to electrophoresis. One µl of these combinations was electrophoresed on a 5% 0.2mm urea-acrylamide (AMRESCO® Page-Plus) gel in an ABI PRISM® 377 DNA Sequencer for 2.5h on module 36D-2400 scans per hour. Each freshly polymerized gel was subject to two separate runs with different sets of samples. A positive control was included on each gel to monitor any differences in fragment sizing between gels. Samples from a female and her pouch-young were loaded onto the gel in non-adjacent lanes to ensure that any leakage between lanes was observed, if present. Fragment sizes were analyzed using Genescan® v3.1.2 (APPLIED BIOSYSTEMS).

Scoring and binning of alleles was performed manually. The genotype of each individual was recorded only after careful scrutiny of the peak profiles. Stutter peaks (2bp apart) were present that were smaller than the allelic peak as is common for microsatellites (Scribner and Pearce 2000). For primer set 1 there was only one or occasionally two stutter bands, but for primer sets 14, 16 and 18 there were 3-4 repeat peaks. The peak patterns for each of these loci were studied carefully to minimize potential mis-scoring. Where the two alleles at a locus for a heterozygote were close in size, heterozygotes could be distinguished from homozygotes due to peak height and pattern. In general there was no more than 0.4bp range in the raw fragment sizes of a single allele for any primer. However, in some samples for primer set 14, raw alleles sized intermediate to binned allele categories (1bp difference). The banding patterns of these samples were examined for smearing or bubbles which may have led to the sizing differences and were binned accordingly, or in the absence of any obvious problem were binned into the nearest allele category.
5.2.4 Analysis of microsatellite data

Allele frequencies, expected heterozygosity (HE, Nei 1987) and observed heterozygosity (H₀), were calculated from the genotypic data using the Excel add-in utility MICRO-SATELLITE TOOLKIT version 3.1 (http://oscar.gen.tcd.ie/~sdepark/ms-toolkit/). Tests for deviation from Hardy-Weinberg Equilibrium (HWE) (Guo and Thompson 1992), tests for linkage equilibrium and calculations of inbreeding coefficients were performed using GENEPOP ON THE WEB, a version of GENEPOP 3.1 (Raymond and Rousset 1995) available at http://wbiomed.curtin.edu.au/genepop/.

Since significant deviations from HWE were detected at three loci (see 5.3.1), tests for heterogeneity in allele frequencies between the two trapping areas (mature vegetation site A and hill area sites B, C and D) were performed in FSTAT (Goudet 1995) to detect if these HWE deviations were due to inappropriate pooling of samples. This test for population differentiation does not assume that the populations are in HWE. The null distribution against which the observed data is tested is generated by randomizing genotypes rather than alleles. The log-likelihood statistic G (Goudet et al. 1996) for each of the loci are combined overall as described in the FSTAT program. Estimated null allele proportions (Summers and Amos 1997) were calculated using CERVUS 2.0 (Marshall et al. 1998).

At the time litters were sampled, young were still in the pouch and therefore the maternal relationship of all young was known prior to genotyping. In order to assess multiple paternity in litters, all mothers were genotyped and maternal alleles were identified in the young. Paternal alleles were identified as either (i) an allele present in the pouch-young that was not present in the mother, (ii) an allele present in homozygous condition in the pouch-young or (iii) one of the two alleles of a heterozygous pouch-young that has a genotype identical to the mother’s (Baker et al. 1999). The level of multiple paternity in litters was assessed, firstly using the minimum number of sires for a litter determined from the number of segregating paternal alleles, and secondly by estimating the number of sires using the relatedness of litter mates (sensu Zane et al. 1999; Kraaijeveld-Smit et al. 2002).
The minimum number of sires method attributed multiple paternity, initially, very conservatively, to those litters with a minimum of 3 paternal alleles at at least two loci (sensu Gardner et al. 2002). This ensured that any errors in genotyping at one locus did not erroneously heighten the level of multiple paternity assessed, and served as an absolute minimum estimate of the proportion of litters with multiple paternity.

Multiple paternity was also calculated using the minimum number of sires method in a less conservative way, by attributing multiple paternity to litters that had a minimum of 3 paternal alleles at any locus. This method is commonly employed in multiple paternity studies (Baker et al. 1999; Zane et al. 1999; Ratkiewicz and Borkowska 2000; Kraaijeveld-Smit et al. 2002). Given that the maximum litter size is four, the maximum number of paternal alleles in litters of honey possums can only be four. Therefore, where multiple paternity was attributed in this way, the minimum number of sires needed to explain those paternal alleles could be no greater than two.

The estimated number of sires was calculated from the likelihood of litter-mates being full-siblings or half-siblings, using the program KINSHIP 1.3.1 (Goodnight et al., http://www.gsoftnet.us/GSoft.html). This approach was adopted by Kraaijeveld-Smit et al. (2002) and uses the relatedness statistic ($r$) formulated by Queller and Goodnight (1989). The primary hypothesis was that the litter-mates were full sibs ($r_m=0.5$ and $r_p=0.5$; maternal relatedness and paternal relatedness respectively) and the null hypothesis was that the litter-mates were half sibs ($r_m=0.5$ and $r_p=0$). KINSHIP uses the pairwise $r$ values, the population allele frequencies and the genotypes of the two individuals to calculate the likelihood that the pair in question could have been produced by the relationship specified in the null hypothesis. The likelihood values presented are the ratio between the primary and null hypothesis. Critical values for rejecting the null hypothesis at three significance levels were calculated by KINSHIP after performing 100 000 simulations using the allele frequency data from all adults genotyped. The rates of Type II errors were also calculated. Pairwise relatedness between all adult individuals in the population were also calculated using KINSHIP.
Finally, paternity exclusion was performed using CERVUS 2.0 (Marshall et al. 1998). The combined average exclusion probability of all loci (Chakravarti and Li 1983) was calculated in CERVUS, and given one known parent was 0.9999. CERVUS was used as a tool to assign paternity to offspring, where the male genotype gave a perfect match to the offspring’s paternal allele at each locus. CERVUS can be used to assign paternity, allowing for a prescribed amount of error in genotyping and therefore some mismatch between parent and offspring. The most likely sire can then be determined based on a log-likelihood score calculated using allele frequencies, but this approach is only reliable if the population is in HWE. In this study, significant deviations from HWE were detected (see next section), and in this case it would be erroneous to rely on likelihood scores calculated using observed allele frequencies. Therefore paternity was assigned according to exact matches. The high allelic diversity (see next section) and the extremely high exclusion probability meant that the power to discriminate between potential sires was high, so that an exact match between father and offspring would be rare in the case that it was not the actual father. In all cases exclusion of fathers gave only one complete match, and the males excluded were usually mismatched at more than one locus, making those excluded unlikely to be the true father, even given some level of error.

5.3 Results

5.3.1 Genetic Diversity

Genetic diversity statistics are presented for the 155 adult honey possums analyzed from all trapping sites, and are made up of the females from the litter samples (40) and the males analyzed for paternity (115). Due to recapture of males and sampling of females in all three years, this data set contained samples spanning the entire three year study and thus was representative of the entire population. All loci were highly polymorphic with between 28 and 50 alleles (Table 5.3). Some alleles were present in the population of young that did not appear in the adult population and therefore the number of alleles within the entire data set was slightly higher. These
extra alleles were allele 308 for locus Tr1, alleles 186 and 254 for locus Tr14, and alleles 182 and 254 for locus Tr16. Allele frequencies reached no greater than 15% for any allele (Figure 5.1a, b). Tests for linkage equilibrium revealed that alleles at each locus were independent of alleles at all other loci.

Table 5.3: Allelic diversity, expected heterozygosity ($H_E$) and observed ($H_O$) heterozygosity for four microsatellite loci.

<table>
<thead>
<tr>
<th>Locus</th>
<th>No. of alleles (adult population)</th>
<th>No. of alleles (adults + young)</th>
<th>$H_E$</th>
<th>$H_O$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tr1</td>
<td>27</td>
<td>28</td>
<td>0.94</td>
<td>0.91</td>
</tr>
<tr>
<td>Tr14</td>
<td>48</td>
<td>50</td>
<td>0.97</td>
<td>0.87</td>
</tr>
<tr>
<td>Tr16</td>
<td>39</td>
<td>41</td>
<td>0.95</td>
<td>0.85</td>
</tr>
<tr>
<td>Tr18</td>
<td>28</td>
<td>28</td>
<td>0.95</td>
<td>0.90</td>
</tr>
</tbody>
</table>

The mean (±s.d.) $H_E$ was 0.95±0.01 and the mean $H_O$ was 0.88±0.01 (Table 5.3) over the four polymorphic loci analyzed. There was a tendency for heterozygote deficits at all loci, and tests for HWE revealed significant deficits at locus Tr14, Tr16 and Tr18 and positive inbreeding coefficients (Table 5.4). This was not due to the pooling of samples (Hartl and Clark 1997) from the different areas, because tests for population differentiation between the hill area and the mature vegetation area (site A) were not significant (over all loci $p=0.933$). Null allele estimates based on the difference between $H_O$ and $H_E$ show values above 0.02 for locus 14, 16 and 18 (Table 5.4). No null alleles were observed by examining mother-offspring groups, and repetition of at least 30 samples gave identical genotypes for each primer set. However, there were a few samples that repeatedly failed to amplify at some loci (Table 5.6). It is possible this was due to a homozygous null allele. It is also just as likely that since some loci amplified more strongly than others, that those particular samples were problematic, but only with those particular primer sets that amplified weakly.
Figure 5.1a: Allele frequencies for microsatellite loci Tr1 and Tr14.
Figure 5.1b: Allele frequencies for microsatellite loci Tr16 and Tr18.
Table 5.4: Results of tests for Hardy-Weinberg equilibrium (HWE) and calculation of inbreeding coefficient (Weir and Cockerham 1984) and null allele proportion (Summers and Amos 1997).

<table>
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<tr>
<th>Locus</th>
<th>p-value</th>
<th>SE</th>
<th>$F_{IS}$</th>
<th>Null</th>
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</thead>
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<td>0.016</td>
</tr>
<tr>
<td>Tr14</td>
<td>0.0004</td>
<td>0.0002</td>
<td>+0.107</td>
<td>0.054</td>
</tr>
<tr>
<td>Tr16</td>
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<td>0.0011</td>
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<tr>
<td>Tr18</td>
<td>0.0003</td>
<td>0.0002</td>
<td>+0.059</td>
<td>0.028</td>
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</table>

Whether the slight heterozygote deficits were actually due to inbreeding or to unamplified alleles could not be determined. Relatedness within the population was calculated in order to provide some assessment of the possibility of inbreeding. Mean pairwise relatedness between all individuals in the population was –0.0003, and mean pairwise relatedness between individual males and individual females was 0.0004. In approximately 2% of comparisons between individual males and females, $r$ was between 0.25 and 0.50. That is, it lay between those values expected for half sibs and full sibs respectively. Therefore, there were some males and females that were related in the population, but overall, relatedness was close to zero, as expected in a panmictic population.

Of the 128 young genotyped, two exhibited mutations. Both of these differed from the mother by one repeat unit (2bp) and were at locus Tr1 and Tr14. The mothers and young involved were all heterozygous, excluding the possibility of allele dropout or null alleles. Identical genotypes were obtained when PCRs of these samples were repeated.

5.3.2 Multiple paternity

From the period June 2000 to July 2002, 59 litters were sampled; 23 were litters with 2 pouch-young and 36 with 3 or 4 pouch-young. Multiple paternity could not be detected from litters with two young unless the number of sires was estimated using the relatedness of the siblings. Therefore, genotyping focused on the litters with 3 or 4 young which were more informative. Forty-one litters and their mothers were...
genotyped, giving a total of 128 pouch-young (Table 5.5). The lower number of litters genotyped in 2001 was due to a greater proportion of the litters (15 out of 23 litters sampled) having only 2 young in that year. Litters analysed were sampled between May and October of each year, mainly from the hill trapping area (mostly from site C, with some from sites B and D; Table 5.6). The smaller number of litters sampled from the mature vegetation area (site A) reflects the lower capture rates at this site (Section 4.2.1).

Table 5.5; Sample sizes for litters analyzed at four microsatellite loci. *This litter had 3 pouch-young, but, only two of the pouch-young samples would amplify. In 2001, one litter with 2 young and a subsequent litter with 3 young were sampled from the same female.

<table>
<thead>
<tr>
<th>Litter size</th>
<th>2000</th>
<th>2001</th>
<th>2002</th>
<th>Total litters</th>
<th>Total new individuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 pouch young</td>
<td>3</td>
<td>2</td>
<td>1*</td>
<td>6</td>
<td>18</td>
</tr>
<tr>
<td>3 pouch young</td>
<td>11</td>
<td>4</td>
<td>9</td>
<td>24</td>
<td>95</td>
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<tr>
<td>4 pouch young</td>
<td>3</td>
<td>2</td>
<td>6</td>
<td>11</td>
<td>55</td>
</tr>
<tr>
<td>Totals</td>
<td>17</td>
<td>8</td>
<td>16</td>
<td>41</td>
<td>168</td>
</tr>
</tbody>
</table>

Multiple paternity was shown by 86% of litters using the minimum number of sires method, or by 74% of litters using a more conservative definition of 3 paternal alleles at more than one locus (Table 5.7). There was no clear difference in the level of multiple paternity in litters from the two trapping areas (Table 5.7). Estimation of the number of sires in a litter, using the relatedness of litter mates, revealed a still greater incidence (95%) of multiple paternity (Table 5.7). Again, there was little difference between the two habitat types in levels of multiple paternity estimated using this protocol.

The incidence of multiple paternity amongst litters was also analyzed separately for each of the three consecutive years of the study. Despite marked changes in the population density, the level of multiple paternity remained high in all three years (Table 5.7).
Table 5.6: Each litter genotyped at four microsatellite loci showing date sampled, the number of paternal alleles at each locus, the occurrence of multiple paternity and the estimated number of sires based on the relatedness of litter-mates. Where the number of paternal alleles within a litter at a particular locus was inconclusive, this indicated that one of the pouch-young had the same two alleles as its mother, and the paternal allele could not then be distinguished. ‘No data’ indicates that the sample from the female or one or more of the pouch-young could not be amplified by PCR for that locus. * The pouch-young in this litter were classified erroneously as full-sibs by relatedness analysis, see text.

<table>
<thead>
<tr>
<th>Female</th>
<th>Site</th>
<th>Date litter sampled</th>
<th>Litter size</th>
<th>No. young genotyped</th>
<th>Paternal alleles Tr1</th>
<th>Tr14</th>
<th>Tr16</th>
<th>Tr18</th>
<th>Multiple paternity based on ≥3 paternal alleles</th>
<th>Estimated no. sires</th>
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</table>
Table 5.7: Incidence of multiple paternity amongst litters determined by the minimum number of sires and by estimating the number of sires through relatedness of litter mates. Data presented for all litters analyzed, and then separately for the two different trapping areas, and the years in high and low population density.

<table>
<thead>
<tr>
<th>Multiple Paternity</th>
<th>All years/areas</th>
<th>Trapping area</th>
<th>Population density</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(2000-2002)</td>
<td>hill area</td>
<td>mature vegetation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(sites B,C &amp; D)</td>
<td>(site A)</td>
</tr>
<tr>
<td></td>
<td>All years/areas</td>
<td>high</td>
<td>low</td>
</tr>
</tbody>
</table>

1. Minimum number of sires method
   - at >1 locus
     - ≥3 paternal alleles: 74% 72% 83%
     - at any locus 86% 86% 83%
   - sample size = 35 29 6

2. Estimated number of sires method
   - 95% 94% 100%
   - sample size = 41 35 6

   95% 100% 100% 88% 100% 100% 88% 100% 100%
   sample size = 41 35 6 17 8 16
Critical values were generated to assess the relatedness of litter mates (Table 5.8). In pairwise tests of offspring in a litter, if the log likelihood ratio was greater than the critical value, then the null hypothesis (half sibs) was rejected and the pair were considered full sibs. In order to keep the type II error rate (accepting the half sibs hypothesis when they were actually full sibs) to a minimum, the p<0.05 level of significance was used, and any log ratio above 0.54 was considered full sibs. The log of the likelihood ratio is symmetrical about zero for favouring the primary or null hypothesis. Most of the log likelihood ratios were either highly negative (supporting half sibs), or were positive and much greater than the critical values given (Table 5.9), giving confidence in the results obtained. There were, however, a very few exceptions to this pattern.

Table 5.8: Critical values for log likelihood ratios (primary/null) at different significance levels for KINSHIP calculations of relatedness of litter mates.

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<th>Type II error</th>
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<td>0.01</td>
<td>0.99</td>
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<tr>
<td>0.001</td>
<td>1.91</td>
<td>0.80</td>
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</table>

For two out of 41 litters (FD and F54) the likelihood ratios indicated that, for example, pouch-young 1 and 2 were full sibs, and 2 and 3 were full sibs, but 3 and 1 were not. In these cases, all pouch young pairs were considered full sibs, thus reducing the rate of type II errors. There were also a small number of cases (9) for which the log likelihood ratio was 0.3-0.44, being less than, but close to, the critical value of 0.54 and these were investigated for the possibility of being type II errors. However, even if those pairs were considered full sibs, it did not change the incidence of multiple paternity, because the number of fathers in the litter remained greater than one. Furthermore, in all these cases, multiple paternity had been shown unequivocally using the minimum number of sires method (Table 5.6).
Table 5.9: Log likelihood ratios of litter-mates being full or half siblings, as calculated by KINSHIP.

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Note: Two consecutive litters from Female 59 were sampled.
The two litters that did not have multiple fathers when analyzed with KINSHIP (FD and F54), were litters that were similarly identified as single-father litters using the minimum number of sires method (Table 5.6). There was a third litter (F57) for which the likelihood ratios were slightly greater than 0.54 (0.59; Table 5.9), indicating a single-father litter. However, this litter had been unequivocally shown to exhibit multiple paternity using the minimum number of sires method (Table 5.6); thus, it was considered that there were two sires for this litter. In general, the results of the KINSHIP tests were congruent with the results from the minimum sires method (Table 5.6).

Sampling of litters was constrained by the very small size of the young, which are born when only about 5 mg and leave the pouch at 1.5-2.0 g. In addition, reduction of the brood from an initial four, to two or three young, further reduced the power needed to detect multiple paternity. The number of young in a litter at the time of sampling thus constrained the number of sires that could be deduced from the litter. In 60% of cases the number of paternal alleles equalled the number of young in the litter (Table 5.6). The number of sires in a litter was categorized according to the size of the litter (Table 5.10). In 59% of cases the estimated number of sires for a litter was equal to the number of young in the litter, but in 41% of cases the number of sires was less than the number of young in the litter. There did not appear to be any detectable difference between the three separate years in the number of sires for a litter, although the sample sizes were small.

Eight litters containing a total of 24 young were genotyped from 2001 and these were the target of paternity assignment. Paternity was assigned for six of these young, and three young from 2002 were also fathered by males in the subsample (Table 5.11). In three of the four litters, one male sired two of the offspring.
Table 5.10: Numbers of litters with 2, 3 or 4 young that had an estimated 1, 2, 3, or 4 sires.

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Female 59 had two different litters, one with two young and one with three young, and both were sampled. Collectively, these litters were fathered by four different males. This can be determined since, the litter with three pouch-young had two sires which were identified as male 19 and 208 (Table 5.11). The litter with two pouch-young was determined by the relatedness analysis to have two sires (Tables 5.6 and 5.9). The two sires of each of these litters must have been different, since male 19 and 208 did not sire all the young.

No firm conclusions regarding paternal success can be drawn from such a small number of sires and formal statistical tests were not attempted for this reason. However, the body measurements of the six known sires were compared to mean measurements taken from all adult males captured in FRNP from 1984 to 2003 (Table 5.11). Males become sexually mature when they reach about 6g and 24mm head length (see Section 3.3). However, all six sires were much larger than this. Four of the six males had a head length greater than the population mean. The scrotal length and scrotal width were greater than the population mean for all males. Scrotal measurements relative to body weight were also generally high compared to the population mean, particularly for relative scrotal length.
Table 5.11: Results of paternity assignment. The individual identification number of the father is given, along with the profile of each father. The body measurements of the father are a mean value from all adult captures of the individual and the number of measurements from which it is taken is given in parentheses. The mean (±SE) measurements of all adult males in the population at all trapping areas in FRNP from 1984 to 2003 is given and the sample size is in parentheses.

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<th>Father</th>
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<th>Site</th>
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<th>Weight (g)</th>
<th>Scrotal length (mm)</th>
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18/05/2001
21,23/07/2001
24/08/2001
23/09/2001
11/04/2002)
12/07/2001
11/04/2002
11/04/2002
11/04/2002
11/04/2002
11/04/2002
11/04/2002
11/04/2002
11/04/2002
11| A    | 29.5 (6)    | 9.4 (6)    | 16.7 (6)               | 9.8 (6)    | 1.8                      | 1.0                      |

Mean of six sires
- 27.9±0.86 (2644)
- 8.2±0.57 (2352)
- 16.9±0.43 (1220)
- 10.4±0.39 (1219)
- 2.1±0.10 (1118)
- 1.3±0.10 (1117)

Population mean
- 26.3±0.03 (2644)
- 7.8±0.03 (2352)
- 14.0±0.08 (1220)
- 9.1±0.05 (1219)
- 1.8±0.01 (1118)
- 1.2±0.01 (1117)
From those sires detected, no single male fathered young from more than one of the litters sampled. The minimum number of sires of all young in all the litters sampled in each of the three years was determined by dividing the total number of paternal alleles at the locus with the greatest number of paternal alleles by two. This gave a minimum of 14 fathers in 2000 for 51 young, nine in 2001 for 24 young and 14 in 2002 for 50 young. The actual number of sires could have been much greater. However, it is clear that fertilizations were not obtained by just a few successful males in the population; rather, many males most likely obtained fertilizations.

5.4 Discussion

The high levels of genetic diversity and multiple paternity shown here for the honey possum must ultimately be interpreted in the light of the life-history, population dynamics and reproduction of this species. However, full discussion of the relationships between these findings will be deferred until Chapter 7. The following discussion will highlight the important trends revealed, and beyond that be confined to specific factors relating to the accuracy of the results, and methodological recommendations for further studies.

5.4.1 Genetic Diversity

The high allelic richness at these four microsatellite loci gave an exclusion probability of 0.9999, indicating that the power to detect multiple paternity was extremely high and that individual identity and paternity assignment would be accurate.

The number of alleles per locus was 27-48 and the mean $H_E$ was 0.95. The level of genetic diversity shown here for the honey possum is therefore amongst the highest known in mammals. Microsatellite studies of other mammals commonly report $H_E$ values ranging from 0.15 to 0.90, with commonly less than 10 alleles per locus (Dallas et al. 1995; Garza et al. 1997; Becher and Griffiths 1998; Surridge et al. 1999; Bouteiller and Perrin 2000; Radespiel et al. 2002). Previously, more than 24 alleles per locus have rarely, if ever, been described. Other marsupials show a similar
range of $H_E$ (0.47-0.89), but greater than 16 alleles has not been reported (Spencer et al. 1995; Houlden et al. 1996; Pope et al. 1996; Taylor et al. 2000). Studies reporting heterozygosities over 0.90 are rare, and are usually only in a few loci (eg. Garza et al. 1997). One other study in a small marsupial, the agile antechinus *Antechinus agilis*, showed levels of variation approaching that of the honey possum ($HE$ 0.84-0.93, number of alleles 10-28: Kraaijeveld-Smit et al. 2002). At least one study in birds has found levels of variation as high as that of the honey possum. Microsatellite loci in the yellow warbler *Dendroica petechia* had between 7 and 46 alleles and heterozygosities all greater than 0.90 (Dawson et al. 1997).

The relationship between multiple paternity and genetic diversity will be discussed further in Chapter 7 (Section 7.4.3).

### 5.4.2 Multiple Paternity

There is clearly a high incidence of multiple paternity, and therefore multiple mating by females in this population of honey possums. Multiple paternity was found in 86% of litters by direct counting of paternal alleles, or 95% of litters by estimating the number of sires. A more conservative method indicates that the lower limit of multiple paternity is still as high as 74%. This more conservative method is sometimes employed to guard against errors in genotyping (eg. Baker et al. 1999; Gardner et al. 2002), but it underestimates the level of multiple paternity and is not employed in most studies (eg. Baker et al. 1999; Zane et al. 1999; Ratkiewicz and Borkowska 2000; Kraaijeveld-Smit et al. 2002). All individuals in the four litters where direct counting indicated 3 or more paternal alleles at only one locus were heterozygous, and therefore this discounts false assignment of multiple paternity due to non-amplifying alleles. Mutation may be another possible source of error where only one locus shows 3 or more paternal alleles, but the observed mutation rate determined through the maternal line (see below), was inadequate to account for four such cases. Therefore, a level of 86-95% multiple paternity has reasonably been
established for the honey possum, with the true value probably close to the upper limit of this range.

Extra-pair paternity (EPP) or multiple paternity is facultative in some mammals and birds (Krokene and Lifjeld 2000). Variation in levels of mixed paternity within species and within populations has been found to be correlated with variation in levels of genetic diversity, female synchrony in breeding and population density (see Hughes 1998; Krokene and Lifjeld 2000). For honey possums, the level of multiple paternity remained comparatively high despite marked changes in population densities. This three-year period had density fluctuations as great as any seen in the entire 19 years (see Section 4.2.1). Numbers fell to 4-10 individuals per hectare in 2001, lower than the long-term average of 8-10 individuals per hectare following very low rainfall. This indicates that the mating system is not density dependent, but rather that multiple mating is a stable feature and has evolved over a long association with the population fluctuations. It may also suggest that multiple mating occurs through behavioural relationships between the sexes, as the level of multiple mating did not fall at times when the probability of a female encountering several males might have been expected to fall. Indeed, the two females that were confirmed as having single-sired litters were both from a high-density year (2000). Since females appear behaviourally dominant (Section 3.8), it is likely that multiple mating is a deliberate strategy, and that they are not coerced into re-mating. Changes in density are related in part to rainfall, and rainfall in the previous year determines the food resources available (see Chapter 4). This suggests that food availability did not impact on the degree of multiple mating either.

Since all ova from one oestrus are released simultaneously in the honey possum (see Chapter 6), as in other mammals (Gomendio et al. 1998), multiple paternity indicates that sperm from two or more different males, capable of fertilizing the ova, must have been present within the tract at the same time. The sperm from different males are therefore in competition to fertilize the same set of ova (sensu Birkhead and Møller 1998; Parker 1998; Anderson and Dixon 2002). Multiple paternity is the
most direct evidence of sperm competition in mammals (Gomendio et al. 1998), and the high incidence of multiple paternity in the honey possum thus confirms that sperm competition occurs in this species.

Female multiple mating and mixed paternity within broods occurs in a wide array of animal taxa (Birkhead and Møller 1998). Molecular evidence for multiple paternity in mammals has shown in some cases that behavioural observations of multiple mating were correct in determining multiple paternity (Gomendio et al. 1998). In other cases, observations of widespread multiple copulations by females led to only a small percentage of litters being multiply sired. It is possible in these cases that copulation does not result in ejaculate transfer, or that some males easily out-compete other males, or are more successful because of female choice. Behavioural observations in the wild were not possible for the honey possum, because of the difficulties common to studies of other nocturnal mammals of small body size, living in habitats with poor visibility (Gomendio et al. 1998). Levels of multiple paternity in small eutherian mammals show levels from zero in the California mouse *Peromyscus maniculatus* and the oldfield mouse *Peromyscus polionotus*, through to 88.9% in the common shrew *Sorex araneus* (Gomendio et al. 1998). Molecular methods have also been employed to study paternity in other marsupial species (Watson et al. 1992; Houlden et al. 1996; Spencer et al. 1998; Fisher and Lara 1999; Sarre et al. 2000; Taylor et al. 2000; Clinchy et al. 2004). Amongst marsupials with a litter size greater than one, multiple paternity has been shown in captivity in the brown antechinus *Antechinus stuartii*, and the brush-tailed phascogale *Phascogale tapoatafa* (see Taggart et al. 1998). Preliminary molecular studies have indicated multiple paternity in the feathertail glider *Acrobates pygmaeus* (Parrot et al. 2002). The only other marsupial in which multiple paternity has been established in the wild is the agile antechinus (Kraaijeveld-Smit et al. 2002, 2003). In this last species, 46 of 47 litters analyzed demonstrated multiple paternity. Aside from this similarity, the reproduction and mating of the agile antechinus is quite different from that of the honey possum. *Antechinus* males are the larger sex, there is a lek mating system and the highly seasonal breeding is followed by male die-off, resulting in females
storing sperm in oviducal crypts for up to 2-3 weeks before ovulation (Taggart et al. 1998; Kraaijeveld-Smit et al. 2002).

In the honey possum, the number of sires estimated for each of the litters based on relatedness of the siblings showed that, in 59% of cases, the number of sires was equal to the number of young in a litter. This indicated that the litter size at the time of sampling constrained the number of sires able to be detected. Thus, in many cases there may be four different fathers, one for each of the four young in the pouch soon after birth; the number of males that a female mated with may have been even greater. However, there were a considerable number (41%) of litters for which the estimated number of sires was less than the number of young in the litter. In three of the four litters to which sires were assigned, one male sired two of the offspring in the litter. This indicates that some males sired more than one of the offspring per female and were thus more successful in gaining fertilizations under sperm competition. Despite this, no more than two offspring in a litter were known to have been sired by the same male.

Paternity was assigned for six out of the 24 young for which paternity assignment was targeted. In addition, three young from 2002 were matched to fathers. These males were all relatively large (>7g and 26-31mm head length), given that males become sexually mature when they reach 6g and 24mm head length. Their scrotal dimensions, particularly scrotal length, were larger than the population mean. This was not due only to their body size, as the scrotal dimensions were also relatively large when body weight was taken into account. Although it is possible that successful sires tend to be larger in their body dimensions, these are only indications based on a very limited number of sires. Conclusions on the characteristics of successful males must therefore await further testing.

A secondary objective of this study was to assess the feasibility of assigning paternity to as many of the pouch-young as possible. It was hoped that this might allow identification of correlates of reproductive success amongst males. Unfortunately,
the magnitude of the difficulties with the microsatellite primers left too little time and resources for this task. An even greater factor was that attempts at assigning paternity to a subset of young met with only limited success, despite the accuracy provided by the highly variable markers. In most of the offspring whose fathers were unassigned, the problem was not misassignment through mismatch at one locus, but at several loci, indicating that there really were no candidate matches amongst those males sampled. The sires, therefore, were simply not trapped.

The subset of litters all came from areas A and C, areas that were sampled at every trip in 2001 and in most months of the year (see Table 2.1). Trapping effort, therefore, was high and failure to capture sires indicates that their movements must have been primarily outside the grid system. Copulations may have taken place either outside the grids or the males did not remain within the grids, such that these males evaded capture. Earlier estimates of home ranges of honey possums had previously indicated that they were generally sedentary (males 1277 m$^2$, females 701 m$^2$) and that most individuals moved less than 30 m over several months (Garavanta et al. 2000). Given this, the grid layout of the present study should have been adequate to capture the majority of sires. However, more recent evidence from the current study has suggested that over 70% of individuals are not recaught, they move further than previously thought and may have mobile home ranges (see Section 4.3). This would explain the low rate of paternity assignment.

Assigning paternity is notoriously difficult in field populations of mammal and bird species and levels of assignment in published studies are commonly between 40% and 70% (Spencer et al. 1998; Alderson et al. 1999; Bouteiller and Perrin 2000; Sarre et al. 2000; Taylor et al. 2000; Kraaijeveld-Smit et al. 2003). In most cases, identification of a large number of sires requires a very large total number of individuals analyzed, since assignment success rates are often not very high. It may have been possible to increase the number of young for which paternity could be determined in this study by analyzing the additional litters consisting of two young, and all males caught over the entire three year period. However, this would have
required genotyping an additional 400 individuals, which was not feasible given the time and resources available.

Thus, whilst this study was clearly able to determine the levels of multiple mating, studies of male reproductive success would be improved by the use of a different trapping regime. Trapping would need to be as intense (with traps 5m apart), but over a much greater area. Litters could be analyzed from a central area, whilst all males from surrounding areas would need to be analyzed as potential sires. The high density of honey possums in this area, and the requirement to analyze males caught much earlier, as a consequence of embryonic diapause, would make such a study extremely large and expensive. In addition, new molecular markers need to be sought (see below).

5.4.3 Microsatellites in the honey possum and recommendations for future study

The most reliable method of determining mutation rates is direct counting from pedigrees; however, there can be biases (see Jarne and Lagoda 1996; Amos 1999; Hancock 1999). For honey possums, at locus Tr1, 123 meiotic events were observed with one mutation, and at locus Tr18, 128 meiotic events were observed, also with one mutation. This corresponds to a rate of $8.1 \times 10^{-3}$ for locus 1, and $7.8 \times 10^{-3}$ for locus 18. Mutation rates at the other two loci (14 and 16) would therefore be lower than these. Caution should be observed when determining mutation rates over few loci (Jarne and Lagoda 1996; Amos 1999). However, these mutation rates lie within the range reported for other organisms, such as $10^{-2}$ for *E.coli*, $10^{-3}$-$10^{-4}$ in mice, $6 \times 10^{-6}$ in *Drosophila*, and $10^{-3}$ in humans (various citations in Hancock 1999). The sample size in the present study appeared adequate for such an assessment, given that a study in humans estimated a rate of $10^{-3}$ using 91 offspring (Weissenbach *et al.* 1992), although this was a combined estimate over 160 loci.

The modest, yet significant heterozygote deficit at three of the four loci, may be due to a level of inbreeding or may indicate anomalies with amplification of the
microsatellite alleles. A Wahlund effect (Hartl and Clark 1997) can be ruled out, because no substructuring within the data between different sampling sites was found. Substructuring was in any case unlikely because the trapping sites were only 3km apart and the mark-recapture study was able to identify a small number of individuals that moved between areas (Section 4.2.3).

There is a small possibility that there is a low level of inbreeding in the population. If future studies could assign paternity to a large sample of offspring, then the incidence of mating between related individuals could be assessed by calculating the relatedness between the parents of all offspring. Overall relatedness in the population was low, and mean $r$ was close to zero, as expected in a panmictic population. Some male-female pairs were identified within the population that were closely related. Although dispersal, as assessed through relatedness (Section 4.2.3), was male biased, perhaps male dispersal is not complete, such that some matings between related individuals occur. Although all loci showed tendencies toward heterozygote deficit, at locus Tr1 this was only very slight and was not significant. This locus had a very crisp banding pattern with minimal stutter and was easy to work with, which may indicate that it was more reliable. Therefore heterozygote deficit at only three of the four loci typed, may be more likely due to non-amplification or non-detection of some alleles at particular loci, rather than inbreeding.

Non-detection of alleles is a possibility because alleles can be mis-scored due to stuttering of microsatellite peaks. The peak profiles were thoroughly checked for each individual, and heterozygotes with alleles close in size were distinguished from homozygotes by peak height and pattern and the difference was obvious in most cases. However, it does remain possible that some individuals were mis-scored due to the stuttering of allele peaks obscuring the true genotype. As mentioned above, Tr1 had negligible stuttering and very clear allele peaks and was also least affected by heterozygote deficit. Whether these unavoidable errors occurred, and were frequent enough to cause the heterozygote deficit is possible but perhaps unlikely given that careful scoring was employed.
Alternatively, non-amplification of alleles is a possibility. The proportion of null alleles presented in Table 5.4, is calculated from the heterozygote deficit, and therefore does not represent evidence for null alleles. No mother-offspring mismatches were observed, although a few samples could not be amplified. Rather than null alleles, it is more likely that there was still some level of allele dropout or differential non-amplification of alleles. Although the re-designing of the primers significantly improved the problem, perhaps some residual problems remained. Some individuals had alleles that were unequivocally detectable, but amplified weakly in comparison to the other allele. It is possible that those alleles were unamplified in other individuals and, either by chance were not alleles corresponding between mothers and offspring, or were in the male population. The multiple paternity data in this study are accurate because of the proven integrity of those pedigrees: the genotypes generated were repeatable and no mother/offspring mismatches were observed. Error in scoring, if it exists, would only alter the estimated level of multiple paternity slightly. In addition, the proportion of non-amplifying alleles was significant, but low (0.02-0.05), and assessment of levels of variation and multiple paternity would be little affected.

It is possible that the flanking regions of microsatellites in the honey possum are unstable or subject to a high mutation rate, independently from the microsatellite locus itself. The quality and quantity of the DNA samples were checked thoroughly and can be ruled out as a possible cause of unamplified alleles. It is likely that there is an inherent feature of honey possum DNA, such as secondary or tertiary structure, GC content or other compounds associated with the DNA, that affect the way the DNA behaves in PCR. There are several other reasons that make this seem likely. A specialized PCR enzyme for difficult templates, a specialized technique with pre-amplification denaturation and long, hot denaturation of the samples before loading on the gel was required to ensure detection of some alleles. PCR products from honey possum DNA have a propensity to have a smear preceding the DNA band when visualized on an acrylamide gel. Small smears occurred regardless of the intensity of the band, the technique (radioactive or fluorescent) and many attempts to
identify and remove the causative factor (for example DNA quantity, sample contaminants, remaining PCR reagents and dilution factors). These characteristics appear to be an inherent difficulty of working with honey possum DNA, and support the idea that the slight heterozygote deficit described here is caused by biological features of the DNA, rather than being related to the technique or locus. In addition, the thorough approach taken to remedy the problem by altering the techniques involved in the DNA analysis indicated that this was not merely a technical problem that could be overcome by common protocol alteration. Congruent with this is that some loci may be more susceptible and indeed, the most reliable locus in this study had a non-significant heterozygote deficit.

Problems with microsatellites when working with particular taxa are undocumented, but anecdotal evidence suggests that difficulties are common (Beaumont and Bruford 1999). Further genetic studies in the honey possum would best be pursued by using new markers. Studies of differentiation of populations could utilize sequencing of regions of the mitochondrial DNA, with use of SSCP (single-stranded conformation polymorphism) techniques to detect which samples differ, so that only a subset of variants need to be sequenced (Sunnucks et al. 2000). For paternity studies new microsatellite loci with tri- or tetra-nucleotide repeats could be sought, since they commonly amplify more reliably and have banding patterns that can be scored more accurately. If a heterozygote deficit was still detected, this would indicate that it could not be attributed to scoring error where heterozygotes were scored as homozygotes. It is entirely likely that a heterozygote deficit could still occur because of the possible inherent difficulties with honey possum DNA discussed above.

Microsatellite markers on the Y-chromosome have been useful in excluding sires of male offspring in human studies, but have their limitations and would need to be used in conjunction with autosomal markers (Jobling et al. 1997). Y chromosomes, in combination with maternal and autosomal markers, have the potential to be informative about the mating system and sex-biased dispersal in mammals (Petit et al. 2002), but locating markers on particular chromosomes requires cytogenetic
techniques. AFLP can be used successfully in paternity studies (Mueller and Wolfenbarger 1999). However, trials of AFLP restriction enzyme/primer combinations used successfully in the quokka Setonix brachyurus (Alacs 2001), was unsuccessful in the honey possum. Smearing and unclear banding patterns resulted, either because of the biological nature of honey possum DNA, or its large genome (2n=24) in comparison to many other marsupials (Hayman and Sharp 1982). Trying other restriction enzyme/primer combinations would take much development time without any guarantee of success. Given the drawback that dominant markers do not yield allele frequency information, and are therefore limited in their application to population genetics (Mueller and Wolfenbarger 1999; Sunnucks 2000), it would be best to channel future development time and resources into characterizing new microsatellite markers in the hope of finding unproblematic ones.

5.5 Conclusion

Microsatellite loci revealed an extremely high level of genetic diversity in the honey possum, amongst the highest seen in any microsatellite study of vertebrates. There was a high incidence of multiple paternity in litters (86-95%), and therefore a high incidence of multiple mating by females, providing evidence that sperm competition occurs. These high levels of diversity and multiple paternity were unaffected by large fluctuations in population densities. Multiple mating is therefore a stable feature of the honey possum mating system.
Chapter 6: Reproduction in the Female Honey Possum

6.1 Introduction

There is much information on the life-history and breeding of the honey possum, which has been presented in Chapter 3. However, there has been very little investigation into female reproduction. There was an early description of reproductive tract anatomy by Hill (1900) based on only two animals, and by de Bavay (1951), also based on only two animals. There has also been a one-page initial report of embryonic diapause involving 16 animals (Renfree 1980). Lack of a detailed description of reproduction in the female honey possum inhibits some aspects of studies into the life-history and mating system. Thus, it is timely to revisit reproduction and embryonic diapause in the honey possum.

Embryonic diapause is widespread and occurs in at least seven orders of animals (Mead 1993). The term embryonic diapause is used to describe delay at the blastocyst stage, and development that resumes some time before implantation takes place (Renfree and Calaby 1981; Renfree and Shaw 2000). There are other types of delay, such as delayed implantation, where implantation immediately follows resumption of development, and delayed development, which describes a delay following implantation. Delay or diapause is widespread in mammals, suggesting that it must have evolved independently many times (Renfree and Calaby 1981). Amongst eutherian mammals it occurs commonly in the orders Carnivora and Rodentia, and less commonly in the Insectivora, Chiroptera, Edentata and Artiodactyla. In marsupials it occurs in the order Diprotodontia, in the families Macropodidae, Burramyidae, Acrobatidae and Tarsipedidae.

Detailed descriptions of the female reproductive physiology, conceptus development and the histology of the reproductive tract throughout the cycle are available for a relatively small group of model species of marsupials. The aim of this chapter is to provide a description of the histology of the reproductive tract, conceptuses and
embryonic diapause in the honey possum. A generalized description of marsupial reproduction facilitates the specific description of reproduction in the honey possum and, for clarity, a brief overview will be given here, concentrating on early development to the blastocyst stage. Current information on embryonic diapause in marsupials will then be reviewed.

6.1.1 Reproductive anatomy and physiology of marsupials

The female honey possum reproductive tract is similar in arrangement to other marsupials, with two separate uteri and a vaginal complex consisting of two lateral vaginae and a median vagina (Figure 6.1). The uteri consist of a glandular endometrial wall, surrounded by a muscular myometrium. The pair of cervices pass into the vaginal cul-de-sacs, where the anterior ends of the lateral vaginae meet (Tyndale-Biscoe and Renfree 1987). In the macropods (Tyndale-Biscoe and Renfree 1987) and in the honey possum (de Bavay 1951), the median vaginal canal, the birth canal, remains open after the first parturition. However, in all other marsupials the birth canal forms prior to each parturition from within a connective tissue strand, that runs from the cul-de-sacs to the urogenital strand. In the one pre-parous honey possum that de Bavay (1951) examined, the median vagina was fully developed until well into the urogenital sinus, and was only separated from the urethra at the posterior end by a solid plate of cells, within which it was evident where a future perforation would occur. In addition, many other species have a partial or complete septum that divides the cul-de-sacs and median vagina, including the closely related feathertail glider Acrobates pygmaeus (Ward and Renfree 1988a).

Changes in the histology of the uteri, vaginae and ovaries occur throughout the oestrous cycle (Tyndale-Biscoe and Renfree 1987). The proliferative phase of the oestrous cycle includes the periods of follicular development and oocyte maturation, oestrus and ovulation. The cells of the uterine epithelium increase in number (hyperplasia) and size (hypertrophy). The stroma of the glandular endometrium can become oedematous. The lateral vaginae and the urogenital sinus enlarge by
hyperplasia and hypertrophy. At oestrus, the median vagina and cul-de-sacs become highly secretory and the lateral vaginas have a squamous epithelium which becomes cornified and sloughed in the lumina. Following oestrus, the vaginal complex decreases in size and the corpus luteum forms in the ovary. The production of progesterone from the corpus luteum causes a ‘luteal phase’ to ensue, regardless of whether fertilization takes place (Tyndale-Biscoe and Hawkins 1977; Renfree 1981). The uterus increases in size due to hypertrophy of the cells, and the glands in the endometrial wall of the uterus deepen and become highly secretory (Tyndale-Biscoe and Renfree 1987). The cuboidal luminal epithelium, and glandular epithelium change to tall columnar cells with small nuclei and secretory vesicles. The demise of the corpus luteum sees the glandular endometrial wall of the uterus regress. The glandular lumina can have degenerating cells and the luminal epithelial cells become smaller. The corpus luteum produces progesterone during pregnancy which declines just before birth. Where post-partum oestrus occurs, the Graafian follicle secretes oestrogens; this, combined with declining progesterone, elicits oestrous behaviour. Positive feedback via the pituitary produces a luteinizing hormone surge which redirects follicle synthesis from oestradiol to progesterone and then leads to ovulation one to two days later.

**Ovary**

Unlike eutherians, the marsupial oocyte grows throughout follicular development in at least some species (Falconnier and Kress 1992; Roger et al. 1992). Final oocytes are generally of similar size in most marsupials (mean diameter 145µm), but the final follicle diameter is positively correlated with body size (Tyndale-Biscoe 1984; Tyndale-Biscoe and Renfree 1987). In developing primary and secondary follicles, the oocyte is surrounded by several layers of follicular granulosa cells and an outer thecal cell layer (Kerr 2000). The oocyte develops a zona pellucida: a protein coat 1-6µm thick (Hughes 1974; Tyndale-Biscoe and Renfree 1987; Selwood 2000). In more developed tertiary follicles or pre-ovulatory Graafian follicles, the oocyte is suspended in a large fluid-filled antral cavity which is surrounded by granulosa cells (Kerr 2000).
Figure 6.1: Ventral view of the reproductive tract of an adult female honey possum showing the pair of uteri, each with connecting oviduct and ovary. In all specimens examined, the long uterine neck projected well into the median vaginal canal as a single papilla, with the cervical canals separated for their entire length. The median vaginal canal is well developed through to the urogenital sinus. The ureters pass between the vaginae to enter the base of the bladder (shown dashed). Transverse sections in positions A, B, C and D correspond to those presented in Figures 6.11 - 6.13.
Maturation of the oocyte itself has been studied in a small number of species (Breed 1994, 1996; Mate 1996). The oocyte within the developing follicle is arrested in meiosis I at diplotene of prophase I. Meiosis I resumes and is completed just prior to ovulation, and sees the extrusion of the first polar body. The chromosomes immediately pass into metaphase II and are arrested in this phase during ovulation. The second meiotic division is not completed until fertilization takes place. Intense cytoplasmic activity occurs during the pre-ovulation period, as well as changes in the zona pellucida.

Oestrus precedes ovulation by one to two days in most marsupials (Lyne and Hollis 1977; Tyndale-Biscoe and Renfree 1987). Oestrus is usually indicated by sloughing of cornified epithelial cells in the vaginal canal (Tyndale-Biscoe and Renfree 1987). After ovulation, the collapsed follicle, the corpus hemorrhagicum, develops into the corpus luteum. The granulosa cells become large ‘puffy’ secretory cells, containing a yellow pigment lutein, and vascularization occurs. The corpus luteum has a role in the regulation of follicular growth and ovulation, oestrous behaviour, conceptus development, preparation of the tract for parturition and of the mammary glands for lactation (Tyndale-Biscoe and Renfree 1987). In general, the progesterone secreted by the corpus luteum increases throughout pregnancy, reaching a maximum in late pregnancy and declining before birth. The role of the corpus luteum in embryonic diapause is discussed below. Corpus luteum degeneration can be recognized by shrinkage of the luteal cells, pyknosis of nuclei and visible areas of connective tissue (Tyndale-Biscoe and Renfree 1987).

_Fertilization, cleavage and conceptus development_

At copulation, sperm are deposited in the urogenital sinus and are thought to travel through the lateral vaginae. They then travel through the cervix, the uterus and into the ampulla region of the oviduct where fertilization takes place (Taggart 1994). Once the spermatozoon has entered the egg (fertilization reviewed in Breed 1994, 1996; Mate et al. 2000), the second maturation division resumes and is completed with extrusion of the second polar body (Selwood 1982; Tyndale-Biscoe and Renfree
Breed 1996). The sperm head decondenses and expands to form the male pronucleus. The male and female nuclei move so they are lying close together before the first cleavage division (Selwood 1982; Selwood and Young 1983). The pronuclei are of similar size and appearance (Renfree and Lewis 1996).

A second acellular egg-coat is secreted within hours of fertilization by non-ciliated cells at all levels of the oviduct (Hughes 1974; Tyndale-Biscoe and Renfree 1987; Selwood 2000). This mucoid coat is an acid glycoprotein and ranges in thickness from 6.7 to 230 \( \mu \text{m} \) in marsupials. A third protein coat, the shell membrane, is secreted by the epithelial cells of the utero-tubal junction and the upper uterus. It reaches maximum thickness during the unilaminar blastocyst stage (range 1-6 \( \mu \text{m} \)), and once reaching the maximum, then remains the same thickness during diapause in macropods. It then gets thinner during expansion and the inner membranes, the zona pellucida and mucoid coat get absorbed during the unilaminar blastocyst stage or expansion into the bilaminar blastocyst, depending on the species (Hughes 1974; Tyndale-Biscoe and Renfree 1987). The shell membrane completely separates the foetus from the maternal uterine tissue, until two thirds of the way through the pregnancy when organogenesis begins at the foetal stage (Hughes 1974; Tyndale-Biscoe and Renfree 1987; Selwood 2000).

To increase clarity in describing early development, Johnson and Selwood (1996) proposed that the term \textit{conceptus} be used for all products of fertilization, and \textit{embryo} be reserved for the stage of development when the embryo-proper starts to develop (primitive streak stage). Thus \textit{conceptus} will be used throughout. Passage down the oviduct takes about one day, and the conceptus enters the uterus in the pronuclear stage (Lyne and Hollis 1977; Selwood 1980; Tyndale-Biscoe and Renfree 1987; Renfree and Lewis 1996). The early conceptus then undergoes a series of cleavage divisions to form the blastocyst (Selwood 1980; Selwood and Young 1983; Renfree and Lewis 1996; Selwood 2001).
The cleaving cells, or blastomeres, divide to form a complete flattened layer against the zona, the trophoblast, with the blastomeres joined at the margins by junctional links (Selwood 1980; Selwood and Young 1983; Tyndale-Biscoe and Renfree 1987). The unilaminar blastocyst is therefore a hollow sphere with a peripheral layer, the trophoblast, and an internal fluid filled cavity, the blastocoele. In marsupials there is no inner cell mass. The number of cells taken to complete the unilaminar blastocyst varies, for example, from 16 cells in the Virginian opossum *Didelphis virginiana*, to 32 cells in the brown antechinus *Antechinus stuartii*, to 60-100 cells in macropods (Tyndale-Biscoe and Renfree 1987: 276). It takes 4-9 days to reach this stage, depending on the species. Until development of the unilaminar blastocyst there is no increase in diameter and the conceptus relies on its own nutrition, but thereafter expansion requires nutrition from uterine secretions (Tyndale-Biscoe and Renfree 1987). The blastocyst rapidly expands by absorbing fluid into the blastocoele as it moves from the unilaminar to bilaminar stage (Tyndale-Biscoe and Renfree 1987).

The cells of the unilaminar blastocyst appear to be identical in macropods (Tyndale-Biscoe and Renfree 1987; Renfree and Lewis 1996). However, earlier during cleavage there is some polarity of cells, and in other groups such as the dasyurids, opossums, brushtail possums and the bandicoots, two cell types are prominent in cleavage through to the unilaminar blastocyst stage (Selwood 1996). As well as trophoblast cells, which give rise to the extra-embryonic ectoderm of the yolk sac and populate two-thirds of the inner surface of the zona, there are pluriblast cells that give rise to the embryonic cells and extra-embryonic membranes, and populate one third of the zona (Selwood 1994; Johnson and Selwood 1996; Selwood 1996). Using the terminology of Johnson and Selwood (1996), this pluriblast (previously embryonic area) is a thickened area of cells, and gives rise to the hypoblast or primary endoderm cells and the epiblast or embryonic ectoderm. The first hypoblast cells have darker-staining, granular cytoplasm, and a compact shape (Tyndale-Biscoe and Renfree 1987: 299). These cells migrate inwards, project pseudopodial filaments, which connect adjacent cells, and in this way form a loose layer beneath the entire inner surface of the trophoblast/epiblast. Cell division makes this layer complete and
therefore a bilaminar blastocyst. All marsupials studied at this stage have an epiblast (cuboidal cells), a trophoblast (squamous cells) and a hypoblast lying within (see diagram below, adapted from Selwood 1994).

This hypoblast is not the definitive endoderm of the foetus (McLaren 1987), and the trophoblast and hypoblast formulate the yolk sac (Selwood 1994). The primitive streak develops from within the epiblast (Lyne and Hollis 1977; Selwood 1980; Hughes and Hall 1984; Tyndale-Biscoe and Renfree 1987; Renfree 1993). At about the time that the notochord is complete, the shell membrane ruptures and close association of the trophoblast to the uterine epithelium occurs. Development up to this stage is relatively slow and takes about two-thirds of the gestation period. Organogenesis is relatively rapid and occupies the remaining third of gestation.

6.1.2 Patterns of reproduction and embryonic diapause

Most marsupials, except the macropods, are polyoestrous and polyovular (Tyndale-Biscoe and Renfree 1987). Apart from in the macropods and the small possums, gestation (taking usually 12.5-19 days) occupies less than 60% of the oestrous cycle and occurs within the luteal phase. If lactation follows, the subsequent follicular phase is suppressed.
The macropods (families Macropodidae and Potoroidae) are polyoestrous and monovular (Tyndale-Biscoe and Renfree 1987). The luteal phase lasts for almost the entire oestrous cycle and the follicular phase is not suppressed. Gestation occurs throughout the luteal phase and is followed by a post-partum oestrus and ovulation (in all but six of the species studied: two pre-partum and four later in pouch life). If lactation follows, then the corpus luteum of pregnancy degenerates, similarly to the non-pregnant female. The new corpus luteum formed at post-partum ovulation is held in a state of quiescence and embryonic diapause occurs. All species of macropod examined, except the western grey kangaroo *Macropus fuliginosus*, exhibit embryonic diapause.

The small possums from the families Burramyidae, except *Burramys*, Acrobatidae and Tarsipedidae, are polyoestrous and polyovular (Tyndale-Biscoe and Renfree 1987). There is a very prolonged luteal phase and gestation which includes embryonic diapause. However, there is some evidence of a different pattern in the eastern pygmy possum *Cercartetus nanus* and the long-tailed pygmy possum *C. caudatus*, where the luteal phase of the oestrous cycle is prolonged into lactation, like that of the bandicoots (Ward 1998).

*Embryonic diapause*

The embryonic diapause of the macropods and that of the small possums, such as the honey possum, is quite different. In the macropods, gestation can be extended for up to 11 months by a period of developmental arrest (see Renfree 1981; Tyndale-Biscoe and Renfree 1987; Renfree 1993). Total stasis of growth occurs in the 70-100 cell conceptus, at the stage of the unilaminar blastocyst (see below). The outer diameter of the shell membrane remains at 0.25-0.33 mm for the entire period and mitoses are never seen. There is always a quiescent corpus luteum present.

This developmental arrest occurs as a direct result of lactation (Renfree 1981; Tyndale-Biscoe and Renfree 1987; Renfree 1993). If the pouch young are removed or lost, the loss of the sucking stimulus causes the corpus luteum and blastocyst to
resume development. In the tammar wallaby *Macropus eugenii*, following reactivation, the diameter of the blastocyst expands exponentially (Tyndale-Biscoe and Renfree 1987; Renfree and Shaw 2000). Following birth, oestrus and mating occurs, and the cycle starts again (Renfree 1981; Tyndale-Biscoe and Renfree 1987; Renfree 1993). In addition to lactational quiescence, two species show a seasonal quiescence. In these species, the corpus luteum and blastocyst remain in quiescence for several months after the weaning of the pouch-young, and they resume development after the summer solstice (see Tyndale-Biscoe and Renfree 1987; Renfree 1993).

The small possums that exhibit embryonic diapause are the feathertail glider, the western pygmy possum *Cercartetus concinnus*, and the honey possum. Renfree (1980) reported blastocysts of between 1.2 and 1.7mm diameter, within the uteri of twelve female honey possums carrying pouch-young of head lengths 5-15mm. The size of the blastocysts within each uterus was variable, but between females with various sizes of pouch-young was similar. There was a slow increase in the diameter of the blastocysts (0.7 to 1.7mm) when plotted against the head length of the pouch-young carried (Renfree 1981). All blastocysts were at the unilaminar stage (Renfree 1980). One female with neonates had sperm in the uterus and a second female with tiny pouch-young had cleaving conceptuses *in utero*. This suggested a post-partum oestrus and embryonic diapause in the honey possum.

Similar patterns of reproduction were also found in the feathertail glider and the western pygmy possum. Ward and Renfree (1988a) found that feathertail gliders had a post-partum oestrus and embryonic diapause. The growth of blastocysts *in utero* in relation to the head length of young concurrently carried in the pouch was in two phases. An initial period of rapid growth to a diameter of approximately 1.6 mm was followed by a plateau phase with little increase in diameter. The beginning of the plateau phase corresponded to about 33 day old pouch-young, with a head length of approximately 10 mm. All blastocysts found were unilaminar.
A study by Clark (1967) found that the western pygmy possum had a post-partum oestrus, and that females with pouch-young of a range of sizes all had blastocysts in their uteri. Clark found that the size of the blastocysts increased with the size of the young in the pouch. However, with the addition of data from females carrying larger sized pouch-young, Ward (1990c) subsequently demonstrated, that the growth of the blastocysts was in two phases, similar to the feathertail glider. The size of blastocysts increased with the size of the young in the pouch to about 0.6 mm, with little expansion of blastocysts then observed. The beginning of the plateau phase corresponded to pouch-young with a head length of approximately 8 mm, and about 20 days old. In the data presented by Renfree (1981) for honey possums, there was indication of a slowing of growth of blastocysts during pouch-life, but not the clear two-phase pattern seen in the feathertail glider and the western pygmy possum.

Single specimens of the feathertailed possum *Distoechurus pennatus*, and the little pygmy possum *Cercartetus lepidus*, investigated by Ward (1990c), indicated that embryonic diapause was probable. The feathertailed possum, had one pouch-young of head length 8.2 mm and had cleaving conceptuses *in utero*, indicating a post-partum oestrus and the possibility of embryonic diapause. The little pygmy possum had two pouch-young of head length 18.1 mm and had blastocysts *in utero*, but because of the late stage of lactation, it could not be confirmed whether there was a post-partum oestrus; again embryonic diapause was possible, but not unequivocal. In the same study, three *C. nanus* specimens, the eastern pygmy possum, were all in anoestrus; however, field observations had shown that females could give birth immediately after weaning the previous litter.

The pattern of diapause in the small possums differs from the macropods, in that there is no complete stasis once the unilaminar stage is reached. There is expansion of the unilaminar blastocyst to a vesicle many times larger than that of macropods, followed by a period of slowed development or stasis. Blastocysts do not grow beyond the unilaminar stage throughout lactation, indicating embryonic diapause.
Role of the corpus luteum in diapause

The state of the corpus luteum and the control of its growth is related to the delay of the conceptus in all species with delay or diapause (Renfree and Calaby 1981). All of the research into the control of diapause in marsupials has been on macropods, and mostly on the tammar wallaby; the following information is based mainly on this species. In macropods, the sucking stimulus inhibits the corpus luteum about 4 to 5 days after oestrus (Renfree 1993). In non-lactating females there is a transient increase in the levels of progesterone on day 6 or 7, and expansion of the corpus luteum and blastocyst follows (Tyndale-Biscoe and Renfree 1987; Renfree 1993). This pulse of progesterone does not occur in lactating females. Although the growth of the corpus luteum is inhibited during diapause and it remains at a diameter of less than 2 mm, it still secretes low levels of steroids. The low levels of progesterone secreted do not change unless lactation is terminated. The oestradiol secreted inhibits follicular growth through negative feedback of the hypothalamus or pituitary (Tyndale-Biscoe and Renfree 1987). In both lactational and seasonal quiescence, signals from the hypothalamus result in the production of prolactin from the pituitary, which inhibits the corpus luteum throughout diapause (see Renfree 1993). The influence of steroids, between ovulation and formation of the diapause blastocyst, on uterine secretions has not been studied (Renfree and Shaw 2000).

Removal of the sucking stimulus by weaning, or removal of the pouch young (RPY), results in a fall in prolactin (Renfree and Shaw 2000), and leads to reactivation (Tyndale-Biscoe and Renfree 1987; Renfree 1993; Renfree and Shaw 2000). However, there is a lag phase. The corpus luteum takes about 3 days to reactivate and the first changes in the corpus luteum are seen around day 4 (Tyndale-Biscoe and Renfree 1987; Renfree 1993). Plasma progesterone, secreted by the corpus luteum, shows the first increases on day 3 (Renfree and Shaw 2000). Some reactivation of the blastocyst, such as mitoses and metabolism, are first seen in the blastocyst on day 4, along with increased uterine endometrial secretions (Tyndale-Biscoe and Renfree 1987; Renfree 1993; Renfree and Shaw 2000). Plasma progesterone levels show a pulse increase about 5 days after RPY and progesterone
is the single hormone needed for reactivation. The first expansion of the blastocyst is seen on about day 8, after which it rapidly increases in diameter.

A corpus luteum is not needed for maintenance of diapause and for survival and development of the blastocyst after full reactivation, in at least some species, for example the tammar wallaby (Tyndale-Biscoe and Renfree 1987; Renfree 1993). However, it is essential for a critical period between day 2 and 6 after RPY for resumption of development of the blastocyst. The progesterone pulse provided by the corpus luteum controls the uterine secretions that are needed to maintain pregnancy, but the need for this progesterone is brief, because ovariectomy after day 7 does not affect the pregnancy. Whilst exogenous progesterone can initiate reactivation in ovariectomized tammar wallabies and quokkas, Setonix brachyurus, (Tyndale-Biscoe and Renfree 1987), diapausing blastocysts have not been reactivated successfully in vitro, suggesting that there may be another controlling factor in maintaining diapause than just the presence of an inhibitory uterine factor (Renfree and Shaw 2000). It is not yet known how specific controls, such as various hormones and growth factors in the uterine secretions, depress the metabolism of the dormant embryo and then reactivate it at a later time (Renfree and Shaw 2000).

After reactivation, the corpus luteum increases steadily in diameter to 4mm (Renfree 1993; Renfree and Shaw 2000). There are some mitoses, but expansion is mostly due to hypertrophy of existing cells. After the pulse increase, plasma progesterone levels, secreted from the corpus luteum, increase from basal levels slowly after day 10 until parturition when they fall.

The corpus luteum clearly plays a key role in the diapause of macropods. The endocrine control of diapause in small possums, however, is unknown (Renfree and Shaw 2000). Whilst the role of the corpus luteum is unknown, it can be an indication of the nature of the quiescence. In the tammar wallaby, the corpora lutea cease to increase in diameter during embryonic diapause (Tyndale-Biscoe and Renfree 1987). The corpora lutea of feathertail gliders showed a two-phase growth pattern, similar to
the blastocysts where, during the second phase as quiescent embryos underwent little growth, the corpora lutea remained virtually constant in diameter (Ward and Renfree 1988a). The corpora lutea of the western pygmy possum expanded slowly with increased head length of pouch young (Ward 1990c). No study has previously been made of the honey possum corpus luteum.

6.1.3 This study of female reproduction

Apart from the gross morphology of the tract described above, and an initial study of embryonic diapause in the honey possum, no detailed description of reproduction exists and no recent studies have been undertaken. This chapter will provide a detailed description of the reproduction and histology of the reproductive tract of the honey possum up to, and including, the stage of the blastocyst. While this study will not attempt to understand the endocrine control of diapause, it will further investigate the nature of the delay in growth of the blastocysts and the corpus luteum, in natural populations of the honey possum in the Fitzgerald River National Park (FRNP).

Sperm storage in the female reproductive tract occurs in isthmic oviducal crypts in some species of dasyurids and didelphids (Taggart et al. 1998). Multiple paternity and sperm competition has been found in the brown antechinus and the agile antechinus Antechinus agilis, both species which exhibit sperm storage (Taggart et al. 1998; Kraaijeveld-Smit et al. 2002). Sperm storage may play a role, at least in invertebrates, in facilitating fertilization of eggs by different males and sperm selection by the female (Birkhead and Møller 1998). Therefore, this study will also determine whether there may be sperm storage within the reproductive tract of the female honey possum.

In addition to a description of female reproduction and embryonic diapause, this chapter provides detail on the reproductive history of individual females captured in the wild. Previously, sequential captures of marked females in the wild, and observations of pouch-young reared (but not born) in captivity, have allowed pouch life to be estimated at 56-65 days (Renfree et al. 1984; Wooller et al. 1999; Wooller
et al. 2000). Sequential captures of marked females in the wild have also allowed the turnover from one litter to the next litter of a similar size to be estimated as 65-67 days (Wooller et al. 2000). These estimates have been based on a necessarily limited number of females because the females need to be recaptured at specific times to provide such high-resolution data. However, when females are sequentially caught at pertinent intervals, this provides the best information on female reproduction under natural conditions. Mark-recapture data from long term trapping in the FRNP are therefore utilized to augment the current information on the reproductive cycle of the female honey possum and the number of litters carried by individual females.

The incidence of females with pouch-young is higher in some seasons of the year and lower in other seasons (Section 3.7). It does not necessarily follow, however, that births in these seasons are synchronous between individual females. The incidence of females in post-partum oestrus in the population would be pertinent to the availability of mates for both males and females. Asynchrony between females would indicate a skew in the operational sex ratio (OSR: the ratio of oestrous-females to sexually active males) and increase the potential for polygamy (Emlen and Oring 1977). Mark-recapture data is used to estimate the timing of births in the population to investigate asynchrony between females.
6.2 Methods

6.2.1 Collection of samples and histology

Female honey possums were collected in a range of reproductive states; from those with tiny neonates in the pouch, through those with various sizes of pouch-young, to those without pouch-young but still lactational, and those with an inactive pouch. Females were collected primarily from the remotely placed grid at site D in order to minimize any impact on those sites used for population monitoring. Any animals that died accidentally during the mark-recapture study at other sites were also preserved. A couple of females were also sacrificed from areas A and B because they represented the specific reproductive stage required to complement the range of samples. Females were sampled primarily in winter 2000 and 2002, which were both years when honey possum densities were high (Chapter 4). This allowed equal comparison across years, free from rainfall and seasonality effects (Tables 6.1 and 6.2). Three females were sampled in March 2003 and one on 1 May 2002, to determine if females carried blastocysts through the March/April period when there is a low incidence of breeding (see Section 3.7).

The females were sacrificed by barbiturate overdose and any pouch-young were placed into the fridge to induce torpor and then into the freezer to sacrifice them. These procedures were all in accordance with Murdoch University animal ethics guidelines and Regulation 17 permits from the Western Australian Department of Conservation and Land Management. The crown-rump length and head length of the pouch-young were then measured using vernier callipers, and when possible the pouch-young were weighed. The head length of tiny pouch-young was measured using the dissecting microscope and an eyepiece micrometer. Pouch-young were preserved in 20% DMSO solution saturated with NaCl, for use in DNA studies.
The reproductive tracts were fixed in situ in Bouin’s fixative for 3-4 hours, and then transferred to 70% ethanol. The reproductive tract was later dissected free from the body and measurements of the preserved tract were taken using an eyepiece micrometer and dissecting microscope. Each uterus was measured in length and width from a dorsal view. The ovaries with attached oviduct were dissected free from the uteri at the utero-tubal junction, and the length, width and depth of the ovaries were measured. Tissues from 24 females were embedded in paraffin wax and sectioned at 4-5 µm thickness. There were three sectioning regimes for the uterus and vaginal complex. For half the samples, the uterus and vaginal complex were kept intact and were sectioned longitudinally, with every fourth section mounted. The other samples were representative of each stage within the range of reproductive states examined (Females AK, AG, AH, C1, G, O, H, M, AN, AP, AQ, Al; Tables 6.1 and 6.2), and the vaginas were dissected free from the uteri at the cervical region. The uteri were sectioned longitudinally as before, but the vaginal complex was embedded vertically and transverse sections were taken at levels 250 µm apart, with approximately five sections taken at each level. For some of the females with neonates (Females AK, AG, AH; Table 6.1) and for the juvenile female Al (Table 6.1), the uteri and vaginas were kept intact, embedded vertically and serially sectioned, with all sections mounted. There were also two sectioning regimes for the ovaries and oviducts. For half the samples, the ovary and oviduct were kept intact, and all sections were mounted. For representative samples throughout the range of reproductive states (Females AK, AG, AH, C1, O, H, M and AQ), the oviducts were dissected free and were embedded separately from the ovary to allow the majority of the sections through the oviduct to be transverse. All sections were stained with haematoxylin and eosin, and for some ovaries every second slide was stained with alcian blue/PAS (Periodic acid-Schiff reaction) to highlight the zona pellucida.

Measurements of embryos and ovarian structures were taken using a compound microscope and an eyepiece micrometer. Corpora lutea, follicles and ova were approximately spherical. From the suite of sections through the largest portion of these structures, approximately the centre of each structure, the maximum length
and maximum width perpendicular to it were measured. The geometric mean of the length and width for each section was calculated and the diameter of the structure was taken as the largest mean value. For ova within the follicle and after ovulation, the internal diameter of the vitellus and the total diameter (including the zona pellucida) were taken. For cleavage-stage conceptuses, the internal diameter and total diameter were measured, and the width of the zona pellucida, mucoid coat and shell membrane were approximated from several measurements to an accuracy of ±0.5µm. Unilaminar blastocysts are fragile because they are only a single layer of cells, and they often partly collapsed during fixation or processing, making them difficult to measure directly. However, the uterine epithelium formed an ‘envelope’ around each blastocyst (Figure 6.4) and, for blastocysts that did not collapse, the shell membrane maintained a close association with the uterine epithelium. These envelopes were thus a defined space equivalent to the actual size of the blastocyst. The diameter of the blastocyst was estimated by taking the largest geometric mean of the maximum length, and maximum width perpendicular to it, of the blastocyst envelope from any section.

To confirm that the envelope measurement accurately reflected the differences in size and stage of the blastocyst itself, the developmental stage of each blastocyst was assessed by estimating the number of cells it contained. This was done by counting the number of nuclei visible in each section, and then totalling them for the suite of sections in which each blastocyst appeared. There were a very large number of sections for most blastocysts. For efficiency, the cell total per section was monitored until there was a clear downward trend, indicating that approximately 80% of the cells had been counted, and then counting was terminated. To compare the number of cells counted per blastocyst to the size of the blastocyst, the length and width measurements of the blastocyst-envelope were converted to a volume. The volume of the blastocyst was calculated using the formula for the volume of an ellipsoid \( (\frac{4}{3}\pi a^2 b) \), where \( a \) and \( b \) represent the radius measurements of the ellipsoid, calculated by halving the length and width measurement from the largest section of the blastocyst. There was a significant positive linear correlation between the
blastocyst volume (mm$^3$) and the total number of cells ($r=0.77, p<0.000$). This indicated that the envelope measurements taken accurately reflected the size and stage of the blastocyst, and the blastocyst diameter was subsequently used for size comparison of blastocysts.

6.2.2 Behavioural observations

In order to obtain some behavioral observations and investigate post-partum mating, some simple mating trials were performed in the field. A maximum of two females were held overnight, after collection from the traps in the morning. Each was held in a separate cage, which also contained one large male (head length >26mm) and one smaller male (head length 24-26mm). The aim was to hold animals suspected to be near post-partum oestrus (ie. those with neonates) for one night, observe their behaviour for 3-4 hours and then release them the next day to allow them to feed naturally. The animals were housed in cages (50mm by 50mm by 38mm) and were supplied with fresh flowers of *Lambertia inermis* and *Banksia baueri* as well as a honey and pollen mixture supplied in syringes held vertically, which allowed the animals to feed freely. Seven females were observed as follows: 1 x female (AL) with pouch-young of crown-rump length (CRL) 3.3mm, 1 x female (AK) with pouch-young of CRL 3.5mm, 1 x female with pouch-young CRL of 3-4mm, 2 x females (AH, AG) with pouch-young CRL of 4mm, 1 x female with pouch-young of CRL 5mm, and 1 x juvenile female with head length of 24mm and pouch tight. Females with identification letters were sacrificed for histology, and all other females were released.
6.2.3 Individual female histories

Long-term mark-recapture data from 1984 to 2003 was utilized in analyzing the reproductive history of individually marked females. Prior to 2000, honey possums were only individually marked in some years. Therefore, data on the reproductive history of females is somewhat limited. This information comes from females captured in trapping areas A, B, C and D. Composite information on the pouch life of young from this study, previous observations of young reared in captivity, and the growth curve in Wooller et al. (1999, see Figure 3.3) were used to age pouch-young. The approximate age of pouch-young was useful for two reasons. Firstly, it provided a time-scale against which the reproductive status of the female could be resolved, since there was a clear relationship between the size of the pouch-young and the development of conceptuses. Secondly, the age of the pouch-young was used to estimate the date of birth of the litter, used in investigating asynchrony.
6.3 Results

6.3.1 Overview of the reproductive stages of the females

Of the 24 females investigated, 22 were adults with head length greater than 24 mm, and none were in anoestrus (Tables 6.1 and 6.2). Females AC and AI were classed as juveniles, with head lengths of 23.2 mm and 21.8 mm respectively (Table 6.1).

 Fifteen of the adult females investigated were carrying pouch young, a further two had swollen mammary glands and elongated nipples indicating they were lactational, and one had elongated nipples indicating that she was in a post-lactational state (Tables 6.1 and 6.2). Four females collected in March 2003 were pouch-empty. All except two of these adult females were carrying conceptuses. Those exceptions, females AK and AL, had the smallest pouch young and had Graafian follicles in the ovaries (Table 6.1). These two females had recently given birth to the neonates in the pouch, and were in a near-oestrus stage, as indicated by the histology of the reproductive tract (Section 6.3.6 below).

 The other adults with pouch-young of head lengths less than 3 mm had either recently ovulated eggs in the oviducts or cleaving conceptuses in utero (Table 6.1). Their ovaries contained corpora hemorrhagica from ovulation and corpora lutea from the previous pregnancy (as described in Section 6.3.5 below). Sperm were found in the tract of three out of the four of these females (Table 6.1).

 Those females with pouch young of head length 3mm or more were all carrying blastocysts in utero, as were all females without pouch-young (Table 6.2). All were unilaminar diapause blastocysts, with the exception of female M (post-lactational), whose blastocysts were reactivated and progressing toward the bilaminar stage (Section 6.3.3 below). Associated corpora lutea of the current pregnancy were prominent in the ovaries.
Table 6.1: Reproductive status of female honey possums with pouch young of head length <3 mm and of two juvenile females. mv=median vagina, ov = oviduct, ut = uterus.

<table>
<thead>
<tr>
<th>Female ID</th>
<th>Date</th>
<th>Pouch Young No.</th>
<th>Pouch Young Head length (mean mm)</th>
<th>Number of eggs/zygotes</th>
<th>Number of corpora hemorrhagica</th>
<th>Number of corpora lutea of previous pregnancy</th>
<th>Number of Graafian follicles</th>
<th>Sperm in tract</th>
<th>Status</th>
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<td>4</td>
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<td>12/07/02</td>
<td>2</td>
<td>1.3</td>
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<td>late pro-oestrus/early post-oestrus</td>
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<td></td>
<td>+</td>
</tr>
<tr>
<td>AH</td>
<td>9/07/02</td>
<td>4</td>
<td>2.1</td>
<td>5 ov</td>
<td>2 ov</td>
<td>5\textsuperscript{i}</td>
<td>3\textsuperscript{i}</td>
<td>3</td>
<td>- (3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>G</td>
<td>4/07/00</td>
<td>3</td>
<td>2.7</td>
<td>1 ut</td>
<td>3 ut</td>
<td>2\textsuperscript{i}</td>
<td>3\textsuperscript{i}</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>

**Juvenile females:**

<table>
<thead>
<tr>
<th>Female ID</th>
<th>Date</th>
<th>No.</th>
<th>(3 inviable ova) mv</th>
<th>Number of eggs/zygotes</th>
<th>Number of corpora hemorrhagica</th>
<th>Number of corpora lutea of previous pregnancy</th>
<th>Number of Graafian follicles</th>
<th>Sperm in tract</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC</td>
<td>1/05/02</td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>3 (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>AI</td>
<td>10/07/02</td>
<td></td>
<td>(1 degen. ovum) ut</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>++</td>
</tr>
</tbody>
</table>

\textsuperscript{1} These neonates weighed soon after sacrifice in the laboratory and were 3.9mg and 4.7mg

\textsuperscript{2} Fluid filled

\textsuperscript{3} Graafian follicle becoming atretic, mature secondary oocyte with 1\textsuperscript{st} polar body and arrested at metaphase II

\textsuperscript{4} One atretic tertiary follicle

\textsuperscript{5} One atretic Graafian follicle and two atretic tertiary follicles
Table 6.2: Reproductive status of female honey possums with pouch young of head length >3 mm or pouch-empty (PE).

<table>
<thead>
<tr>
<th>Female ID</th>
<th>Date</th>
<th>Pouch Young</th>
<th>Number of conceptuses degenerating in parentheses</th>
<th>Number of corpora lutea</th>
<th>Sperm in tract</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>4/07/00</td>
<td>3</td>
<td>Head length 4.0 (mm)</td>
<td>Crown-rump length 7.7 (mm)</td>
<td>Left Uterus: 3</td>
<td>Right uterus: 3</td>
</tr>
<tr>
<td>E</td>
<td>4/07/00</td>
<td>4</td>
<td>3.6</td>
<td>7.9</td>
<td>3 (1)</td>
<td>5</td>
</tr>
<tr>
<td>L</td>
<td>4/07/00</td>
<td>4</td>
<td>4.0</td>
<td>6.9</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>J</td>
<td>4/07/00</td>
<td>4</td>
<td>4.3</td>
<td>7.2</td>
<td>4 (1-2)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>AJ</td>
<td>11/07/02</td>
<td>4</td>
<td>5.7</td>
<td>10.3</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>F</td>
<td>4/07/00</td>
<td>2</td>
<td>8.5</td>
<td>14.7</td>
<td>2</td>
<td>1 (1)</td>
</tr>
<tr>
<td>I</td>
<td>4/07/00</td>
<td>3</td>
<td>9.5</td>
<td>13.8</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>O</td>
<td>4/07/00</td>
<td>2</td>
<td>11.5</td>
<td>16.2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>P</td>
<td>4/07/00</td>
<td>5</td>
<td>10.4</td>
<td>15.5</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>H</td>
<td>4/07/00</td>
<td>PE lactational</td>
<td>4</td>
<td>3</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>K</td>
<td>4/07/00</td>
<td>PE lactational</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>M</td>
<td>4/07/00</td>
<td>PE post-lactational</td>
<td>1 (1)</td>
<td>3</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>AN</td>
<td>5/03/03</td>
<td>PE lactational</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>AP</td>
<td>6/03/03</td>
<td>PE lactational</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>AQ</td>
<td>6/03/03</td>
<td>PE lactational</td>
<td>1 (1)</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>AD</td>
<td>1/05/02</td>
<td>PE lactational</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

* These ovaries were cut too far in at the start of the sectioning and were missing the first one-third of the ovary which probably accounts for the missing corpus luteum

† Right uterus for this female was abnormal
The two juvenile females were both reproductively mature, although not carrying pouch young. The vaginal morphology of female AI suggested she was pre-parous (see Section 6.3.7 below). Inviable or degenerating ova were found in both of these juvenile tracts, and the associated corpora lutea from that previous ovulation event were present in the ovary. The larger of these two females (AC) had Graafian follicles in the ovaries and was in oestrus or just post-oestrus (see Section 6.3.6 below) and had sperm in the oviducts. The smaller of these two females (AI) had only secondary follicles, along with the corpora lutea, in the ovaries.

6.3.2 Size of conceptuses in relation to pouch-young

The sizes of conceptuses were related to the size of the young in the pouch. The females with the smallest pouch-young (<3mm head length) had ova in the follicles, ovulated eggs and cleaving conceptuses, all less than 150 µm diameter. The growth of unilaminar blastocysts was in two phases (Figure 6.2). For females with pouch-young of head length 3-6mm, the blastocyst diameter increased in proportion to the head length of the young from approximately 0.8 to 1.7mm. For females with pouch-young of head lengths greater than 6 mm, there was a plateau in the size of the blastocyst around means of 1.3-1.7mm diameter, with none becoming larger than 1.8mm. The two females captured which had no pouch-young, but were still lactational, (H and K), had blastocysts with mean diameters of 1.5mm and 1.7mm respectively, and therefore represent a continuation of the plateau stage. There was a marked increase in size before any changes toward the next stage of development were observed in the blastocysts. Female M was in a post-lactational state and had reactivated blastocysts which had expanded to 2.6mm, and differed in appearance to the plateau phase unilaminar blastocysts and these are described below in Section 6.3.3. The females captured during autumn were all without pouch-young and had unilaminar blastocysts ranging from 1.3 to 1.6mm, again falling along the plateau in growth.
Figure 6.2: The growth of conceptuses carried by females with young in the pouch. Values are the mean diameter (± s.d.) of all conceptuses within each individual female. Error bars do not appear where the s.d. was negligible. Females that were not carrying pouch-young but were still lactational or in a post-lactational state were placed on the graph according to an approximate size of their free-living young. Females sampled in autumn were not carrying pouch-young, but the size of their blastocysts is indicated by an arrow. The time-scale provides an approximation (in days) of the size and stage of development of young reached, and was compiled from observations on pouch-young reared in captivity, along with observations from honey possums caught in the field (see Section 6.3.10 below).
Intrauterine variation in the size of the blastocysts was usual. The range in size of unilaminar blastocysts carried by individual females varied from about 200 to 600 µm. The expanded blastocysts of female M, however, varied in diameter by approximately 760 µm. There did not appear to be any difference between the uteri of individual females with respect to intrauterine variation.

6.3.3 Conceptus descriptions

The ova/single-celled conceptuses found in the oviduct were usually found close together in the ampulla region of the oviduct. All were an irregular ovoid shape and were surrounded only by a zona pellucida. One exception was an ovum found further toward the uterus, in the isthmus region of the oviduct in female AG which had a distinct mucoid coat. The conceptuses in female AH had a purple-staining mucoid coat secretion surrounding them, that had not yet adhered to the egg to form a distinct mucoid coat (Figure 6.3). The zona pellucida of ova/single-celled conceptuses was approximately 1.5-2.0 µm in thickness. In the one ovum that had a distinct mucoid coat this was approximately 1.8 µm. No shell membrane was present around any of the ova/ single-celled conceptuses. The ova/ single-celled conceptuses in the oviduct had a slightly smaller mean diameter than the oocytes in the Graafian follicle (Table 6.3). However, there was greater variation in the oviducal cells and the difference from those in the ovary may be because of some loss of central turgidity once released from the follicle.

Table 6.3: Diameters of ova and conceptuses recovered from the ovary, oviduct or uterus (mean ± s.d.).

<table>
<thead>
<tr>
<th>Stage</th>
<th>n</th>
<th>Total diameter (µm)</th>
<th>Vitellus diameter (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>oocytes (ovary)</td>
<td>14</td>
<td>132 (±7.4)</td>
<td>129 (±7.3)</td>
</tr>
<tr>
<td>single-cell (oviduct)</td>
<td>8</td>
<td>128.3 (±19.7)</td>
<td>121 (±18.9)</td>
</tr>
<tr>
<td>2-cells (uterus)</td>
<td>4</td>
<td>139 (±2.7)</td>
<td>120 (±6.5)</td>
</tr>
</tbody>
</table>
Figure 6.3: Early conceptuses. [a] Single-cell conceptus in female AH oviduct, with mucoid coat secretion (mcs) surrounding the zona pellucida (zp). This conceptus had two dense pronuclei of which one is visible (pn). Numerous sperm (arrows) were lying against the zp. [b] Single-cell conceptus in female C1 oviduct. This conceptus had two large, expanded pronuclei lying close together, within which strands of chromatin and droplet-like granules were visible. Two polar bodies were visible at edge, one more compact than the other. [c] Two-cell conceptuses in the uterus of female G, with distinct shell membrane (sm), mucoid coat (mc) and zona pellucida. Nucleus (n) of both cells visible.
Characteristics of each cell found in the oviduct of each female are described in Table 6.4. Female AG was kept overnight for mating trials, but no mating behaviours were observed. Sperm was absent from the tract. The ova in the oviducts of this female all had two areas of condensed chromosomes appearing as short rods. One set was at the edge of the cell, appearing as a polar body. The other set was nearby, but away from the edge of the cell, and arranged in metaphase II. Often the metaphase arrangement was so distinct that the spindle fibres were visible. In other species of marsupial, meiosis II does not recommence until fertilization takes place (Breed 1994, 1996; Mate 1996). The characteristics of ova in the oviducts of female AG suggest that the ova were all arrested in their second maturation division, and were therefore unfertilized.

Female C1 was not part of the mating trials, and had sperm in the oviducts and surrounding the conceptuses. Only the sperm head usually remained, although occasionally sperm tails were still present. There were approximately 3-5 sperm heads per section in close association with the zona pellucida of the conceptus (Figure 6.3). Conceptuses usually appeared in about 10-15 sections, and sperm nuclei appeared only in one section each; therefore each conceptus had approximately 30-75 sperm surrounding it. Four of the five conceptuses in the oviducts of female C1 had two large pronuclei and therefore were fertilized (Table 6.4; Figure 6.3b). Polar bodies were not always visible. The fifth conceptus only had one visible nucleus. This nucleus was fully formed and not undergoing meiosis. One polar body was visible along with another area of compact nuclear material. It could not be confirmed whether this was a decondensing sperm nucleus; however, it is most likely that this cell was fertilized. Female AH was held overnight for mating trials, when no significant behavioural observations were made. She must have mated before capture or in the enclosure unobserved, because sperm were present in the oviducts (Table 6.4). The conceptuses were in various stages. Four of the seven conceptuses were fertilized as they either had resumed meiosis, or had two pronuclei (Figure 6.3a). The stages of the other three conceptuses could not be
determined conclusively, but because none were arrested in metaphase II, it is possible that they too were fertilized.

Two forms of pro-nucleus were observed in the conceptuses, similar to those described by Selwood (1982). Small, dense pro-nuclei were observed with very condensed chromosomal material, as in female AH (Figure 6.3a). These may represent early formation of the pronuclei, as in Selwood (1982). Large, expanded pronuclei contained droplet-like granules and thin strands of chromatin (Figure 6.3b). These may represent late-stage pro-nuclei, as in Selwood (1982). Two conceptuses in the tract of female AH had a very elongated droplet-like granular nucleus. It is possible that this represents the fusion of the two pronuclei.

The two-cell conceptuses in the uteri of female G all had; a zona pellucida (approximately 1.5-2.0\(\mu\)m), a mucoid coat (approximately 3.5-5.5\(\mu\)m), and a shell membrane (approximately 1.5-3.5\(\mu\)m) (Figure 6.3c). These had a mean diameter only slightly larger than the single-cell in the oviduct (Table 6.3).

All unilaminar blastocysts consisted of a shell membrane (approximately 2.0-7.0\(\mu\)m), with the ribbon of flat trophoblast cells closely associated with the inside of the shell membrane (Figure 6.4). Mitotic cells were not observed. No mucoid coat or zona pellucida could be seen in any blastocysts, regardless of size. The shell membrane of the blastocyst was in close association with the uterine epithelium and the endometrium developed an envelope around each blastocyst. The thin projections of the endometrial wall extend around the blastocyst to create a partial barrier between the blastocysts within one uterus (Figure 6.4). Where the blastocysts abutted, their shell membranes were attached to one another, and if dissected fresh from the uterus, they stuck together and could not be separated without damaging them.
Table 6.4: Description of the ova and single-cell conceptuses from the oviduct. Structures noted are those that were clearly observable. See text for description of ‘large’ and ‘dense’ pronuclei, and ‘granular’ nuclei. Sperm penetration and decondensing of the sperm nucleus was difficult to decipher and remains speculative. Cells with two distinct pronuclei were clearly fertilized, and resumption of meiosis II beyond metaphase II also suggested fertilization had taken place.

<table>
<thead>
<tr>
<th>Female</th>
<th>AG</th>
<th>C1</th>
<th>AH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Left oviduct</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egg 1</td>
<td>Condensed chromosomes (metaphase II), polar body.</td>
<td>2 large pronuclei. Egg fragmented.</td>
<td>2 dense pronuclei, 2 polar bodies.</td>
</tr>
<tr>
<td>Egg 2</td>
<td>Condensed chromosomes (metaphase II), polar body.</td>
<td>2 large pronuclei. Two polar bodies. Egg fragmented in parts.</td>
<td>Female nucleus in anaphase II. Area of compact nuclear material, possibly sperm penetration. Egg collapsed.</td>
</tr>
<tr>
<td>Egg 3</td>
<td>Condensed chromosomes (metaphase II), polar body.</td>
<td>2 large pronuclei. Egg fragmented.</td>
<td>Female nucleus in anaphase II. 1 polar body. Area of compact nuclear material, possibly sperm penetration. Egg collapsed.</td>
</tr>
<tr>
<td>Egg 4</td>
<td>Condensed chromosomes (metaphase II), polar body. Egg collapsed.</td>
<td>-</td>
<td>1 elongated granular nucleus. 2 polar bodies.</td>
</tr>
<tr>
<td>Egg 5</td>
<td>-</td>
<td>-</td>
<td>2 large pronuclei.</td>
</tr>
<tr>
<td><strong>Right oviduct</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egg 1</td>
<td>Condensed chromosomes (metaphase II), polar body. Egg collapsed.</td>
<td>1 large nucleus, 1 polar body visible along with a second area of compact nuclear material, possibly sperm penetration.</td>
<td>1 elongated granular nucleus. 1-2 polar bodies. Egg collapsed.</td>
</tr>
<tr>
<td>Egg 2</td>
<td>Condensed chromosomes (metaphase II), polar body. Egg collapsed.</td>
<td>2 large pronuclei, 2 polar bodies.</td>
<td>1 large nucleus.</td>
</tr>
<tr>
<td>Egg 3</td>
<td>Condensed chromosomes (metaphase II), polar body. Egg collapsed.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Egg 4</td>
<td>Condensed chromosomes (metaphase II), polar body. Egg collapsed.</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sperm surrounding ova?</th>
<th>No</th>
<th>Yes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Status</td>
<td>All arrested in metaphase II, unfertilized ova</td>
<td>All fertilized single-cell conceptuses</td>
</tr>
</tbody>
</table>
Figure 6.4: Unilaminar blastocysts. [a] Longitudinal section through a uterus that contained three unilaminar blastocysts. The trophoblast (tb) was closely associated with the interior of the acellular shell membrane (sm). The uterine epithelium (ut) formed an 'envelope' around all blastocysts and the endometrial layer was packed with uterine glands. [b] The shell membrane of a blastocyst was closely associated with the uterine epithelium. The trophoblast was a flat ribbon of cells with nuclei (n) clearly visible. Uterine glands (gl) can be seen in the endometrial layer. [c] Portion of a reactivated blastocyst from female M, with shell membrane (sm) lying next to the uterine epithelium, beneath which glands of the uterine endometrium are visible. The trophoblast cells around the entire perimeter of the blastocyst had become rounded and compact, with many cells dividing (arrows).
One female (M) had blastocysts that were more advanced than the unilaminar stage, but were not yet bilaminar. These blastocysts had plump round cells, with round nuclei lying inside the shell membrane (Figure 6.4c). These appeared to be ‘active’ blastocysts: mitoses were very common, there were many more cells than a unilaminar plateau-phase blastocyst, and they were much larger than any at the unilaminar stage (Figure 6.2). There was no trophoblast layer between these cells and the shell membrane. Therefore these cells were not the first hypoblast (endoderm) cells, but rather were the changing trophoblast layer. There did not appear to be any region where the cells were cuboidal or different in appearance to indicate that they were the precursor cells of the hypoblast, as described by Selwood for other marsupials (1994; 1996).

In female K, which did not have pouch-young but was still lactational, the unilaminar blastocysts were of a size within the plateau phase. In only some of these blastocysts, at the region of the junctional link between it and the next blastocyst, some trophoblast cells were round squamous cells, similar to that described for female M. This appeared to be an early stage of transition of the blastocyst.

6.3.4 Degenerating conceptuses and reproductive amortization

For 7 out of the 16 females with blastocysts, there were more corpora lutea in at least one of the ovaries than there were blastocysts in the ipsilateral uterus. This was presumably because of unfertilized ova or degenerated conceptuses. Indeed, degenerating conceptuses were found in five of the females. These conceptuses usually were collapsed but had a mucoid coat and shell membrane, and were commonly the size of eggs, or occasionally small blastocysts. They were found being resorbed into involutions in the uterine epithelium.

Ova/conceptuses in the oviduct were also found collapsed or broken (see Table 6.4). Within each oviduct for each female, usually only some of the cells were collapsed, and these were always lying within millimetres of intact specimens. It is likely that
these cells were naturally degenerating, since the same dissection, fixation and processing chemicals were applied to all ova/conceptuses. The exception may be the left oviduct of female C1 where all conceptuses were fragmented, and this may indicate some destruction. Five out of nine ova were collapsed in female AG, and this natural attrition may be because the ova were unfertilized. In females C1 and AH, attrition occurred in both fertilized ova and those ova where fertilization could not be confirmed. If natural attrition occurred, it was independent of fertilization status.

The numbers of ova or conceptuses found in the oviducts or uterus were equal to the number of corpus hemorrhagica from which they came (Table 6.1). There were, however, two cases where there was one less conceptus than corpus hemorrhagica, and this was most likely due to a loss of a conceptus close to the point of dissection during histological processing.

Reproductive amortization was evident from the progressive reduction from the mean number of corpora lutea, to the mean number of embryos, through to the mean number of pouch young (Table 6.5). Loss was calculated between ovulation and the blastocyst stage as: (mean number of corpora lutea or hemorrhagica – mean number of blastocysts)/ mean number of corpora lutea or hemorrhagica (Stockley 2003). Similarly, loss between the blastocyst stage and neonate stage was calculated as (mean number of pouch-young after birth – mean number of blastocysts)/ mean number of pouch-young after birth, using the mean number of pouch-young from Wooller and Richardson (1992). There was a 13% loss between ovulation and the blastocyst stage, and a 36% loss between the blastocyst and neonate stage. The mean number of blastocysts (5.5) was greater than the number of teats (4). Overall loss, calculated in a similar manner as described above, was 42% from ovulation to the neonate stage, and 61% from ovulation to weaning.

The mean number of blastocysts for females in the plateau-phase, which corresponded to those with young of >6mm head length, was smaller than the mean number of blastocysts for females with pouch-young of <3mm head length (Table 6.5). To test if this was significant and determine whether degeneration occurred
during the blastocyst stage, an analysis of covariance was performed with the category of the female (pouch-young head length) as the factor, the number of conceptuses as the dependent variable and the number of corpora lutea as the co-variable. Neither the stage of diapause ($p=0.082$) nor the number of corpora lutea ($p=0.17$) were significant in predicting the number of viable blastocysts. Inspection of the residuals indicated that the data were well conditioned and variances were homogeneous, and therefore no transformation was applied. Loss of conceptuses thus probably occurs before and after the blastocyst stage of development, rather than during diapause. However, the power of the test was not high due to the small sample sizes.

Table 6.5: Reduction amortization from the mean number ($±$s.d.) of eggs ovulated as indicated by the corpora lutea of current pregnancy, the mean number ($±$s.d.) of viable blastocysts, to the mean number ($±$s.d.) of pouch-young. Data are presented separately for the size-classes of pouch-young, and then overall. For females with pouch-young of head length $<$3 mm, the number of corpora lutea correspond to the previous ovulation which produced the current set of young. The number of corpora hemorrhagica and corpora lutea for these females are included as independent ovulation events in the overall calculation. Sample sizes are in parentheses, and differences in sample sizes within size classes are due to the inclusion/exclusion of pouch empty (PE) females, or exclusion of females with abnormal or missing structures (refer to Tables 6.1 and 6.2).

<table>
<thead>
<tr>
<th>Head length of pouch young</th>
<th>Corpora lutea</th>
<th>Viable blastocysts</th>
<th>Pouch-young</th>
</tr>
</thead>
<tbody>
<tr>
<td>$&lt;$3 mm</td>
<td>5.8 ±1.07</td>
<td></td>
<td>3.3 ±0.75</td>
</tr>
<tr>
<td></td>
<td>(6)</td>
<td></td>
<td>(6)</td>
</tr>
<tr>
<td>3-6 mm</td>
<td>7.7 ±1.20</td>
<td>7.0 ±0.90</td>
<td>3.8 ±0.40</td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td>(5)</td>
<td>(5)</td>
</tr>
<tr>
<td>$&gt;$6 mm or PE</td>
<td>5.8 ±1.03</td>
<td>4.7 ±1.27</td>
<td>2.5 ±0.50</td>
</tr>
<tr>
<td></td>
<td>(11)</td>
<td>(10)</td>
<td>(4)</td>
</tr>
<tr>
<td>Overall</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corpora lutea/hemorrhagica</td>
<td>6.2 ±1.31</td>
<td>5.5 ±1.60</td>
<td>3.6 ±1.5</td>
</tr>
<tr>
<td></td>
<td>(24)</td>
<td>(15)</td>
<td>(15)</td>
</tr>
</tbody>
</table>

* Mean ($±$s.d.) before and after brood reduction (Wooller and Richardson 1992), sample size not given.
6.3.5 Ovarian structures

The ovaries of all females contained primary and secondary follicles and were often packed with primordial follicles (Figure 6.5). All developing follicles had nuclei in the form of a germinal vesicle, with narrow threads of chromatin, indicating they were in the diplotene stage of prophase I. Graafian follicles (Figure 6.5) had a mean follicle diameter of 425 £m (s.d. 65.6, n=15), with the oocytes inside had a mean total diameter of 132 £m (s.d. 7.4, n=14). The stage of maturation of the Graafian follicle oocytes (eg. Figure 6.6d) for each female is described below in Section 6.3.6.

Corpus luteum

There were two different types of corpora lutea. The corpus luteum from the previous pregnancy was present in females who had recently given birth and had pouch-young of <3mm head length (Table 6.1). This was different in appearance to the corpus luteum from females with blastocysts (Figure 6.5). The luteal cells of the recently active corpus luteum were large, puffy cells, that stained light in colour. Scattered throughout the gland and at its centre, were more compact cells with darkly staining nuclei (Figure 6.5c). The luteal cells of diapause corpora lutea, associated with plateau-phase blastocysts were all compact and darkly staining (Figure 6.5d). This was also true of female M which had reactivated blastocysts.

After ovulation the follicle collapses totally, as in females AG and C1 (Figure 6.6a). The central space then fills with fluid and expands, leaving several layers of follicular cells around the edge (Figure 6.6b and c). For example, the fluid-filled corpora hemorrhagica (mean ± s.d.) of female G (458 £m±9.6, n=5), with cleaving cells in utero, were larger than those of female AH (325 £m±28.8, n=8), with less advanced, single-cell conceptuses in the oviduct.
Figure 6.5: Ovaries and corpora lutea. [a] Section through the ovary of a near oestrus female with 2mm pouch-young. Two Graafian follicles (GF) are visible, each containing an oocyte (Oo). An ‘active’ corpus luteum (CL) of previous pregnancy also present, as well as secondary (2F), primary (1F) and primordial (pF) follicles. [b] Section through the ovary of a female with unilaminar blastocysts in diapause. Diapause corpus luteum present, along with secondary and primary follicles and many primordial follicles. [c] Enlarged light-staining cells of an ‘active’ corpus luteum. [d] Compact cells with dark-staining nuclei of diapause corpus luteum.
Figure 6.6: Post-ovulation follicles and a pre-ovulation oocyte. [a] Collapsed follicle immediately after ovulation in female C1. [b] The corpus hemorrhagicum partially filled with fluid in female AH, whilst single-cell conceptuses still traversing the oviduct. [c] The corpus hemorrhagicum of female G, fluid filled and as large as a graafian follicle. This female had cleaving eggs in utero. [d] Oocyte within the antrum of a Graafian follicle, the zona pellucida (zp) is surrounded by granulosa cells. The oocyte was arrested in its second maturation division just prior to ovulation in female AC. The condensed, chromosomes appeared as short rods arrested in metaphase II (arrow), with the first polar body (pb) already extruded.
Fully formed corpora lutea were much smaller than these fluid-filled corpus hemorrhagica (Figure 6.7). The mean diapause corpus luteum diameter varied slightly between the females from 226 to 312 µm, and remained constant regardless of the head length of the pouch-young (Figure 6.7), or of the blastocyst size. The corpora lutea associated with the reactivated blastocysts of female M had not increased beyond the size range mentioned (290±12.5). The females captured in the autumn, all without pouch-young, had mean corpora lutea diameters smaller than most of the other females (192 to 237 µm). The mean diameter of ‘active’ corpora lutea of the previous pregnancy decreased in size with increasing size of the young in the pouch and development of the new conceptuses (Figure 6.7). Females AK and AL with grafiaan follicles in the ovaries, had the largest active corpora lutea (mean diameter 350-400 µm). Those of females AG, C1 and AH, with ova/conceptuses in the oviducts were smaller (mean diameter 300-350 µm). Those in female G were smaller again (mean diameter 295 µm), had 3 to 10 pyknotic cells per section, and connective tissue was becoming visible, indicating that they were degenerating and forming corpus albicantia.

*Degenerate structures and interstitial tissue*

Female G was the only female that had corpora albicantia. However, areas of exposed connective tissue, often associated with pyknotic luteal cells, indicated the remains of degenerated corpora. Follicles that failed to ovulate either became atretic, or sometimes developed luteal cells, and were indistinguishable to a corpus luteum, except for the presence of an oocyte (Figure 6.8a). Atretic follicles of all sizes were very common in the ovaries of all females. The granulosa cells became pyknotic and degenerated (Figure 6.8b). Clusters of cells that appeared to be interstitial tissue were often seen, and some had what appeared to be a degenerating oocyte in the centre (Figure 6.8b). Structures that appeared intermediate between an atretic follicle and interstitial tissue were observed which clearly contained a large oocyte. Interstitial tissue was common, occurred throughout the ovary and ranged in size from similar to a primary follicle, to the size of a large tertiary follicle. In one female there was numerous, long clusters of such tissue (Figure 6.8d).
Figure 6.7: The mean (±s.d.) diameter of corpora lutea in the ovaries of all females. Females that were not carrying pouch-young but were still lactational or in a post-lactational state were placed on the graph according to an approximate size of their free-living young. Females sampled in autumn were not carrying pouch-young, but the size of their corpora lutea is indicated by an arrow.
Figure 6.8: Degenerate follicles and interstitial tissue. [a] A follicle that failed to ovulate, indistinguishable from a corpus luteum, except for the presence of a degenerating oocyte (Oo). The zona pellucida of the oocyte stained purple under alcian blue/PAS staining. [b] An atretic follicle also stained with alcian blue/PAS. The granulosa cells were becoming pyknotic and degenerating. The zona pellucida and nucleus (n) of the oocyte are clearly visible. [c] Interstitial tissue, possibly with degenerating egg still at the centre (arrow), indicating its possible origin as a follicle. [d] One female had long clusters of interstitial tissue.
6.3.6 Histological characteristics of the tract at different stages of reproduction

Two post-partum females (AK and AL) and the juvenile female AC (head length 23.2mm), were close to oestrus with characteristic histological appearances in the uterus and ovaries. The exact stages of these three females could not be determined because the relation between the timing of behavioural oestrus, mating and ovulation is unknown and the extent of cornified cells in the vaginae at each stage is unknown. The juvenile female, AC, was most likely in oestrus or immediately post-oestrus, and close to ovulation. The vaginae had deep layers of squamous epithelial cells. Enlarged intermediate cells with nuclei and cornified cells were both being shed, and there were pyknotic cells in the lumina. This is consistent with the presence of sperm in the tract and Graafian follicles in the ovaries. Four of the six oocytes were all at the same stage, and the remaining two had their nuclear material missing. The first polar body was extruded and the chromosomes appeared as short rods at metaphase II (Figure 6.6d).

Female AL was either entering oestrus or just post-oestrus, and female AK was less advanced and in a pro-oestrus stage. Female AK had deep layers of squamous epithelium in the vaginae, with cells at the luminal edges becoming pyknotic and being shed. The oocytes in the Graafian follicle were all at the same stage and had nuclei with no nucleoli and with condensed chromosomes. They were clearly at a more advanced stage than the diplotene of those in developing follicles, indicating recommencement of meiosis and presence in late prophase I. Female AL had many cells being shed from the vaginal epithelium, some cornified and some intermediate cells with nuclei. The oocytes in the Graafian follicles had no polar bodies, but all had resumed meiosis I, and the condensed egg chromosomes appeared as short rods in approximately metaphase I. The stage of female AL appeared slightly earlier than that of the juvenile female AC because the first maturation division of the eggs was incomplete and because there were less cornified cells in the vaginae. In addition, there was no sperm in the tract (unlike AC), although this female was held overnight for mating trials and no significant interest in the female by the males was
observed. It is possible then, that this female was in early post-behavioural oestrus, prior to ovulation, hence the disinterest from the males. Alternatively, the lower proportion of cornified cells compared to AC, and the presence of the eggs still in the first maturation phase indicate she was just prior to behavioural oestrus, and the artificial environment of the cage may have inhibited interest from the males.

In female AC, degenerating eggs from the previous oestrus event were found in the tract and the non-pregnant uterus had a thick, vascular endometrial layer with a small lumen (Figure 6.9a). Both females AK and AL had debris in the uterus from the recent birth consisting of cells, red blood cells and membranous material (Figure 6.9b). Both had a thin glandular endometrium, with some pyknotic cells. In all three, there was a stratified squamous epithelium with large cells with large round nuclei and mitotic cells were seen. There were many red blood cells within this epithelial layer, and a few pyknotic epithelial cells were being shed into the lumen. In females AK and AL this epithelium was deeply layered and highly folded, particularly in female AL, such that it formed folded ‘branches’ into the uterine lumen (Figure 6.9b). This excessive folding may have been from pregnancy. In female AK the epithelium had been destroyed in many places, possibly during birth. The glandular epithelium was columnar and there were pyknotic cells in the glandular epithelium and those cells adjacent to it. The least pyknosis was seen in the uterus of female AK, more in AL and the most in AK, indicating that degenerative cells increase toward oestrus.

The uteri of post-ovulatory females (AG, C1, AH, G) were in varying states of degeneration and proliferation. The endometrial layers of the uteri were much deeper than that of the near-oestrous females described above and were oedematous and vascular. There was a simultaneous process of proliferation and degeneration of the uterus, and a continuum of changes, from females AG and C1, which had most recently been through post-partum oestrus and ovulation, to female AH whose eggs were accumulating a mucoid coat, to female G with cleaving conceptuses in utero. Female AG had much cell debris in the vaginae, and the other females exhibited some shedding of pyknotic epithelial cells in the vaginae.
In the uteri of AG and C1 the hypertrophied epithelial cells had extremely enlarged cell nuclei, taking up 60 to 90% of the cell volume (Figure 6.10a). The epithelium varied from squamous to cuboidal to columnar, and mitoses were frequent. There were pyknotic cells in the epithelium, glandular epithelium and sub-epithelial stroma, seen to the greatest extent in C1, and some cell debris in the central lumen. The glands and the stroma of the endometrial layer were regressing. Many glands had enlarged luminal spaces containing cell debris and pyknotic cells, and occasionally there were areas of empty space in the stroma. In AG, the glandular epithelial cells were still mostly columnar, whereas in C1 they had regressed to flat cells and the glandular lumenina had merged and were densely packed with debris. In AH the epithelium was still variable, but with more columnar cells and the nuclei were smaller than in AG/C1. The epithelial cells were ciliated and there were many secretions in the central uterine lumen. Pyknotic cells and mitoses were still common, but less frequent than in C1. There was some shedding of epithelium and stroma into the central lumen. There was some enlargement and regression of glands, but less so than C1. In G, the epithelium was cuboidal to columnar, with secretory ciliated cells (Figure 6.10b). However, it was missing in places where it was being shed into the central lumen along with sub-epithelial components. Mitoses were rarely seen and many of these shed cells were pyknotic, although overall there were fewer dying cells than in AH and C1. Female G had many free red blood cells in the sub-epithelial layer. In one uterus there was a very large glandular lumen with flat epithelial cells, possibly a consolidation of several glands and it had some cell debris within. However all other uterine glands had columnar epithelia, similar to those in females with blastocysts.
Figure 6.9: [a] Section through uterus of female AC, in oestrus, showing the uterine lumen (L), and the deep endometrial layer had scattered glands (gl) and capillaries (c). Inset shows close-up of the uterine epithelium (ut) with enlarged cells with large round nuclei. Black 'inkspots' of pyknotic cells present throughout, particularly in the glandular epithelium. [b] Section through the uterus of female AK, post-partum and pro-oestrous. Debris (d) from birth present in the lumen. The uterine epithelium was highly folded, and the glandular endometrial layer relatively shallow. Inset shows close up of layered epithelium. There were fewer pyknotic cells than in female AC.
Figure 6.10: [a] Section through the uterus of female C1, post-ovulation. Breakdown of glands (gl) and stroma of the endometrial layer was evident. Glandular lumina merged, and contained pyknotic cells and debris. Cells of the luminal epithelium were enlarged (ut), with very puffy cell nuclei, taking up 60-90% of the cell volume (see inset of enlarged area). Mitotic cells (arrows) occurred in the epithelium. [b] Section through the uterus of female G, which contained cleaving conceptuses. The glandular epithelium was columnar. The luminal epithelium (ut) was in parts cuboidal (see inset of enlarged area), but in some places the epithelium and sub-epithelial components were being shed (s) into the uterine lumen (L).
Uteri carrying blastocysts had endometrial walls that were densely packed with uterine glands. The glandular epithelial cells were columnar, having large basal nuclei. There were occasional secretions in the small glandular lumina. Uteri carrying growth-phase blastocysts had epithelia that varied from cuboidal to columnar to tall columnar (approximately 8.0-31.9 μm high) with large oval shaped nuclei taking one-half to two-thirds of the volume of the cells. Mitoses were seen in the epithelium. Secretions were frequently seen, and the endometrial layer was of medium thickness. In uteri with plateau-phase blastocysts the central lumen became very large and the endometrial wall thin. The epithelia were tall-columnar, approximately 11-25μm high with large oval shaped nuclei. Secretions were only occasionally seen.

6.3.7 Morphology of the reproductive tract

The uteri were separated distally, deflecting away from each other (Figure 6.1). In specimens from females which had recently given birth and from the single specimen with bilaminar blastocysts, the uteri lay adjacent to each other, abutting along their entire length. The preserved uterus measured (mean±s.d.) 5.0±3.1mm long by 2.5±0.8mm wide, ranging from 2.0 x 1mm for the juvenile females, to 9.2 x 4.8mm for females which had recently given birth. The ovaries were ovoid and held in close contact to the uteri. The preserved ovary measured (mean±s.d.) 1.4±0.3mm long by 1.1±0.3mm wide by 0.9±0.2mm deep. The fresh weight of the ovaries was obtained for one female that was sacrificed in the laboratory (Female AL). The right ovary weighed 2.3mg and the left ovary weighed 3.0mg.

The morphology of the vaginal complex was examined in 12 females at a variety of reproductive stages, in which transverse sections of the vaginal complex were taken (Females AK, AG, AH, C1, G, O, H, M, AN, AP, AQ, AI). In addition, clear longitudinal sections were obtained during sectioning of the attached uterus from one female (AL).
For all females the cervices protruded well into the vaginal culs-de-sac and ended in the median vagina (as represented in Figure 6.1) below the level where the lateral vaginal canals separated from the median vaginal canal (Figure 6.11a). In only three cases did the cervices end at the level of separation, such that there was an open vaginal cul-de-sacs. Even then, the lateral vaginal canals were beginning to separate. The cervices were separated into two canals for their entire length and held within a common papilla (Figure 6.11b). There was a vestigial septum extending from the base of the cervices to the walls of the culs-de-sac (Figure 6.11c), as found by de Bavay (1951). However, this was only present part of the way down the cervices, and to different extents in different females. Where the cervices ended in the culs-de-sac they were always unsupported in parous females (Figure 6.11d).

There was no septum completely dividing the vaginal cul-de-sacs or the median vagina in any female examined. For most females, across a broad range of reproductive stages, there was no septum whatsoever. However, in three specimens there were partial septa. In females AG and AQ there was a small vestige left only in sections shortly before the vaginal canals merge near the urogenital sinus. In the juvenile female AI, there was a full septum where the cervices enter the cul-de-sacs (Figure 6.11e), but this was broken into a partial septum in the median vaginal canal (Figure 6.11f), and then was lost completely for most of the median vaginal canal, and reformed as a partial septum shortly before the vaginal canals merged near the urogenital sinus.

In all females examined, including the juvenile female AI, the median vagina was well developed, as a large open canal running between the cervices and the urogenital sinus (Figure 6.1). The lateral vaginal canals curve around until they become adjacent canals and finally merge to a posterior vaginal sinus (Figure 6.12). In recently parous females the lateral vaginal canals and the median vaginal canal had wide lumina and were clearly open through to the urogenital sinus (Figure 6.12a). In females with pouch-young of head length <6mm and/or with plateau-phase blastocysts, the posterior portion of the lateral vaginal canals was occluded, and the
median vaginal canal remained open through to the urogenital sinus, but had regressed to a narrow lumen (Figure 6.12b). In the juvenile female AI, the median vaginal canal became totally occluded at its distal end, shortly before the urogenital sinus (Figure 6.12d). As in the specimen described by de Bavay (1951), there was a sheet of cells blocking the passage of the canal, but it could clearly be seen where a perforation would open the canal during the first parturition. This juvenile female was therefore pre-parous, consistent with the degenerating ova present in its tract. In parous females the median vaginal canal joined the distal posterior vaginal sinus, and this common canal joined the urethra shortly after to form the urogenital sinus (Figure 6.13).

6.3.8 Absence of sperm storage crypts

Serial sections were made of each oviduct of each female. For females AK, AG, AH, C1, O, H, M and AQ, these were embedded separately to allow clear transverse sectioning; for all other females, oviducal sections were examined as they appeared attached to the ovary. There were no sperm storage crypts in any specimen in any region of the oviduct. In the oviducts that had sperm inside them, the sperm were scattered throughout the lumen. In a few instances, sperm were observed in clusters with their heads closely associated with the oviducal epithelium (Figure 6.13d). The oviduct consisted simply of a highly folded, pseudo-stratified, tall-columnar epithelium with ciliated cells, similar to other marsupials (Tyndale-Biscoe and Renfree 1987). There did not appear to be any storage areas in any other part of the reproductive tract. In females that had sperm present in the tract, none were found in the vaginal complex, and few were found in the uterus. An accumulation of degenerating sperm was observed in one near-oestrous female adjacent to the opening of a uterine gland.
Figure 6.11: Vaginal morphology. [a] Transverse section through 'A' in Figure 6.1. The cervices (c) protrude well into the median vaginal canal (mvc), such that they are still present when the lateral vaginal canals (LV) are separate. Bl = bladder, ur = ureter. [b] Longitudinal section through the cervices and vaginal culs-de-sac. The cervical canals are separated for their entire length. [c] Transverse section showing supporting septum (s) between cervical papilla (c) and the wall of the mvc. [d] The supporting septa disappears before the end of the cervical papilla. [e] In the juvenile female AI, there was a septum dividing the median vaginal canal. [f] This septum in the juvenile female became only partial further down the mvc - see transverse section 'B' in Figure 6.1.
Figure 6.12: Vaginal canals. Transverse sections through 'C' in Figure 6.1. [a] The lateral vaginal canals (LV) run adjacent to each other on the dorsal side of the median vaginal canal (mvc) and the urethra (Ua). This female was recently parous. [b] In females that have larger pouch-young or have weaned young, the lateral vaginal canals were occluded (LV occl.) and the median vaginal canal was narrow, but remained open. [c] The lateral vaginal canals merge to form a posterior vaginal sinus (pvs). [d] In the pre-parous juvenile female Al, the median vaginal canal is occluded (mvc occl.) and separated by a sheet of cells from the urogenital sinus.
Figure 6.13: Urogenital sinus (Sections through 'D' in Figure 6.1) and an oviduct with sperm. [a] The posterior vaginal sinus (pvs) merges with the median vagina (mvc). [b] The common vaginal sinus becomes merged to the urethra (Ua), to form [c] the urogenital sinus (ugs). [d] Longitudinal section through the oviduct of female AC, showing sperm with their heads closely associated with the oviducal epithelium. The epithelium is simple, but highly folded. This section does not go through the centre of the oviducal lumen, so the luminal space seen is limited.
6.3.9 Behavioural observations

No matings were observed, but other behaviours were seen. Animals were often in torpor, or were preoccupied with escape from the cage, reinforcing the artificial nature of any mating trials. Sniffing was often observed between individuals. The two males in each cage were observed sniffing each other, but, more commonly, males were observed sniffing females. This occurred in the trials with the near oestrous female AL with pouch-young of CRL 2-3mm, the post-ovulation female AG with 4mm pouch-young, and another female with 5mm pouch-young. In the trial with female AK, who was in pro-oestrus, both the large and small male in the enclosure showed significant interest in the female. They took turns in sniffing her underbelly, pouch and cloaca, and sitting beside her, sitting on her, or crawling over her whilst she was in torpor, but neither attempted to mount. Occasionally the female would stir and push the males away with her forelimb. After intermittently checking the female, the males would either feed, or return repeatedly to their individual corners of the cage to groom. Initially, the larger male was aggressive toward the smaller male. A 'fixed stare' followed by jumping toward the smaller male was observed, and the smaller male would leap away and retreat (sensu Russell 1986). Aggression between males was not observed in any other trial. After some time, these two males tentatively approached each other, engaged in nose-to-nose sniffing (sensu Russell 1986), and thereafter were tolerant of each other.

In the enclosure females often excluded males from food by chasing them away, jumping at them which made them leap away, or very occasionally, by making squealing sounds. Sometimes, after a period of time, the male and female would become acquainted by nose-to-nose sniffing, and thereafter feed on the same flower or the adjacent syringe.
6.3.10 Individual female histories

During the long-term trapping studies of the honey possum in the FRNP prior to 2000, animals were only marked with individual identification numbers in some years. Detailed observations of the pouch status have been recorded in Figure 6.14 for those individually marked females that were most informative. Observations from the field on development and growth of the young between successive captures corresponds well with the data recorded from a limited number of individuals from previous studies in captivity. The observations of individual females in the wild suggest that pouch-life lasts for approximately 55-60 days, but that this may vary slightly between individual females. This corresponds closely to the duration previously reported for six females of 56, 60, 60, 62, 64, and 65 days (Wooller et al. 2000). A large number of pouch-young were observed in the current study, and the young became furred between a crown-rump length of 15 and 20mm.

Some individually marked females were captured carrying pouch-young, and then were later recaptured with a second set of young (Table 6.6). Entries 1-4 in Table 6.6 indicate that the duration of this entire cycle is about 64-68 days. This corresponds well with the eight females previously described by Wooller et al. (2000) with intervals of 65-67 days. Entries 5-11 indicate that in some cases turnover between litters may take longer (Table 6.6). Based on the size of the first and second litters, these durations are all about 80-100 days.

The number of litters that a female carried is recorded in Table 6.7 for those females caught at least twice between 2000 and 2003. The number of litters recorded for a female is obviously limited by the number of times she was captured and the interval over which she was captured. Many females were only recorded with one or two litters, but were only captured 2-3 times over their lifetime. Those females that were captured more than three times tended to have a greater number of litters. Some females carried three or four litters in the time over which they were captured. One female at site A was first caught in March 2000 without young, and again in June without young. She was then captured in July with 8mm pouch-young, but was again
lactational in October. She was not caught again until July 2001 when she was again lactational, and was last captured in September 2001 with four recently-born young in the pouch. This female clearly had had at least four sets of young.

Table 6.6: The duration between captures of females with successive litters, and the size in crown-rump length of the pouch-young (py), or the status of the pouch. PE=pouch-empty.

<table>
<thead>
<tr>
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<th>2nd capture/2nd litter</th>
<th>Duration</th>
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</thead>
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<tr>
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<td>7mm py</td>
<td>9mm py</td>
<td>68 days</td>
</tr>
<tr>
<td>2.</td>
<td>8mm py</td>
<td>7mm py</td>
<td>66 days</td>
</tr>
<tr>
<td>3.</td>
<td>PE lactational</td>
<td>PE lactational</td>
<td>61-64 days</td>
</tr>
<tr>
<td>4.</td>
<td>PE post lactational</td>
<td>fully furred py</td>
<td>60-62 days</td>
</tr>
<tr>
<td>5.</td>
<td>17mm py</td>
<td>5mm py</td>
<td>57-58 days</td>
</tr>
<tr>
<td>6.</td>
<td>18mm py</td>
<td>6mm py</td>
<td>44 days</td>
</tr>
<tr>
<td>7.</td>
<td>20mm py</td>
<td>12-15mm py</td>
<td>67 days</td>
</tr>
<tr>
<td>8.</td>
<td>19mm py</td>
<td>8-10mm py</td>
<td>68 days</td>
</tr>
<tr>
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<td>15-20mm py</td>
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<td>10.</td>
<td>19mm py</td>
<td>8mm py</td>
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<tr>
<td>11.</td>
<td>10mm py</td>
<td>7mm py</td>
<td>99 days</td>
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</tbody>
</table>

Table 6.7: Females marked between March 2000 and September 2002, and recaptured between sessions 1-4 months apart. Only females that were captured as adult are included. Most females were caught 2-3 times over the given period. A small number of females were captured 4 or more times and where each of these animals (5 individuals) is included an * is followed by the number of times they were captured.

<table>
<thead>
<tr>
<th>Number of litters</th>
<th>26-61 days (1-2 months)</th>
<th>62-180 days (2-6 months)</th>
<th>181-365 days (6-12 months)</th>
<th>366-540 days (12-18 months)</th>
<th>541 days (18 months+)</th>
</tr>
</thead>
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<tr>
<td>0</td>
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<td>6</td>
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<td>4</td>
</tr>
<tr>
<td>1</td>
<td>12</td>
<td>9 (*4)</td>
<td>7</td>
<td>2</td>
<td>2</td>
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<tr>
<td>2</td>
<td>7</td>
<td>2</td>
<td>5 (*4)</td>
<td>(*6)</td>
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<td>3</td>
<td>1 (*7)</td>
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<td></td>
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<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td>1 (*6)</td>
<td></td>
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</table>
Figure 6.14: The interval between sequential captures of individual females with pouch-young, and the size and development stage of the young at each capture. Each bar represents an individual female. Similar reproductive stages are aligned and resolved against a timeline in days. The growth of the young as documented from previous studies in captivity (Wooller et al. 2000) corresponds with that in the wild and the composite of this information provides an estimate of the stage reached at a particular age. The time after birth within which furring and pouch exit may occur in the wild is indicated below the timeline. The duration of weaning may vary.
Other instances of females carrying three or four litters have been recorded prior to 2000. One female carried a succession of three litters over about 8 months. She was first captured in March and then April without pouch-young, but had small pouch-young by June, had large pouch-young in September, and new-born young in December. She was captured for the last time in the following March without pouch-young. Wooller et al. (2000) described another female that had at least four different litters in one year.

6.3.11 Asynchrony of female cycles

Since females clearly have a post-partum oestrus and ovulation, an indication of when births occurred in the population would approximate the spread of oestrus over time. The birth dates of litters were estimated for females captured within two trapping periods: July 1985 to March 1987 and April to October 2001. These periods were among the only times when trapping was carried out at least every two months. Estimation of births was restricted to periods of high resolution trapping in order to maximize the chance that all trappable females with litters were recorded at some time during the pouch-life of the young (about two months).

Births, and therefore females in post-partum oestrus, were scattered over time (Table 6.7 and 6.8). There were times when there would be a female in oestrus for a series of successive days, but there was no clear pattern to such periods. On any one day there was only one, or very occasionally two, females that had given birth and would therefore be in oestrus soon after. There were also periods, where no females gave birth for a large part of a month. Numbers of females caught without pouch-young were also recorded for each trapping session to indicate that females were often present in the population, even when pouch-young were not recorded. Births therefore were not absent for lack of data. The numbers of births presented here were probably an underestimate, given that the birth dates of females without pouch-young, but still lactational, were not able to be estimated, and that there were probably females in the population that were not trapped (see Section 4.3). Nonetheless, oestrous females were obviously scattered through time.
Table 6.8: Estimated date of birth of litter for each female captured with pouch-young at sites A and C from July 1985 to March 1987. Trapping sessions were held in each area simultaneously. Each ‘x’ represents one female has given birth, and will therefore soon be in oestrus. Months underlined are those in which a trapping session was held. The number of females captured without pouch-young and without pouch-young but still lactational are indicated at the end of each month in which a trapping session was held.

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Table 6.9: Estimated date of birth of litter for each female captured with pouch-young at sites A and sites C and D from April to October 2001. Areas are presented separately since trapping session were not always held in both areas simultaneously. Each ‘x’ represents one female has given birth, and will therefore soon be in oestrus. Months underlined are those in which a trapping session was held. The number of females captured without pouch-young and without pouch-young but still lactational are indicated at the end of each month in which a trapping session was held.

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6.4 Discussion

Reproduction in the female honey possum will be discussed in three broad categories. Firstly, specific descriptive details of the histology and reproductive anatomy of the female will be discussed. This will be followed by a synthesis of the available information on the mating behaviour of the honey possum. Finally embryonic diapause, cycle length, lifetime reproduction and reproductive amortization will be discussed.

6.4.1 Reproductive histology and anatomy

Many histological aspects of reproduction in the honey possum generally appear similar to that described for other marsupials. The oocytes were arrested in prophase I, and resumed meiosis prior to ovulation, with the chromosomes arrested in metaphase II for those females closest to ovulation. The chromosomes remained in arrest in unfertilized eggs and had resumed and completed meiosis II for fertilized conceptuses. All conceptuses in the oviduct were single-celled and the exact stage of fertilization and the maturation divisions differed between them. The smaller size of eggs in the oviduct has also been reported by Selwood (1982). It seems likely that this difference is not significant, but rather is a result of the more irregular shape of the egg in the oviduct after histology, due to the loss of support from the fluid that surrounded it in the follicle. Deposition of the mucoid coat occurs in the oviduct, very much the same as that described in the brown antechinus ‘strands of mucoid material…found around, but not closely applied to the surface of each egg’ (Selwood 1982).

The zona pellucida and the shell membrane of conceptuses were approximately 1.5-2.0µm and 1.5-3.5µm respectively and were within the range of 1-6µm described for other species (Hughes 1974; Tyndale-Biscoe and Renfree 1987; Selwood 2000). The mucoid coat was thin and measured approximately 3.5-5.5µm. The mucoid coat ranges from 6.7-230µm in other species (Hughes 1974; Tyndale-Biscoe and Renfree 1987; Selwood 2000). The trophoblast cells of the unilaminar blastocyst were
uniform in their gross appearance under the light microscope. This was also true after reactivation, when all trophoblast cells became round. The honey possum is similar to the macropods (Renfree and Lewis 1996) in this way and distinct from the dasyurids, which have two distinct cell types at the blastocyst stage (Selwood 1996).

The uterine histology during pregnancy in the honey possum was typical of that in marsupials generally (Tyndale-Biscoe and Renfree 1987), and for those that exhibit diapause (Shaw and Rose 1979; Tyndale-Biscoe and Renfree 1987; Ward and Renfree 1988a). Many uterine glands were present in the glandular endometrium. The luminal epithelium of the uterus and the glandular epithelium consisted of tall-columnar cells. The post-partum uteri appear to show proliferation of cells characteristic of follicular development, oestrus and ovulation, simultaneously with the degeneration that occurs after birth. Proliferation of the luminal epithelium was greatest for females near ovulation, where it was squamous, but changed toward cuboidal cells with increased time after ovulation. The nuclei of the luminal epithelial cells continued to increase in size post-ovulation and were similar to that described for pro-oestrous in the long-nosed potoroo Potorous tridactylus, where they “bulge out into the lumen” (Shaw and Rose 1979). Degeneration of cells from the stroma, luminal epithelium and glandular epithelium increased after ovulation to a peak, before declining slightly in the female with cleaving conceptuses. In some marsupials, the whole epithelium degenerates and is replaced after oestrus (Tyndale-Biscoe and Renfree 1987), but honey possums are similar to macropods (Tyndale-Biscoe and Renfree 1987) in that the glandular lumina fill with cellular and amorphous debris, but the epithelium is not replaced. Stretching of the glands containing debris, with flattening of the epithelial cells was noted in the long-nosed potoroo (Shaw and Rose 1979), similar to that found in the honey possum.

Formation of the corpus luteum in the honey possum is different to that described for other marsupial species. Several patterns have been described for other species (Tyndale-Biscoe and Renfree 1987). In some, the basement membrane supporting the granulosa cells is penetrated by the theca interna, and blood and connective
tissue may fill the antrum of the follicle and, as the granulosa cells expand, they fill the gaps in this matrix. In other species the expansion of the granulosa cells occurs before this extrusion happens. The follicle may collapse completely and the granulosa cells hypertrophy, as in the brush-tailed possum *Trichosurus vulpecula*. In the honey possum, the follicle collapsed completely, but the basement membrane remained intact, and the antral space filled again with fluid after ovulation. These fluid-filled corpora hemorrhagica were much larger than the corpora lutea of all females with unilaminar blastocysts, so the corpus luteum must form rapidly during cleavage and formation of the blastocyst, and involve a decrease in size. Detailed study of a series of ovaries within this period would be needed to determine how the corpus luteum forms. Similar to that of the western pygmy possum (Clark 1967), the honey possum corpus luteum does not degenerate until after parturition; this is different from the macropods, in which decline and pyknosis occurs before birth (Renfree and Tyndale-Biscoe 1973).

Interstitial tissue in mammals has two origins. It may develop in juveniles during differentiation or development of the ovary (Tyndale-Biscoe and Renfree 1987; Mulling et al. 1998; Eckery et al. 2002), or it may develop from the theca interna of atretic follicles (Tyndale-Biscoe and Renfree 1987; Perez et al. 1999). In the honey possum, structures that appeared to be at an intermediate stage between follicular atresia and interstitial tissue were observed. Some interstitial tissue had what appeared to be degenerating oocytes at their centre. This may suggest that interstitial tissue arises from atretic follicles in adult honey possums, but these observations need further study and at this stage conclusions can not be drawn.

The morphology of the vaginal complex was consistent with that found by de Bavay (1951). In particular, the median vaginal canal was well developed and was open through to the urogenital sinus in all but the juvenile, pre-parous female. The protrusion of the cervices well into the median vagina found in all specimens examined, is slightly different to the representation of the tract of the two females described by de Bavay (1951), and to the representation of the tract in Tyndale-
Biscoe and Renfree (1987). Both these diagrams show the cervices ending well above where the lateral vaginal canals segregate. This may be subject to some individual variation, but the cervices opened below the level of lateral-vaginal separation in most females.

Stored spermatozoa were not found in the honey possum reproductive tract. In several sections sperm were observed embedded in the oviducal epithelium. In eutherian mammals, this functions to prolong the life of the sperm (Gomendio and Roldan 1993b; Gomendio et al. 1998).

The characteristics of the reproductive tract of each of the two juvenile females was consistent with previous notions of female sexual maturity (Section 3.3). Females were categorized as adults based on size, as the majority of females with pouch-young had a head length of >24mm and weighed > 6g. However a few females carried pouch-young at a smaller size. The histology of the reproductive tract of both juvenile females examined indicated that they had undergone at least one previous oestrus cycle which had not led to pregnancy. The morphology of the vaginal complex in one of these females, AI, indicated that she was pre-parous. Although oestrous and ovulation may occur at a size of less than 24mm head length, and this may lead to pregnancy in some females, it appears that mating and pregnancy most commonly occur on a subsequent cycle when the female is older. Indeed, female AC with a head length of 23.2mm was near ovulation, and the presence of sperm in the oviduct indicated that she had mated recently.

6.4.2 Mating behaviour

The behavioral observations of animals held in captivity overnight were consistent with general patterns previously noted (see Section 3.8; Russell 1986; R.D. Wooller pers. comm.). Females were dominant to males and supplanted them from food. Olfactory communication appeared important in social interaction. No matings were observed, but two males were observed continuously monitoring a pro-oestrous
female by sniffing her. Russell (1986) also noted that male honey possums monitor the reproductive status of females by sniffing their pouch and cloaca. Russell (1986) noted that it was clear when a female was attractive to males in the group because all males, particularly the largest male, would persistently follow and sniff her. In macropods, the males can detect changes in the females several days before oestrus, and they investigate the pouch and genital area of the female (Tyndale-Biscoe and Renfree 1987). In the wild, male honey possums would likely monitor the reproductive status of females, and remain near a female in pro-oestrus to monitor her continuously.

In the current study, the female pushed the males away, and Russell (Russell 1986) observed females aggressively chasing the interested males away. This suggests that females actively choose to allow copulation. Two copulation events have independently been observed in the honey possum, and both were very brief (less than 30 seconds) (Russell 1986; R.D. Wooller pers. comm.).

6.4.3 Embryonic diapause and reproduction in the female honey possum

Post-partum oestrus and the nature of embryonic diapause

The uterine and ovarian reproductive stages of female honey possums showed a sequence from near-oestrus, through single-cell conceptuses in the oviducts, to cleaving conceptuses in utero, in parallel with the increasing size of young in the pouch. Females with young greater than 3 mm head length all had unilaminar blastocysts. These results confirm the findings of Renfree (1980) and show that a post-partum oestrus occurs. Clearly, a pre-partum oestrus or an oestrus later in pouch life are inconsistent with these results.

The sequence of events following birth could be resolved by determining the approximate ages of the pouch-young carried by a female, and relating this age in days with the reproductive status of the female as determined from the histology of
the tract. Pouch-young of different sizes were aged according to the timeline presented in Figure 6.14.

The two females with the tiniest pouch-young were near oestrus. Female AK had four pouch-young with a mean head length of 1.7mm, about 3-4 days old and was in pro-oestrus. Female AL had two pouch-young with head lengths of 1.3mm, about 2-3 days old was in late pro-oestrus or early post-oestrus. Renfree (1980) examined one female with sperm in the tract that had neonates with a head length of 1.3mm. The size of the neonates produced at birth must be slightly variable between females, and/or the timing of post-partum oestrus may be slightly variable between females. Any variability in the sizes of neonates would inevitably reduce the resolution of these estimates, but not greatly. Oestrus probably occurs around 2 and 4 days post-partum. Females with single-cell conceptuses and unfertilized ova in the oviduct had young with mean head lengths of 2.0-2.2mm, giving them an approximate age of 4 to 5 days. One female with cleaving conceptuses in utero had pouch-young of head length 2.7mm, giving an approximate age of 5-6 days. Given that it is unknown exactly how long the eggs had been in the oviduct, or conceptuses in utero, we can approximate the time of ovulation at about 3-5 days post-partum, approximately one day after oestrus, with approximately one day taken to traverse the oviduct. This is consistent with most well-studied marsupials in which ovulation occurs 1-2 days after onset of behavioural oestrus and it takes about 24h for passage of the egg down the oviduct (Lyne and Hollis 1977; Selwood 1980; Tyndale-Biscoe and Renfree 1987).

Oestrus in the tammar wallaby occurs within 8h of birth and lasts for less than 12h (Tyndale-Biscoe and Renfree 1987). For other macropods it occurs 1-10 days after birth (Tyndale-Biscoe and Renfree 1987). In feathertail gliders, ovulation was estimated at 1-3 days post-partum, because a female with 4-5 day old pouch young had cleaving eggs in utero (Ward and Renfree 1988a). This is very similar to that estimated for the honey possum. The length of oestrus is likely to be as brief as that in the tammar. Captive studies of the honey possum may provide further resolution
on the timing of oestrus, although repeated handling of such a sensitive species may disrupt normal cycling patterns. Measuring hormone levels in the faeces (eg. Oates et al. 2004) may be a more effective way of monitoring the oestrous cycle.

There appeared to be rapid formation of the unilaminar blastocyst, given that a female with pouch-young of head length 2.7 mm had cleaving conceptuses, and all females with pouch-young of head length greater than 3.6 mm had unilaminar blastocysts. Therefore, between day 5-6 after birth, and day 10 cleavage to the unilaminar blastocyst took place. In other marsupials formation of the blastocyst takes 4-9 days depending on the species (Tyndale-Biscoe and Renfree 1987: 276). This 5 day period of cleavage to form the unilaminar blastocyst also corresponded to formation of the corpus luteum.

It is clear that the growth of the unilaminar blastocysts occurred in a two-phase manner (Figure 6.2). Rapid growth occurred from a oocyte diameter of 0.13mm to a unilaminar blastocyst of about 1.7mm diameter, with no subsequent increase in size, nor development beyond the unilaminar stage for the entirety of lactation and weaning. This therefore represents a true period of diapause, similar to that seen in the feathertail glider (Ward and Renfree 1988a) and the western pygmy possum (Ward 1990c).

This two-phase pattern of growth appears unique to the small possums. In macropods, from an oocyte diameter of approximately 0.12-0.14 mm, the unilaminar blastocyst forms and developmental arrest occurs at approximately 0.25-0.33 mm (Tyndale-Biscoe and Renfree 1987). Thus, there is little expansion before diapause. Slow expansion of the blastocyst throughout diapause does occur in other species, such as the roe deer Capreolus capreolus, the European badger Meles meles, and the western spotted skunk Spilogale putorius latifrons (Renfree and Shaw 2000).

Preserved honey possum blastocysts within the plateau phase measured between 1.2 and 1.8 mm. This is the same as the range found by Renfree (1980).
feathertail glider blastocysts in this phase were 1.6-2.0 mm in diameter (Ward and Renfree 1988a), although preserved plateau-phase blastocysts corresponded to 0.75 mm (Ward 1990c). Preserved western pygmy possum blastocysts in the plateau phase measured approximately 0.6 mm (Ward 1990c).

The beginning of this plateau phase corresponded to pouch-young of head length about 6 mm, at about 18-20 days (Figure 6.15). The plateau phase for the western pygmy possum was estimated for when the pouch-young had a head length of 8 mm and were 20 days old (Ward 1990c). The plateau phase for the feathertail glider was estimated for when the pouch-young had a head length of 10 mm and were 33 days old (Ward and Renfree 1988a). The onset of the slow-phase growth of blastocysts appears at a similar time in the pouch life (30-50%) and lactation (25-33%) of the young in the pouch, despite the more rapid growth of pygmy possum young. Early in lactation the pouch-young are small and presumably demand the least resources. This corresponds to the time of rapid growth of the blastocyst. The period of diapause corresponds to a time when the demands of lactation would be the greatest. Reactivation, and therefore organogenesis, does not occur until after lactation.

Unilaminar blastocysts held within the plateau phase were all associated with a corpus luteum of a similar size. In some mammal groups with diapause, the corpus luteum remains active but in some it becomes quiescent (Renfree and Calaby 1981). In the western pygmy possum the corpus luteum expands slowly throughout diapause (Ward 1990c), whereas in the feathertail glider it shows a two-phase growth similar to the blastocysts (Ward and Renfree 1988a). This may indicate different mechanisms of control in these three small possum species.

Tyndale-Biscoe (1968) has argued that delays in development, linked to signals from the corpus luteum, may be a normal feature of embryonic development. The embryo can reach the unilaminar blastocyst stage on endogenous energy reserves, and brief pauses of several days have been described for the brown antechinus (Selwood
Synchrony between the uterine secretions and the conceptus would ensure that adequate nourishment was available for expansion and embryogenesis (Tyndale-Biscoe 1989). What is unique for macropods and small possums is the overlap of successive litter production by means of the post-partum oestrus and a period of quiescence (Tyndale-Biscoe 1989). Once the luteal phase extended into the follicular phase, and therefore post-partum oestrous occurred, extension of close synchrony of the corpus luteum, uterus and embryo, likely led to embryonic diapause (Tyndale-Biscoe 1989). Embryonic diapause in macropods was facilitated by the adaptation of the luteal cells of the corpus luteum to develop receptors for prolactin and thus become sensitive to inhibition.

Inhibition of the corpus luteum may be responsible for the maintenance of diapause in the honey possum, as it is in macropods and other animals (Renfree and Calaby 1981). Reactivation of diapause presumably results eventually in expansion of the corpus luteum, because those corpora lutea in post-partum females that had been active were morphologically distinct and much larger than those in diapause. However, the smaller corpora lutea associated with unilaminar blastocysts were either capable of secreting hormones, or blastocyst growth must be controlled by another pathway. There are two reasons for these suggestions. Firstly, the blastocysts were in a rapid growth phase initially, even though the corpus luteum appeared morphologically quiescent. Secondly, reactivation had begun in female M, yet the corpus luteum had the same appearance as other females with blastocysts in diapause. Perhaps this female was in the very early stages of reactivation, or perhaps the blastocyst responds directly to some other signal, rather than reactivation being mediated through the progesterone secretion of the corpus luteum.

**Cycle length, reactivation and embryonic diapause**

All females with pouch-young of 3mm head length or greater, and indeed all females without pouch-young examined in this study, were carrying unilaminar blastocysts. It is likely therefore that oestrus routinely follows birth and gives rise to a diapause pregnancy. Only the early signs of reactivation of unilaminar blastocysts were
observed in females whose young had left the pouch. One female, still lactational, was carrying plateau-size blastocysts which showed a change in the morphology of a few of the trophoblast cells in one region of the blastocyst. A second female, which was post-lactational, had blastocysts in which the trophoblast cells around the entire perimeter of the blastocyst had become round and compact. Mitoses were very common and these blastocysts were greatly expanded. Since collection of animals was focused in winter, reactivation at times of plentiful resources, may progress toward the end of lactation, or after weaning.

Analysis of individual female reproductive histories has shown consistency between the cycle length estimated in this study and that of previous studies based on honey possums from the FRNP and from those in captivity. The length of pouch life is therefore well established and is between 55 and 65 days. Variation is likely to be due to individual variation between females.

Turnover between one litter and the next litter of a similar size can be as little as approximately 65-68 days. This suggests that weaning may be relatively rapid once the young have left the pouch. Russell (1986) noted that young reared in captivity ate some honey one week after pouch-exit. As reactivation does not appear to occur until after pouch-exit, this also suggests that organogenesis is very rapid. This is a possibility, since in some marsupials gestation is as short as 12.5 days (Tyndale-Biscoe and Renfree 1987). The gestation length of the honey possum remains unknown. If it is short, this would still involve reactivation of blastocysts while weaning occurred. The brief period of association with the mother by the young that have recently exited the pouch (Section 3.5) may thus overlap with birth of the next litter.

In other females, the turnover duration was between 80-100 days. In captivity lactation continued for some time after pouch exit and weaning occurred at around 90 days (Russell 1986; Russell and Renfree 1989). Cycle durations of 80-100, are more consistent with the situation described in captivity, whereby lactation may
proceed for longer. Nevertheless, the length of lactation may still be variable in the honey possum. Perhaps reactivation occurs after pouch exit, just before or after weaning, but lactation was of longer duration. An alternative explanation may be that females with a shorter cycle duration abandoned their previous litter before weaning, leading to the rapid turnover of 65-68 days. Conversely, it is possible that females with a longer cycle duration (80-100 days) did not reactivate their diapaused blastocysts immediately after lactation. The available resources and body condition clearly impact the incidence of breeding throughout the year, and from year to year in the FRNP (Chapters 3 and 4). If a female is not in a condition to breed, perhaps the blastocysts remain in diapause and, in that situation, the female allows lactation to continue for a longer period.

Of the females sampled in autumn, none had pouch-young, and all had unilaminar blastocysts within the plateau-phase size. All had corpora lutea slightly smaller, but similar in appearance to the other females with diapause embryos. This suggests that these blastocysts were indeed in embryonic diapause and that the female carried these fertilized conceptuses through from a post-partum oestrus in January or February. The alternative is that the blastocysts were in this stage as part of a normal progression of expansion, but it is perhaps unlikely that all four would be in the same stage. Reactivation clearly can occur once the young have left the pouch. However, it apparently does not occur as a matter-of-course if the pouch-young are removed. Removal of pouch young did not result in an increase in blastocyst diameter up to 93 days later, suggesting that removal of a sucking stimulus is not the single cue for reactivation in the honey possum (Renfree 1981; Renfree et al. 1984; Russell and Renfree 1989). The incidence of unilaminar blastocysts, apparently in diapause, being carried through autumn, corresponds with a time when the flowering food resources are the poorest, body condition is also low and the incidence of breeding is low, or even absent in some years (Section 3.7).

The cue regulating reactivation of diapaused blastocysts may be related to the availability of food and be mediated through the body condition of the female.
Females not in a condition to breed may hold the blastocysts in diapause until such time as further investment in reproduction is physiologically possible. This would explain why, in some years, some females are capable of breeding in autumn, yet in other years, with lower resource availability, they are not. Those females that meet the required nutritional threshold may reactivate their blastocysts once the previous litter have left the pouch. In years following low rainfall, the incidence of breeding females was lower in all seasons, but particularly low in autumn (Table 4.2). In these years the number of females that do not meet the required nutritional threshold may be greater. This may result in females carrying diapaused blastocysts for longer periods, especially over autumn. Indeed, 2001 was a year following low rainfall and an associated population crash, and, the normal low incidence of breeding in autumn extended into May and June.

Variation of the interval between successive litters has been reported for the feathertail glider and the eastern pygmy possum. Periods longer than the length of pouch-life and weaning were possibly due to extension of diapause (Ward 1990a, 1990b). Shorter intervals between litters occurred at times when most births occurred and when the fat-stores in the base of the tail were greatest.

The endocrine control of reproduction and embryonic diapause may involve several criteria that need to be met in order for reproduction to occur. Certainly, in the tammar wallaby, there is a complex interaction between sucking stimulus and photoperiod (Renfree 1993; Renfree and Shaw 2000). Recently, the effect of photoperiod on the endocrine state of the honey possum has been examined (Oates et al. 2004). A change from long to short daylength increased the level of progestagen and, to a lesser extent oestradiol, assayed from the faeces. This indicated a link between photoperiod and endocrine state. Females that were changed to the shorter daylength regime also had larger blastocysts (Oates et al. 2004). Unfortunately, the level of oestradiol in these females was already significantly greater at the beginning of the experiment than among individuals kept under unaltered photoperiod. The reproductive status of all females was therefore
not equally comparable. Studies are continuing to elucidate the effect of hormone levels on blastocyst development (J.E. Oates pers.comm.).

The change from long to short daylength occurs fairly rapidly in south-western Australia around March/April. This is consistent with the time when the incidence of pouch-young is lowest in the FRNP, and may indicate a role for photoperiod. However a total stasis in breeding in autumn does not occur in all years and, since breeding is continuous throughout the rest of the year, the role of photoperiod appears less important. In earlier studies at Manypeaks, the only time when no pouch-young were recorded was in December of two of the three years studied (Wooller et al. 1981; Wooller et al. 2000). Manypeaks is only 100km west of the FRNP and its photoperiod would not differ. Differences in the flowering phenologies of the varieties of *Banksia nutans* appear more consistent with the geographical differences in breeding. The exact role of the changed endocrine state with photoperiod found by Oates (2004) on breeding and blastocyst development thus requires further study. It is likely that a combination of factors may be involved in the regulation of reproduction in the honey possum.

Despite winter generally being a time when there is a high incidence of females carrying young, individual females are not synchronous in their reproductive cycles. Depending on the length of the cycle, a female honey possum may only return to oestrus every 65-100 days. Even then, this may depend on the food availability and body condition of the female. A female may extend the period of diapause. Oestrus would therefore be difficult for males to predict. Accordingly, the pattern of births and therefore oestrous females in the FRNP, was scattered over time (Section 6.3.11). There was no period either over days or weeks, where oestrous females were predictably accumulated. Indeed, there were times when there was a female in oestrus for several consecutive days, but other times when only one or no females were in oestrus for many days. On any one day there was only one, or very occasionally two, females at all trapping sites that would have been in oestrus. It was not possible to assess the number of males in the population at the exact time of
oestrus for each female (eg. Waterman 1998), but there are sexually active males in the population all year round (Scarlett and Woolley 1980; Rutter 2001). The proportion of adults trapped that are male was 58%, but this may be due to their greater movement (Section 4.3). Since not all females are in oestrus over one defined period, there would be many more sexually active males than females, and therefore the OSR would be skewed. The implications of this for the mating system are discussed in Chapter 7.

**Lifetime reproduction**

The ability of trapping studies to resolve iteroparity in the honey possum is limited by the number of times a female is caught. Some females certainly have at least three or four litters over their lifetime. However, the growth rate of the young is slow, the size of the litter is small and the relative size of the young is small compared to other small mammals (Sections 3.3 and 3.4). Evolution of a post-partum oestrus and embryonic diapause would have allowed pouch-young to be produced in succession, when appropriate conditions prevailed, and decreased the interval between litters. A rapid return to production of young, following unfavourable conditions, would have increased lifetime reproductive output in this short-lived species. The distant relationship between the small possums (Section 1.1.1) suggests that embryonic diapause may have arisen through convergent evolution, as it has many times amongst mammals (Renfree and Calaby 1981).

It is generally accepted that, given the pervasive nature of embryonic diapause in the macropods, it evolved early and was retained. Tyndale-Biscoe (1989) suggests that the ancestral and most common, mode of reproduction in the macropods was continuous breeding, through post-partum mating, lactational quiescence and reactivation of the corpus luteum and conceptus at the end of lactaction of the previous young. For small, forest-dwelling marsupials this would have maximized fecundity and allowed rapid return to reproduction after a dearth of resources (Tyndale-Biscoe 1989). The other patterns of macropodid reproduction were
probably derived from this basic pattern as the descendants exploited other climates and habitats.

Despite the honey possum being able to produce litters in succession, its lifetime reproductive output is moderate compared with other small mammals. After taking approximately 4-6 months to reach sexual maturity, the breeding lifespan of a female honey possum is probably 1-1.5 years. If she produces four litters within that time, with an average of 2.4 young being weaned (Wooller and Richardson 1992), this gives a total fecundity of 9.6 offspring. The Australian house mouse *Mus musculus domesticus* is known as being particularly fecund (Strahan 1995). It has a post-partum oestrus and can produce successive litters of 4-8 young. The common shrew also has a post-partum oestrus and can produce two or sometimes three litters of 6-10 young in its single breeding season (Searle and Stockley 1994; Stockley and Macdonald 1998). The larger litter sizes of the dasyurids (see Table 3.1 and McAllan 2003), and production of more than one litter in the breeding season means that, even without embryonic diapause, their lifetime reproductive potential may be greater (Russell 1986).

Smith and Lee (1984) found that the product of annual fecundity and maximum reproductive lifespan tended to increase with decreasing body size in phalangeroid possums and dasyurids. The small possums generally have smaller litter sizes than similarly sized dasyurids (Table 3.1; Smith and Lee 1984). However, the western pygmy possum, the eastern pygmy possum and the mountain pygmy possum *Burramys parvus* have rapid production of young compared to dasyurids (Smith and Lee 1984) and other small possums, and shorter periods of lactation than other small possums (Ward 1998). Ward (1990b) suggested that a female eastern pygmy possum may have up to 40 offspring in a lifetime if she lives four years; the modal litter size is four, all four young are often weaned, an average of 2.5 litters are produced each year, and the lifespan is 3-5 years. The feathertail glider, the feathertailed possum and the long-tailed pygmy possum *Cercartetus caudatus*, have smaller litter sizes and longer lactation and are further toward the ‘slow’ end of the
fast-slow life-history continuum in terms of fecundity (Ward 1998). Despite a short lifespan, early maturity and embryonic diapause, the honey possum is also a ‘slow’ species. If a female honey possum has four litters in a lifetime, each litter would represent approximately 25% of total reproductive output. This may increase to 50% if she only manages to have two litters in her lifetime.

Reproductive amortization

The sizeable variation between the viable uterine blastocysts found within each female honey possum was also recorded by Renfree (1981; 1984). This variation was not purely due to fluid intake into the blastocoele, because variation in cell number also occurred. The blastocysts in diapause for the western pygmy possum were also highly variable (Ward 1990c), but any variation present was not indicated for feathertail gliders (Ward and Renfree 1988a). Selwood (1980) found intrauterine variation in cell number and diameter (of several hundred μm) between unilaminar blastcysts of the brown antechinus. In tammar wallabies, although they are monotocous, comparison between females shows that the primitive streak develops in vesicles of different sizes, and the early stages of development are the most variable in terms of size reached by a specific day (Tyndale-Biscoe and Renfree 1987). Size variation in blastocysts may therefore be common, and may not be related to their subsequent survival.

In the honey possum there was a progressive reduction during reproduction as a result of overproduction of ova and of brood reduction after birth. Reproductive amortization was 61% from ovulation to weaning. Before lactation, there was a 13% loss from ovulation to the blastocyst stage and a 36% loss between the blastocyst and neonate stage. Every female examined had a greater number of corpora lutea than teats. Overproduction of ova by the honey possum was also noted in a paper by Ward (1998) which presented previously unpublished data by Renfree. The mean numbers (mean ±sd, sample size) of corpora lutea (6.0±2.1, 4) and conceptuses therein (≈5.0±1.5, 55) was similar to this study.
Attrition may occur soon after fertilization, since collapsed fertilized conceptuses were observed in the oviduct, and in most cases this collapse was thought to be natural. No degenerating blastocysts were observed, and significant loss was not detected during this stage. However, the power of the test was low due to small samples sizes. Loss between the blastocyst and neonate stage could occur before or after birth. Some blastocysts, perhaps those of a smaller size, may not develop to full term. Alternatively, all blastocysts may develop to full term. Marsupial pouch-young remain permanently attached to the teat for at least the first few weeks of life (Russell 1982), and therefore teat number limits the maximum litter size. Not all young born may make it to the pouch and onto a teat. Excess neonates in the pouch were not observed in this study and have not been documented in the long-term studies of honey possums.

Overproduction of embryos occurs in other species of small marsupial (Table 6.10). The western pygmy possum and the eastern pygmy possum both ovulate more eggs and have more conceptuses than teats (Ward 1998). The western pygmy possum and the mountain pygmy possum _Burramys parvus_ produce supernumerary young (Mansberg and Broome 1994; Ward 1998). Many species of dasyurid produce more eggs than they have teats (Taggart _et al._ 2003) and two examples are presented in Table 6.10. Supernumerary young have also been reported for dasyurid species and may be common (Whitford _et al._ 1982; Strahan 1995). However, the feathertail glider, the feathertailed possum and the long-tailed pygmy possum do not overproduce young (Ward 1998, and Table 6.10).

As marsupial neonates are born at an altricial stage compared with eutherians, there is little investment prior to birth (Hughes and Hall 1988)(Tyndale-Biscoe 1984, 2001). Ward (1998) suggests that there may be less selective pressure on marsupials to limit the number of young at birth, given the long period of lactation over which reduction can occur. The cost for a female honey possum of over-production of ova and embryos would be small, and may function as an insurance policy against loss.
Loss may occur because of chromosomal abnormalities or genetic incompatibilities (Zeh and Zeh 1996, 1997) and is common amongst mammals (Zeh and Zeh 2001; Stockley 2003). Alternatively, in marsupials there may be a risk that all young will not complete the journey to the pouch after birth. Since each litter represents a large proportion of the reproductive potential in the honey possum, the advantage of giving birth to at least four (or more) young, such that a full litter of four young occupy the teats, would be great in this short-lived species. There is evidence, however, that females do give birth to less than four young. Two females only had three blastocysts in utero. Perhaps these females secured too few matings, some conceptuses were inviable or the cost of giving birth to the maximum of four young was too great. If the conceptuses differ in fitness, then perhaps those fertilized by the most fit males are those that survive. The role of selective processes operating in pre-lactational amortization is discussed in Section 7.3.2 in relation to multiple paternity in the honey possum.

Table 6.10: The number of ova, conceptuses, teats and pouch-young in species of small marsupial. Values for small possums are means (±s.d.) except in the case of the honey possum pouch-young where it is mean (±S.E.). Data for the honey possum from this study, and average litter size taken before and after brood reduction from Wooller and Richardson (1992). Data taken from Ward (1998) for all other small possums, and the s.d. estimated from Figure 3 in that study. Litter size from this study is presented as a single average value, and sample sizes ranged from 3-59. Data for the two dasyurids, the agile antechinus Antechinus agilis and the fat-tailed dunnart Sminthopsis crassicaudata taken from McAllan (2003) and Taggart et al. (2003); maximum values for ova/teats given and a range litter size.

<table>
<thead>
<tr>
<th>Species</th>
<th>Corpora lutea/ova</th>
<th>Conceptuses</th>
<th>Teats</th>
<th>Pouch-young</th>
</tr>
</thead>
<tbody>
<tr>
<td>Honey possum</td>
<td>6.2 (±1.3)</td>
<td>5.5 (±1.6)</td>
<td>4</td>
<td>3.6 (±1.5)</td>
</tr>
<tr>
<td>Western pygmy-possum</td>
<td>7.7 (±1.0)</td>
<td>7.7 (±1.5)</td>
<td>6</td>
<td>4.6 (±1.0)</td>
</tr>
<tr>
<td>Eastern pygmy-possum</td>
<td>7.8 (±3.0)</td>
<td>7?</td>
<td>4</td>
<td>3.8 (±0.6)</td>
</tr>
<tr>
<td>Feathertail glider</td>
<td>3.7 (±0.8)</td>
<td>3.4 (±0.8)</td>
<td>4</td>
<td>2.8 (±0.8)</td>
</tr>
<tr>
<td>Feathertail possum</td>
<td>1.3 (±0.4)</td>
<td>1.3 (±0.4)</td>
<td>2</td>
<td>1.2 (±0.4)</td>
</tr>
<tr>
<td>Long-tailed pygmy possum</td>
<td>2 (±0.4)</td>
<td>-</td>
<td>4</td>
<td>2.1 (±0.4)</td>
</tr>
<tr>
<td>(New Guinea populations)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agile antechinus</td>
<td>max 19</td>
<td>-</td>
<td>max 10</td>
<td>6-8</td>
</tr>
<tr>
<td>Fat-tailed dunnart</td>
<td>max 14</td>
<td>-</td>
<td>max 8</td>
<td>7-10</td>
</tr>
</tbody>
</table>
Producing a full litter of four young would be advantageous if they could all be weaned. However, in the honey possum the litter is reduced further during lactation. Reduction of the litter throughout pouch life appears to be a common phenomenon in polytocous marsupials (Lee and Cockburn 1985), and amongst the other small possums it occurs in the feathertail glider (Ward 1990a), with the tendency noted in the western pygmy possum (Ward 1990c) and the mountain pygmy possum (Mansberg and Broome 1994). It does not appear to occur in the eastern pygmy possum. Brood reduction may allow the mother facultatively to alter her lactational load according to the prevailing conditions and her future survival, raising to independence the maximum number of young possible (sensu Lack 1954; Charnov and Krebs 1974; Temme and Charnov 1987; Kozlowski and Stearns 1989).

In honey possums some females are capable of weaning four young, and it follows that a full litter would be produced in case the conditions allow all of them to be weaned. However, litter size does not appear to be related to the prevailing conditions. In the three years examined, litter size was unrelated to whether the year was one of high or low resources (Section 4.2.2). The incidence of breeding was however lower in low resource years, and as discussed above females may not initiate breeding until they reach a certain nutritional threshold. The most sustainable strategy may be to maximize survival and delay reproduction until food increases. Diapaused blastocysts could then be reactivated, and perhaps any breeding attempt then progresses with maximal investment, as evidenced by litter size remaining equal over 2000, 2001 and 2002 (Section 4.2.2). Therefore, those females that do breed in years of low resource availability may only be those capable of the average output. The downward adjustment in brood size may thus be more related to the individual circumstances of the female. Larger mothers are possibly capable of rearing a larger number of pouch-young, but this remains tentative (see Section 4.3). Perhaps the foraging abilities of females are individually variable regardless of the prevailing conditions. Brood reduction may represent optimism on the part of the female, and the extent of the reduction occurs in line with the success of each individual.
6.5 Conclusion

Female honey possums were observed that were in reproductive stages from near-oestrus, through to ovulation, cleavage and the unilaminar blastocyst. The histology of the honey possum female reproductive tract is broadly similar to other marsupial species, with some specific differences. The gross morphology of the tract was similar to that previously described, including a patent median vaginal canal. Sperm storage crypts were not found in the tract.

All females examined with and without pouch-young were either close to oestrus, had ovulated or were carrying conceptuses. The honey possum has a post-partum oestrus and embryonic diapause proceeded in a two phase manner similar to other small possum species. The unilaminar blastocyst expanded rapidly, and then from about 18-20 days following birth, the blastocyst remained at a constant diameter of 1.2-1.8mm. No growth, nor development beyond the unilaminar stage, was observed during pouch-life. The first signs of reactivation occurred during lactation after pouch exit, and expansion of the blastocyst had only occurred in one post-lactational female. The diameter of the corpus luteum remained constant throughout diapause.

Females without pouch-young sampled in autumn, a time of low food availability in the FRNP, had unilaminar blastocysts *in utero* that appeared to be in diapause. Length of pouch-life in the honey possum is 55-65 days, and the interval between litters of the same size is approximately 65-100 days. The length of diapause is probably variable and reactivation may be linked to the availability of food. Embryonic diapause may reduce the time between production of successive litters in the honey possum, but lifetime reproductive potential is reasonably low. Each litter represents a substantial proportion of a female’s lifetime reproductive output. Reproductive amortization occurred from ovulation through to weaning.

Females are not synchronous in their sexual receptivity and the OSR at any one time would therefore be skewed. Males monitor the reproductive status of females through smell.
Chapter 7: General Discussion - The Mating System of the Honey Possum

7.1 The mating system of the honey possum and sexual selection

Mating systems are the composite of both male and female reproductive strategies and the outcome of sexual selection (sensu Emlen and Oring 1977; Reynolds 1996; Gomendio et al. 1998). Sexual selection is a continuous process which takes place at a number of stages of reproduction, from mate acquisition and subsequent fertilization, to differential abortion and investment in offspring (Møller 1998). The large testes and long sperm of the male honey possum were taken to imply that sexual selection was operating upon males and the finding of multiple mating by females confirms this. The female strategy of multiple mating contributes to sexual selection of paternal traits (Møller 1998). Sexual selection of male traits may occur through the pre-copulatory mechanisms of intrasexual competition for mates or intersexual selection (female mate choice) (Eberhard 1998; Møller 1998). It may also occur through post-copulatory mechanisms of sperm competition and cryptic female choice of sperm or embryos. The following sections interpret the mating and reproductive strategies of female honey possums, determined in previous chapters, in view of its consequences for males. In the light of the life-history traits and population dynamics determined for the honey possum, a provisional model of the mating system is proposed. This is approached separately for the sexes, firstly from the male perspective and then from the female perspective, before a final synthesis of the mating system of the honey possum is proposed.

7.2 The male perspective

The incidence of multiple paternity in the honey possum is evidence of sperm competition, as it is in other mammals (Gomendio and Roldan 1993b; Gomendio et
al. 1998; Parker 1998). At ovulation, all ova were released simultaneously and were lying within millimeters of each other in the oviduct, the site of fertilization (see Chapter 6). Therefore, for fertilization to be effected by several males, the sperm from several males must have to be present in the tract at the same time, and therefore they would be in direct competition.

Sexual selection may operate upon honey possum males in several ways, and these may not be mutually exclusive, but also cumulative. Multiple mating by females means that sperm would be in direct competition in the female tract. Sexually active males are present all year round. If conditions prevail, females may produce successive litters, because they exhibit embryonic diapause and breeding occurs year-round. Females are not synchronous in their breeding, nor in the timing of oestrus, and the young grow relatively slowly, such that a female may only return to oestrus every 65-100 days (Chapter 6). Oestrus is unpredictable and may be brief in duration. At any one time, there would be more males available to mate than females in oestrus and the operational sex ratio (OSR) would be strongly skewed (sensu Emlen and Oring 1977). Intrasexual competition between males would occur for access to mates and for paternity of the young. Emlen and Oring (1977) suggest that the greater the skew in OSR, the greater the variance expected in reproductive success among members of the limited sex.

7.2.1 The female tract as an ‘arena’ for sperm competition

The female reproductive tract, as the ‘arena’ in which sperm competition occurs, has the potential to influence which sperm are more successful than others (Eberhard 1998; Gomendio et al. 1998). For example, if the reproductive tract is large, swimming speed or endurance may be favoured. To negotiate thick egg membranes, acrosomal enzyme production may be favoured. In female mammals, the reproductive tract is a hostile environment and may influence sperm competition at a number of levels (Eberhard 1998; Gomendio et al. 1998). Of the millions of sperm ejaculated, most die or are phagocytozed before they reach the site of fertilization (Gomendio et al. 1998). Acidity in the vagina probably evolved to protect
against micro-organism growth, but is also hostile to sperm (Eberhard 1998; Gomendio et al. 1998). Leucocytes invade the uterus and cervix after copulation to phagocytose sperm. In one female honey possum, an accumulation of sperm was observed degenerating at the entrance to a uterine gland (Section 6.3.8).

In both eutherian and marsupial mammals, peristaltic muscular actions and ciliary action play a large part in the transport of sperm (Taggart 1994; Gomendio et al. 1998). In marsupials, sperm is deposited in the upper regions of the urogenital sinus and moves up the lateral vaginae (Taggart et al. 1998). The cervices may act as a selective barrier to sperm transport. In eutherian mammals, the sperm must actively swim through the uterotubal junction into the oviduct, which creates a secondary barrier (Gomendio et al. 1998), although the limited evidence available suggests that this may not be the case in marsupials (Taggart 1994). In eutherians, sperm survive in the lower isthmus of the oviduct for a period of hours before fertilization (Taggart 1994; Gomendio et al. 1998). During this time, sperm attach to invaginations in the epithelial wall, which enhances their survival (Gomendio et al. 1998). Sperm that fail to attach lose their fertilizing capacity. This ‘reservoir’ is quite different from the sperm storage crypts of some species of dasyurid and didelphid marsupials (Taggart et al. 1998). Sperm transport has been studied in those marsupials that have sperm storage crypts. In the fat-tailed dunnart *Sminthopsis crassicaudata*, only sperm in the crypts closest to the site of fertilization were ‘t-shaped’ and travelled toward the ova after fertilization. Specialized sperm storage crypts were not observed in the honey possum (see Section 6.3.8), but the oviduct was highly folded and sperm were observed attached to the oviducal epithelium. This may provide a similar function to that in eutherians. However, a dedicated study of sperm transport in the female tract of the honey possum would be required to confirm this.

In those eutherian mammals for which sperm transport has been studied *in vivo*, Møller (1998) notes that the ratio of sperm to ova at the time of fertilization is 1:1, and that the concept of numerous sperm competing to penetrate the egg is a misperception. Rather, all dasyurid and didelphid marsupials that have been studied
in regards to sperm transport have isthmic sperm storage crypts, and efficient sperm transport delivers approximately 1:10 to 1:20 sperm to the oviduct, compared to approximately 1:10 000 in eutherians (Taggart et al. 1998). In the honey possum female reproductive tract, an estimated 30-75 sperm surrounded each egg at the time of fertilization (Section 6.3.3).

Although most authors agree that characteristics of the female tract determine how postcopulatory selection occurs, the question of female ‘control’, or whether particular features of the female tract have evolved in order to facilitate sperm competition and sperm selection, remains contentious (Birkhead 1998; Eberhard 1998; Parker 1998). Females may actually facilitate sperm competition by having ‘obstacles’ for the sperm to overcome (Eberhard 1998; Gomendio et al. 1998). If direct selection on the part of the female was not possible, these obstacles may provide a test of ‘ability’ of different sperm (Gomendio et al. 1998). Selection of sperm would be adaptive if the female could pass on these traits to her sons (see the female perspective in Section 7.4 below). Similarly, poorly performing or abnormal sperm may reflect poor quality males, since sperm abnormalities are influenced by inbreeding, diet and stress (Gomendio et al. 1998).

It can be difficult to distinguish between sperm competition and cryptic female choice (Simmons et al. 1996) and they are not mutually exclusive (Eberhard 1998). Successful paternity can only occur if a male’s sperm can overcome barriers to sperm transport and successfully fertilize the egg, and if the conceptus can develop and the offspring be successfully weaned. Divergent female reproductive tract morphologies have influenced the evolution of male anatomical features used to compete in sperm competition, particularly in insects (Eberhard 1998). Indeed, the design of the female tract may have evolved to actively select for competitive males, rather than merely being a passive receptacle for sperm. Further experimentation and evidence is needed to show that cryptic female choice is widespread (Birkhead 1998), but there are strong suggestions that sperm and/or embryos are subject to female-initiated selective forces in the tract (Eberhard 1998; Gomendio et al. 1998;
Birkhead et al. 2004). Studies are accumulating which show that female genotype influences the outcome of sperm competition (Olsson et al. 1996, 1997; Stockley 1999; Bretman et al. 2003). Abortion of conceptuses is one mechanism by which the paternity of offspring may be influenced after sperm competition.

7.2.2 Adaptations to sperm competition

Clearly sperm numbers, motility and ability to penetrate the egg are important if a male’s sperm is going to be competitive. There is an allometric relationship between adult testes mass and body mass in eutherian and marsupial mammals (Harcourt et al. 1981; Harvey and Harcourt 1984; Kenagy and Trombulak 1986; Rose et al. 1997; Taggart et al. 1998; Taggart et al. 2003). A greater number of sperm is thought to counteract the increased loss when sperm have to traverse greater distances. Indeed, male mammals produce a greater number of sperm when the female reproductive tract, in particular the oviduct portion, is longer (Gomendio and Roldan 1993a). In addition, since the testes are endocrine glands, they may need to be larger in larger bodied animals to maintain hormone concentrations (Harcourt et al. 1981).

Sperm production is a costly process. This is evidenced by, among other things, seasonal reduction in testis size and sperm depletion after frequent mating (Harcourt 1991; Gomendio et al. 1998). Across species, a positive relationship is predicted between sperm production and the risk or intensity of sperm competition (Parker 1998). When there is a low risk of sperm competition, more resources should be spent on mate acquisition, but when the risk is high, and/or there are many competing ejaculates, virtually all resources should be spent on reproduction. It has been shown experimentally that selection via sperm competition can bring about a rapid evolutionary response of increased testes size in insects (Hosken and Ward 2001).

Amongst mammals, all comparisons indicate that taxa in which females mate with more than one male have larger testes per unit of body mass than in single-male
breeding systems or monogamous taxa (Gomendio et al. 1998). This relationship holds for primates (Harcourt et al. 1981; Harvey and Harcourt 1984), bats (Hosken 1997, 1998), ungulates (Ginsberg and Rubenstein 1990), other eutherian mammals (Kenagy and Trombulak 1986), marsupials (Rose et al. 1997; Taggart et al. 1998), birds (Møller and Briskie 1995), fish (Stockley et al. 1997), frogs (Jennions and Passmore 1993) and butterflies (Gage 1994).

Gomendio et al. (1998) suggested that, amongst mammals, this relationship was so strong that it was a good predictor of mating system. The relative testes mass in the honey possum is extremely high (4.2% body weight, Renfree et al. 1984) and, as predicted, it has a mating system in which the incidence of females mating with more than one male is very high. Predictions derived from the testes mass of the small number of marsupials for which information about the mating system is known, also support this trend (Rose et al. 1997; Taggart et al. 1998). For example, the brown antechinus *Antechinus stuartii*, has large testes for its body size and also exhibits multiple paternity. The feathertail glider *Acrobates pygmaeus*, also has large testes for its body mass, and preliminary indications suggest multiple paternity in this species (Parrot et al. 2002). Larger testes have a higher ratio of seminiferous tubules to connective tissue (Harvey and Harcourt 1984). The large testes in the honey possum, therefore, indicate selection for a large investment in spermatogenic tissue. Increased spermatogenic investment can occur by producing a greater number of sperm and/or by producing larger sperm cells (eg. Møller 1988; Gomendio and Roldan 1991; Gomendio et al. 1998; Parker 1998). Each of these possibilities will be discussed in turn.

If sperm competition is a ‘raffle’, then to produce more sperm would be an advantage, since males that ejaculate a greater number of sperm are more likely to fertilize the eggs (Gomendio et al. 1998; Parker 1998). Species with relatively large testes for their body weight have more sperm-producing tissue and this allows production of a larger ejaculate volume and greater numbers of sperm per ejaculate in mammals (Harvey and Harcourt 1984; Møller 1988, 1989). Also, Møller (1988)
found that species of primate with multi-male breeding systems had a greater number of motile sperm in the ejaculate. However, in primates, the relationship between testes weight and number of sperm remains in question (Gomendio et al. 1998). Nonetheless, greater testes mass or epididymal sperm count does correlate with greater paternity success in some mammals in which sperm competition is the major form of post-copulatory selection (Stockley et al. 1996; Stockley 1997a).

It has been previously assumed that if a large number of sperm per ejaculate was an adaptation to sperm competition, then there may be a trade-off between the number and size of sperm produced (Gomendio and Roldan 1993b). However, in rodents and primates, Gomendio and Roldan (1991) showed that species in which females mated with more than one male had longer sperm than species where females mated with a single male. This appeared to indicate that there is no such trade-off amongst mammals, but later findings have contradicted this. Gomendio et al. (1998) found no relationship between sperm length and sperm competition in primates when relative testes size was used as an indicator of polyandry. Anderson and Dixon (2002) found no relationship between female mating patterns and sperm length in primates, and Gage and Freckleton (2003) found no relationship between relative testes size (as an indicator of sperm competition) and sperm length in mammals. Yet a positive association between sperm competition and sperm length has been found in birds (Briskie et al. 1997; Johnson and Briskie 1999), butterflies (Gage 1994), and cichlid fishes (Balshine et al. 2001). Further, one study of fish taxa showed a decrease in sperm length with increasing sperm competition (Stockley et al. 1997). A microevolutionary study of invertebrates showed that high levels of sperm competition select for larger sperm (LaMunyon and Ward 2002). Paternity studies of invertebrates in some cases provide support for the large sperm hypothesis (Radwan 1996; Oppliger et al. 2003) and in other cases do not (Morrow and Gage 2001; Gage and Morrow 2003). The conditions under which sperm size would be selected in sperm competition may be restrictive and could favour increased, decreased or no change in sperm size (Parker 1993, 1998). This may account for the different results observed in the various studies. Nevertheless, increasing sperm size with risk of
sperm competition is found amongst taxa as diverse as birds, butterflies and some fish. These are taxa with widely different physiologies and female anatomy, but have sperm competition in common (Gomendio et al. 1998). This indicates the potential influence that sperm competition may have on the evolution of sperm length.

Rather than just a raffle, sperm competition can also be seen in terms of a ‘race’ to fertilize the ova, in which case sperm that can swim faster would be adaptive, since the sperm that reaches the ova first after ovulation is most likely to fertilize it (Gomendio et al. 1998). Longer sperm have been shown to swim faster in mammals (Gomendio and Roldan 1991). Longer sperm can mostly be attributed to an increase in tail length (Cummins and Woodall 1985), and longer flagella generate greater forces (Katz and Drobnis 1990).

The honey possum has large testes and a high incidence of female multiple mating, both of which indicate of sperm competition. This points to the potential role of sperm competition in the evolution of long sperm in this species, the longest sperm known amongst mammals (356µm). Studies of sperm number would be necessary to determine whether honey possums also have a relatively large number of sperm per ejaculate. Gomendio and Roldan (1991) suggested that both increased sperm numbers and elongated sperm may evolve in response to the same selective pressures. Theoretical models show that particular circumstances are required for long sperm to be selectively favoured (Parker 1993, 1998); one of which may be high sperm density in the female tract.

Sperm motility has been studied in marsupials from the family Dasyuridae, that have characteristically long sperm (Taggart 1994). In the brown antechinus, a species that exhibits multiple paternity, the mean total sperm length is 271µm (Taggart 1994) and mean sperm tail length is 259.7µm (Taggart et al. 1998). However, there is a negative correlation between sperm tail length and body mass in marsupials, and the only two species studied thus far to have long sperm tails for their body size are the dusky antechinus Antechinus swainsonii (mean sperm tail length: 260µm) and the
kowari *Dasyuroides byrnei* (mean sperm tail length 242.1µm) (Taggart *et al.* 1998). Dasyurid sperm moves in a sinusoidal motion (Taggart and Temple-Smith 1990). This gives rapid progress in viscous vaginal fluid, but slower, ineffective movement in standard culture medium. The sperm of the honey possum is similar to the dasyurids in its midpiece structure, and is also long. As this structure relates to movement, a similar pattern of movement has been suggested for the sperm of the honey possum (Harding *et al.* 1982; Taggart and Temple-Smith 1990), although this has not been observed directly. Sperm from American marsupials also have radial displacement of midpiece dense fibres, similar to the honey possum and dasyurids (Taggart 1994). Paired sperm of the American marsupials, also moves more efficiently through viscous fluids. The structure of the long sperm of the honey possum may therefore have been selected for its high motility.

The similarity of honey possum sperm to that of the dasyurids and didelphids may be the result of selection for sperm motility, but for a different underlying reason. High sperm motility has been implicated in efficient sperm transport and storage in these taxa (Taggart and Temple-Smith 1990; Taggart 1994). Interestingly, male dasyurids and didelphids produce fewer sperm than other marsupials and eutherian mammals (Taggart and Temple-Smith 1991; Taggart 1994). In *Antechinus*, males exhibit spermatogenic failure prior to the mating season and total male die off occurs a few weeks after mating. Multiple paternity occurs in the brown antechinus, but probably not in some other species of dasyurid (Taggart *et al.* 1998). It is likely that coevolution of male and female reproductive biologies has influenced the characteristics of sperm in dasyurids, as it does in eutherian mammals, independent of sperm competition.

In marsupials, viscous mucus occurs in the vaginal culs-de-sac and cervices after insemination (Taggart 1994). In eutherians it also occurs in the isthmus of the oviduct prior to ovulation, but it is not known whether this is true for marsupials. It is in these two regions that independent movement of sperm may be most critical (Taggart 1994; Gomendio *et al.* 1998). As explained above, the cervices represent a
selective barrier to sperm. Since the first sperm to reach the egg may be the one most likely to fertilize it, swimming speed after release from oviducal isthmic temporary ‘holdings’ or from storage crypts may also be crucial in sperm competition (Gomendio et al. 1998). It may be that the structure and size of sperm in the honey possum has been selected for greater motility within these regions.

Large sperm size may have trade-offs in sperm survival. In mammals (Gomendio and Roldan 1993a) and fish (Stockley 1997), sperm survival is inversely related to sperm length. Gomendio and Roldan (1993a) tentatively suggested an energetic argument, based on Cummins and Woodall’s (1985) observation that increase in length was usually achieved through increase in the principal piece of the tail and, to a lesser extent, in the midpiece of the tail. The midpiece contains the mitochondria, the source of energy for the cell. Large sperm may be energy limited because of the smaller amount of energy per unit size and the cost of greater swimming speeds. Coevolution between male ejaculates and female reproductive biology may also explain the fertile lifespan of sperm (Gomendio and Roldan 1993a). In eutherian mammals, there is a strong positive relationship between the fertile lifespan of sperm and the interval between the onset of oestrus and ovulation (Gomendio and Roldan 1993a). This is thought to have developed since males have little ability to determine when the best time to mate with the highest likelihood of conception will be.

Available evidence suggests that copulation is very brief in the honey possum (see Section 6.4.2) and this may allow for several copulations to occur in a short oestrous period, without adversely affecting the survival of sperm from those males who are second or third to mate. The length of the honey possum sperm may result from selection through sperm competition, but may be enabled by a short oestrous duration and a relationship between the sperm head and the epithelial lining of the oviduct. This provides the potential for a female to influence sperm competition (sensu Eberhard 1998) and to synchronize the sperm population from several matings (Gomendio et al. 1998).
Studies of sperm transport in the honey possum may help to determine the motility and ejaculate numbers compared to other species. This might confirm whether there is a trade-off between sperm size and sperm number in a species with the longest sperm of any mammal. Studies might also concentrate on the movement of sperm through the tract and the possible function of reserving sperm in the oviduct prior to fertilization. Detailed information on the length of oestrus and the timing of the non-pregnant oestrous cycle would be needed to facilitate a successful study of actual mating.

7.2.3 Competition before and after copulation amongst male honey possums

The incidence of multiple paternity in the honey possum is between 86-95%, with the true value likely to be close to 95%. This indicates that the risk of sperm competition for a male is very high. The estimated number of sires per litter was often three or four, and the number of sires detected in 59% of litters was equal to the number of young, indicating that detection of sires was constrained by the number of young at the time of sampling. The number of males with which a female mated could often be three, four, or more. It follows that there is not only a high risk, but a high intensity of sperm competition in this species. The size of the sperm and testes are extreme compared to most other mammals and this, together with the high incidence of multiple paternity, indicates that sexual selection by means of sperm competition is likely to be intense in the honey possum. The relative importance of components of sexual selection, in this case pre- and post-copulatory selection, would be expected to be reflected in the magnitude of expression of the sexual characters (Møller 1998).

In sperm competition theory, it is assumed that males have a fixed energy budget for allocation to reproduction (Parker 1998). The fitness of an individual will thus be a trade-off between gaining matings, for example by mate searching or fighting for access to females, and between expenditure on the ejaculate. Parker (1998) suggests that, under intense sperm competition, it may not pay to overtly ‘fight’ for mates. Given that honey possum males are smaller than females, investment in
body size by males does not appear to be important. Aggression, noted by Russell (1986) between males and females, and between two males, was basic and unritualized (see Section 3.8). Furthermore, males have no ornaments, nor any armaments, having only vestigial teeth and no claws. Thus, agonistic encounters between males to ‘win’ dominance of the female, such as in polygynous mating systems are not evident. Rather, competition between males probably occurs mostly during post-copulatory sperm competition. However, there may be some scramble-competition between males for access to an oestrus female and this possibility is discussed later in this section.

Amongst mammals, between-species comparisons indicate that relative investment in body size or adaptations to sperm competition, such as testes mass, can be indicative of the breeding system. Amongst primates, body size dimorphism (males larger), relative canine size and testes size vary according to the mating system (Harvey and Harcourt 1984). In polygynous species in which females mate with a single male, the males compete physically for access to females, but the risk of sperm competition is small. In multi-male breeding systems, the risk of sperm competition is high. The males of single male breeding systems have greater sexual dimorphism in body size and greater relative canine size, whereas males from multi-male breeding systems have greater relative testes mass. In monogamous mating systems, investment in body size and testes mass is low. Similarly, Sachser et al. (1999) found that between two species of caviomorph rodents, the promiscuous species, the yellow-toothed cavy *Galea musteloides*, had a high relative testes mass (1.86% body weight) and females were larger than males. In contrast, a polygynous species, the wild cavy *Cavia aperea*, had a low relative testes mass (0.58%) and males were significantly larger than females. Invertebrate taxa show similar trends (Poulin and Morand 2000).

There are exceptions to this pattern, such as in the grey mouse lemur *Microcebus murinus*. This species lacks sexual dimorphism and the relative testes size is high (Andres et al. 2001). This indirectly suggested sperm-based scramble competition,
but paternity studies and behavioral observations in captivity have shown that dominant males achieve the most matings and father almost all litters of young. Multiple paternity was not found. In this species the mating system relied on pre-copulatory aggression-based competition, despite the lack of sexual dimorphism (Andres et al. 2001). This study indicates the need to confirm with direct evidence that sperm competition exists.

The honey possum invests heavily in spermatogenesis and has sperm competition, but the asynchrony of females in oestrus would mean that males would need to actively locate receptive females. The greater movements of males during their daily activities (see discussion, Section 4.3) may reflect their displacement from rich food resources by larger females and their need to forage further afield, but may also reflect their search for receptive females. Limitations to studying the movement of honey possums using either radiotracking or trapping, have been noted (see Section 4.3), and direct observation of social interactions in the field have proved almost impossible because of their sensitive and cryptic nature (Garavanta 1997). Nevertheless, Garavanta (1997) used trapping data and found that the home ranges of males overlapped or intersected with other males more often than with other females. In contrast, the home ranges of females overlapped with males more often than with other females. This is consistent with males searching for females, and females mating with several males. Males are clearly very tolerant of each other, as also reflected in captive studies (Russell 1986; R.D. Wooller pers. comm.), and do not defend territories. In captivity, males interact little with other individuals, either male or female. The overlapping home ranges of males, therefore, probably reflects their greater movement in general.

Given the skew in OSR, it is likely that honey possum males compete to gain matings through their knowledge of the location of receptive females. Males assess the reproductive status of a female through smell (see Section 6.4.2). The timing of oestrus would be difficult for males to predict. The interval between oestrous events for each female may vary, depending on a variable length of embryonic diapause,
unpredictable availability of food resources affecting a female’s ability to breed, and the possibility that a female may lose a litter. This would require males to monitor the reproductive condition of solitary-living females that are spread over time and space. From observations in captivity in this study and in Russell (1986), males are able to identify females that are close to oestrus and persistently follow and monitor them. Males probably remain nearby when a female is near oestrus.

Competitive mate searching has been documented in other promiscuous mammals in which overt male physical combat does not occur (Schwagmeyer et al. 1998; Waterman 1998; Jackson 1999). In thirteen-lined ground squirrels *Spermophilus tridecemlineatus*, sperm competition favours the first male to mate (Schwagmeyer et al. 1998). Males that arrived at a female’s activity range first, were those males that had spent more time with the female on the day before oestrus and were more persistent in their searching. In Cape ground squirrels *Xerus inauris*, males live in all-male bands in which established dominance relationships are maintained with little aggression (Waterman 1998). Males compete by searching, repeatedly copulating and disrupting copulations by other males. Dominant males were more successful at locating females, were usually first to mate and mated more often.

In the honey possum, in 41% of litters for which multiple paternity was assessed, the number of sires was less than the number of young in the litter, indicating that some males fathered more than one offspring and were more successful than others (Section 5.3.2). Amongst the modest sample of offspring to which paternity was assigned, all the sires weighed more than 7g and had head lengths of 26mm or greater. This was despite males being capable of siring offspring from a size of 6g and 24mm (Section 3.3). Scrotal dimensions appeared large compared to the population mean. Caution must be applied to interpreting any noted trends because of the limited sample size. However, if males that successfully gain fertilizations are generally of a large body size, then this may be due to at least four possibilities. Firstly, females may show a preference to mate with larger males or select them through cryptic female choice. Their size may indicate their foraging success,
longevity and better overall quality. Secondly, larger males are older and their greater reproductive success may stem from their greater experience and skill at locating females in oestrus. Thirdly, size was noted as important in encounters between male honey possums in captivity (Section 3.8 and Russell 1986). It may be that larger males may gain more matings by chasing smaller males away from oestrous females. Finally, larger males may compete more effectively in sperm competition. Male body size covaries with ejaculate size within many insect and fish species, and larger body size may therefore be important in sperm competition (see review in Møller 1998). If male size indicates quality or condition, then these males may invest more in their ejaculate (eg. Simmons and Kotiaho 2002). In the present study, four of the six sires had greater scrotal dimensions relative to body size than the population mean. Further studies to determine the attributes of males with higher reproductive success compared to others in the population would be needed to assess the direction of sexual selection in the honey possum. Several of these factors may determine male reproductive success. Although there may be variance in male reproductive success, it was clear that fertilizations were not obtained by just a few successful males in the population.

With regard to the dominance of larger males over smaller males, if this is a factor in the mating behaviour of the honey possum, it would still not allow larger males to monopolize females. As suggested previously (Section 5.4), the larger size and dominance of females over males, suggests that females would actively choose to mate with several males and might not allow this to be compromised by mate-guarding from particular males. A multiple mating strategy by females conflicts with a male strategy of maximizing the number of offspring sired (Stockley 1997b; Gomendio et al. 1998). Males may therefore have evolved some other strategy, such as multiple copulations, to bias the outcome of multiple mating. In eutherian mammals, the male that copulates closest to the time of ovulation, but within enough time for sperm transport and maturation, is most likely to effect fertilization (Gomendio et al. 1998). Male size may influence the order of mating and number of copulations with a female, and may thus influence their fertilization success.
A limited number of observations of mating behaviour were made in captivity during the current study and also by Russell (1986). In the present study, one large and one small male were observed with a pro-oestrous female in captivity (Section 6.3.9). The males continuously monitored the female by sniffing her pouch, cloaca and underbelly. The two males would take turns at sniffing the female, and then return to their preferred corner of the cage to groom. The larger male initially showed aggressive tendencies toward the smaller male, but these subsided after they became acquainted by nose-to-nose sniffing (sensu Russell 1986). The female was in torpor, but occasionally stirred and pushed the males away.

This information, albeit limited, allows speculation on the mating behaviour of the honey possum. The most likely scenario is that males competitively search for receptive females and, upon location of a pro-oestrous female, competitively monitor her, but are broadly tolerant of one another. During oestrus, several males may be present simultaneously and at this time larger males may aggressively discourage smaller males from copulating. However, they would be unlikely to succeed as the female would remain dominant. As females begin to breed before they are fully grown, smaller females may be less able to dominate the situation during oestrus, in which case interplay between the males might be more significant. Alternatively different males may visit a female at different times during her oestrus, mate briefly and disperse.

Although changes in population density do not affect the incidence of multiple mating by females, they may affect the strategies of males. In section 4.3 it was proposed that, even at times of the lowest population densities, encounters between individuals of the opposite sex are still feasible, particularly if movements are slightly greater during these times. This was based on the current estimate of home ranges in this species, which as discussed, probably represent a minimum value (Section 4.3). It may be that a lower density of females causes males to move further in their search for receptive females.
There are few detailed studies of mating behaviour in marsupials and none of the small possums (see review in Taggart et al. 1998). Amongst small marsupials, the brown antechinus is probably the species most extensively studied. Multiple paternity has been recorded in this species in captivity and for a closely related species, the agile antechinus *Antechinus agilis*, in the wild (Taggart et al. 1998; Kraaijeveld-Smit et al. 2002). However, in this species, males are the larger sex and exhibit lekking behaviour, and are therefore very different to the honey possum. The mating of the yellow-toothed cavy (Sachser et al. 1999) shows close similarities with the behaviour predicted for the honey possum, in that it shows competitive mate searching behaviour. Individual yellow-toothed cavies are broadly tolerant of each other and huddling activity occurs in groups, with animals present that had previously been involved in agonistic encounters with each other. Males show clear dominance hierarchies, not seen in honey possums. During oestrus, the dominant male tried to guard the female, but the larger female avoided this by racing around and often changing direction, thereby attracting the attention of other males. Females mated with up to five different males.

This study of honey possums has clearly shown that the incidence of multiple mating by females is very high, but without direct evidence that males mate with several females. The lack of synchrony of oestrus and the effectively continuous mode of reproduction make it possible for males to access several females over a space of time. Male honey possums do not provide any parental care. The home ranges of females in the wild overlap with males more than with other females, but also overlap with several other females (Garavanta 1997). Males range more widely than females. There is no social structure to suggest that females monopolize certain males. Indeed, females carrying pouch-young in captivity were extremely intolerant of males (Russell 1986). Males would be free to mate with many females over their lifetime and would be expected to do so in order to increase their lifetime reproductive success.
7.3 The female perspective

7.3.1 Benefits of multiple mating – the hypotheses

It has been shown that multiple paternity, and therefore multiple mating, occur with high frequency in the honey possum. This occurs regardless of marked fluctuations in population density and, together with the observation that females are behaviourally dominant, suggests that females actively choose to mate with several males. Since the advent of DNA analyses of paternity, multiple paternity has proved to be widespread in animal taxa (Birkhead and Møller 1998). Female multiple mating is widespread amongst mammals, occurring in approximately 133 species from 33 families and nine orders (Wolff and MacDonald 2004). The benefit for males mating with several females is an increase in the number of offspring sired. Females are limited by offspring production per unit time. For species that invest their parental care in relatively few young (e.g. birds and mammals), the benefits of multiple mating may come from increasing the quality and reducing the loss of offspring in which they have invested (Hosken and Stockley 2003).

It is thought that females incur costs from copulating with several males. These include energetic costs of multiple copulations, increased risk of predation, parasitism, risk of sexually transmitted diseases, (Møller 1998) and physiological interactions between the ejaculates of different males within the female (Eberhard 1998). The costs of multiple mating must therefore be balanced by some advantage. The adaptive significance of polyandry has been much debated in the literature and the various hypotheses advanced may have more or less relevance for particular life-histories (Hosken and Stockley 2003). The following sections identify the prevailing hypotheses and are followed by a discussion of those that may be considered most relevant for the honey possum.

Benefit hypotheses are separated into the broad categories of direct material benefits and indirect genetic benefits (see Jennions and Petrie 2000; Hosken and Stockley 2003). Material benefits are most often applicable to insects and fish which have...
large numbers of offspring and invest little in each set (Hosken and Stockley 2003). Examples of material benefits include male-donated nuptial gifts that increase the female’s lifespan or fecundity, sexually transmitted anti-predator defense chemicals and infertility avoidance by ensuring an adequate sperm supply. Other direct benefits include increased paternal care (Briskie et al. 1998), cost minimization when forced copulations occur, or infanticide avoidance (Wolff and MacDonald 2004), and are also not applicable to the honey possum.

There is much evidence that multiple mating is adaptive to females through the genetic benefits they pass on to offspring (Jennions and Petrie 2000). Manipulative experimental designs in invertebrates (Tregenza and Wedell 1998; Newcomer et al. 1999; Fedorka and Mousseau 2002), fish (Evans and Magurran 2000) and mammals (Keil and Sachser 1998), that control for material and maternal effects, have shown that polyandrous females pass on genetic benefits to their offspring and thereby increase their reproductive success. Potential sires may be chosen by means of precopulatory mate selection, followed by sperm competition or cryptic female choice (Jennions and Petrie 2000; Hosken and Stockley 2003). Alternatively, paternity may rely on post-copulatory mechanisms, such as sperm competition and cryptic female choice, as a ‘filter’ to choose the most viable male, the best sperm or the most compatible sperm or conceptus (reviews in Jennions and Petrie 2000; Hosken and Stockley 2003). Finally, it has been suggested that polyandry may be advantageous by increasing offspring diversity per se, as a bet-hedging strategy, where reliable indicators of the best male do not exist (Watson 1991).

**Benefits of mate choice**

Females in socially monogamous species may be unable to pair with a high quality male, and may choose to ‘trade-up’ through extra-pair copulations with high quality males. Field studies in birds have shown that extra-pair mates are often more ‘attractive’ in their secondary sexual traits or are more dominant males (see reviews in Møller 1998; Jennions and Petrie 2000). In populations of the blue tit *Parus caeruleus*, 31% of nests had extra-pair young (Kempenaers et al. 1992). Males that
fathered all young in a clutch were larger, survived better, received more female visits and recruited more young. In the socially monogamous allied rock-wallaby, *Petrogale assimilis*, one-third of young were from extra-pair males (Spencer *et al.* 1998). These males had longer arms, an indicator of fighting ability, than the long-term partners of females seeking extra-pair copulations. Females that showed a mixed-mating strategy weaned the greatest proportion of their young. Females that remained faithful to their mate, weaned a lesser proportion and females that only had extra-pair young weaned the lowest proportion of their young. This suggested that polyandrous females gained genetic benefits for their offspring (Spencer *et al.* 1998). Most of the available evidence for the ‘trading up’ hypothesis is drawn from birds, and from a few other socially monogamous species, and its applicability to other taxa is unknown (Jennions and Petrie 2000; Hosken and Stockley 2003).

**Benefits of mechanisms that bias post-copulatory paternity**

Several hypotheses involve either sperm competition or cryptic female choice to bias paternity. However, the effect of these two processes can be difficult to separate, and females may exert influence over the outcome of sperm competition (Eberhard 1998). The ‘intrinsic male quality’ hypothesis suggests that females can obtain ‘good genes’ for their offspring if the fittest male performs best in sperm competition, or is chosen by selection of sperm or selective abortion of conceptuses (Madsen *et al.* 1992; Birkhead and Moller 1993). Yasui’s (1997) model determined that, if males with greater general viability can invest more in sperm competition and are therefore more competitive, polyandrous females should have greater viability of both sons and daughters than monandrous females. This assumes that post-copulatory male traits that bias paternity are correlated with heritable viability (Jennions and Petrie 2000).

In the dung beetle *Onthophagus taurus*, Simmons and Kotiaho (2002) found that testes weight, sperm length, and ejaculate volume had high heritability, and that there was significant genetic variation in testes weight and ejaculate volume. Condition indices also had high heritability and males in good condition had larger testes, suggesting they would have greater fertilization success (Simmons and Kotiaho 2002).
Madsen et al. (1992) found that polyandrous sand lizards Vipera berus, had a greater proportion of offspring that were viable than monandrous females. They originally interpreted this as evidence that multiple mating promoted sperm competition and therefore fertilization by higher quality males, but these results could also be interpreted as selection for genetically compatible males (Olsson et al. 1996). In a few species of birds and fish, suggestive evidence comes from positive correlations between secondary sexual traits or phenotypic cues indicating viability, as well as testes mass and ejaculate volume (see Jennions and Petrie 2000). There is some evidence that parasite resistant males may be capable of producing an ejaculate of higher quality (see Jennions and Petrie 2000). Sperm are recognized as non-self in the testes. Parasite resistant males are more likely to be able to afford to produce immunosuppressive hormones such as testosterone and androgens. Immunosuppression would mean that sperm are less subject to attack and that a greater number are then available for ejaculation.

The ‘sexy sperm’ hypothesis suggests that by promoting sperm competition females produce sons that are more successful at sperm competition (Keller and Reeve 1995) and is largely an adaptation of Fisherian runaway theory. Such an explanation relies on sperm competitive ability being heritable. Some evidence of this is available. For example, in the bulb mite Rhizoglyphus robini, some males were consistently better than others at gaining paternity through sperm competition, and there was a significant correlation between the paternities gained by fathers and sons (Radwan 1998). Pai and Yan (2002) provided some support for the sexy sperm hypothesis in red flour beetles Tribolium castaneum, but only under a high intensity of sperm competition, where females mated with 16 males. There was also some evidence of ‘good genes’ effects. Simmons (2003) found that a sexy sperm hypothesis could not be supported in the field cricket Teleogryllus oceanicus. Currently, there appears to be only limited evidence for the sexy sperm hypothesis (Jennions and Petrie 2000; Hosken and Stockley 2003).
It is becoming apparent that the genome of any female in the population can not be combined with that of all males to produce a viable embryo capable of developing to sexual maturity, but rather that the ideal genetic partner varies amongst females (Zeh and Zeh 1996, 1997, 2001). Since genetic incompatibility is generally not obvious at the phenotypic level (Zeh and Zeh 2001), females may mate multiply to promote post-copulatory mechanisms that bias paternity toward genetically compatible sperm and/or ova to produce viable offspring, such that ova are not wasted (Zeh and Zeh 1996, 1997). Polyandry for incompatibility avoidance may undermine any directional selection acting on males (Tregenza and Wedell 2000; Zeh and Zeh 2001).

Incompatibility can arise because of genetic relatedness between mates, dominance and overdominance, intra- and intergenomic conflict, fetomaternal interactions, and immune system function (Tregenza and Wedell 2000; Zeh and Zeh 2001). To illustrate that such incompatibility may be widespread, some examples are given below (full reviews in Zeh and Zeh 1996, 1997; Jennions and Petrie 2000; Tregenza and Wedell 2000; Zeh and Zeh 2001, 2003).

The advent of DNA technology has revealed selfish genetic elements that establish conflict within the genome and between the foetus and the mother. Such intragenomic conflict can be caused by cellular endosymbionts, transposable elements, segregation distorters, maternal-effect lethals and genomic imprinting (Zeh and Zeh 1996; Tregenza and Wedell 2000). Genomic imprinting results in differential expression of genes at maternally and paternally inherited loci. For example, the insulin-like growth factor (IGF II) in mice stimulates nutrient transfer from the mother, and is only expressed from the paternal copy of the gene Igf2 (see Zeh and Zeh 1996). In contrast, the Igf2r locus codes for a product that degrades IGF II, and this gene is expressed only from the maternal copy. Individual variation in the production of these factors occurs through variation in modifier loci, and successful development of the embryo relies on the correct balance from maternal and paternal genomes (Zeh and Zeh 1996). A maternal locus with high suppression activity would only produce normal offspring when paired with paternal genotypes which stimulate a high level of nutrient transfer (Zeh and Zeh 1996). Nutrient transfer from mother to embryo relies
on a complex interplay between numerous imprinted and other genes. Zeh and Zeh (1996) suggest that this would result in selection for females to fertilize their eggs with compatible male genotypes.

The immunologically hostile female reproductive tract has the potential to provide a physiological screening process for incompatible genotypes in sperm and/or ova (Zeh and Zeh 1997). This may occur through several mechanisms. Some cell-surface proteins on sperm are produced through haploid expression. In humans and mice it has been found that such sperm antigens induce anti-sperm immune responses that lead to defective sperm, embryos or infertility. In eutherians and marsupials, there is a large production of leucocytes in the female tract after copulation and a massive reduction in numbers of viable sperm. Evidence is accumulating that females are capable of sperm selection based on similarity or compatibility of mates (Bishop et al. 1996; Olsson et al. 1996, 1997; Stockley 1999). Other paternal genes are expressed at the stage of blastocyst development, and have the potential to cause abortion of the embryo (Zeh and Zeh 1997). Viviparous females have the opportunity to reallocate resources from defective to viable embryos, particularly if they produce more conceptuses than can survive to birth. This may be reflected in the fact that mammalian species regularly suffer reproductive loss. In humans, 70% of conceptions later fail; in other mammal species, up to 40% and sometimes 60% wastage of ova/conceptuses can occur (Zeh and Zeh 1996, 2001; Stockley 2003). Multiple mating, followed by selection of sperm/conceptuses, may be a tactic to avoid loss from incompatibility. Consistent with this is the finding that polyandrous taxa appear to suffer a lower rate of reproductive loss (Stockley 2003).

In viviparous females, the conceptus represents a foreign body, and success of the embryo relies on complex immunological interactions between the foetus and the mother (Zeh and Zeh 2001). The vertebrate major histocompatibility complex (MHC), a cluster of genes involved in immune response, is typically extremely polymorphic (review in Tregenza and Wedell 2000). MHC haplotype similarity can cause foetal
loss in humans and primates, and paternity bias based on MHC has been shown for mice, rats, humans (Tregenza and Wedell 2000) and fish (Landry et al. 2001; Kurtz et al. 2003). In mice, selection based on MHC haplotype can occur through sperm selection at the ova, or completion of the second meiotic division of the ovum may be determined by sperm haplotype (Wedekind et al. 1996; Rüllicke et al. 1998). An optimal level of heterozygosity at MHC confers advantages in parasite resistance (eg. Kurtz et al. 2003). Therefore, choice of sperm with an optimal level of dissimilarity may lead to incompatibility avoidance and increased genetic quality of offspring through heterozygosity (Brown 1997; Tregenza and Wedell 2000). MHC differences between individuals may reflect general dissimilarity, and so selection based on genetic relatedness or MHC may be difficult to separate (Tregenza and Wedell 2000).

There is growing evidence that bias in paternity based upon incompatibility and relatedness is widespread. In a captive population of the yellow-toothed cavy, females mated to a single male suffered significantly more stillbirths and higher mortality of young before weaning than females that were multiply mated (Keil and Sachser 1998). Since both categories of females had the same male partners, the genetic quality of males was controlled. This suggested that post-copulatory mechanisms allowed female cavies to avoid failed genetic combinations. In pseudoscorpions Cordylochernes scorpiodes, polyandrous females had a much lower rate of spontaneous abortion than monandrous females (Newcomer et al. 1999). The experiment controlled for material benefits and male quality, suggesting that genetic incompatibility selects for polyandry in this species. Similarly, in the field cricket Gryllus bimaculatus, controlling for male quality showed that increased hatching success of polyandrous females was due to avoidance of genetic incompatibility (Tregenza and Wedell 1998), thereby avoiding the costs of inbreeding (Bretman et al. 2003). In the sand lizard Lacerta agilis, higher offspring viability of polyandrous females, compared to monandrous females, was associated with selection of sperm on the basis of genetic similarity, with distantly related mates siring more of a female’s offspring (Olsson et al. 1996, 1997). This was interpreted
as an inbreeding avoidance mechanism, since females often mate with siblings and such matings were known to result in reduced offspring viability.

Kempenaers et al. (1999) suggested that higher hatching success in extra-pair nests of the tree swallow *Tachycineta bicolor* was consistent with genetic compatibility or diversity, rather than ‘good genes’. Foerster et al. (2003) found that both processes were operating in blue tits. In the latter study, individual heterozygosity or diversity was associated with increased fledgling success, which has been shown to be a strong predictor of fitness in other studies (Coltman et al. 1998; Hansson et al. 2001; Hansson and Westerberg 2002). Increased success may come not only from inbreeding avoidance, but also from maximizing the dissimilarity of mates. Long-term data on the genetic and reproductive success of long-lived vertebrates (albatrosses, seals and whales) revealed that there was a negative relationship between reproductive success and parental similarity (measured as heterozygosity or gene diversity Amos 2001). These effects are true across a full range of parental similarity, rather than being due to a small number of closely related individuals. However, another study, in the red-winged blackbird *Agelaius phoeniceus*, found no such relationship between heterozygosity and fitness (Weatherhead et al. 1999).

In natural populations of the common shrew *Sorex araneus*, females regularly incur costs of mating with related individuals because of limited juvenile dispersal (Stockley et al. 1993b; Stockley and Macdonald 1998). However, common shrews appear unable to select unrelated males to fertilize their eggs through mate choice, sperm selection or selective abortion of embryos (Stockley 1997a). In this instance, it appears that multiple paternity, and overproduction of young with varying levels of genetic fitness, may function to promote competition within the litter to select the fittest individuals (Stockley and Macdonald 1998).

**Genetic diversity and bet-hedging**

It has been suggested that where criteria for choosing a high quality or genetically compatible mate do not exist, or are unreliable, females may mate multiply in order to
increase the genetic diversity of the litter *per se*, and thereby hedge their bets (Watson 1991). This may be advantageous for females if there were perceptual errors in the assessment of mates, such that mate choice was fallible. Secondly, if there were temporal fluctuations in the environment, producing a genetically diverse brood may mean that at least some of them will be able to survive under the future selective environment. However, theoretical models predict that the selective advantage of such a strategy for females may only be optimal under a limited set of conditions (Hosken and Blanckenhorn 1999; Yasui 2001). A random mating strategy may only be favoured in an unpredictable environment where the most fit male genotype is variable, the cost of female re-mating is small and the population size is relatively small (< 400 females) (Yasui 2001). Even in an unpredictable environment, if there is a slight increase in cost, multiple mating is no longer advantageous above female population sizes of 100. There is little evidence that bet-hedging alone can explain multiple mating in natural populations (Jennions and Petrie 2000). The exception may be among insects, in which genetic variability within a colony may be advantageous to reduce competition, or to increase parasite resistance (Jennions and Petrie 2000; Hosken and Stockley 2003).

### 7.3.2 Possible benefits of multiple mating for female honey possums

Offspring production by female honey possums appears limited by the prevailing conditions. From year to year, the incidence of breeding is lower following years of low rainfall, an effect thought to be mediated through the availability of food. Although breeding can occur all year, the incidence of breeding within any particular year appears constrained by the flowering phenologies of foodplants and the availability of food in that year. The slow production rate of young appears congruent with nutritional limitations associated with an unusual diet, consisting solely of nectar and pollen (Wooller et al. 2000). Their small litter size cannot, therefore, be increased beyond the teat number of four. Females are iteroparous, and continuous reproduction through embryonic diapause may increase a given female’s lifetime reproductive output. Nevertheless, lifetime production of young by each female may still be relatively small compared to other species of small marsupial and eutherian
mammal (Section 6.4.3). Each set of young, therefore, represents a large proportion of lifetime reproductive effort (25% or greater). Multiple paternity probably represents a strategy by which females can improve the genetic quality of their limited number of offspring. This would thereby minimize the risk that offspring will be lost, and maximize offspring survival and subsequent reproduction.

Although, at present, the exact method by which female honey possums may increase the genetic quality of their offspring through multiple mating can not be determined, several features of their life-history and reproduction lend themselves more to some explanations than others. Females would be capable of exerting a preference for mates, since they are behaviourally dominant. However, investment in male body size appears limited and males do not appear to compete overtly for mates, and have no ornaments or armaments upon which mate choice might be based. Mate choice based on size is a possibility, as indicated by preliminary trends in paternity discussed above, although there are several other explanations for this apart from mate choice. The grey mouse lemur, lacks sexual dimorphism, but it was suggested that females may use olfactory cues to choose mates (Andres et al. 2001). Such a preference may exist in the honey possum, but it seems unlikely that choice of reproductive males is a limiting factor for females. Therefore ‘trading up’ would not adequately explain multiple mating in the honey possum.

Variation probably exists between honey possum males in their sperm competition abilities, as in other taxa (Ginsberg and Huck 1989). Indeed, in 41% of litters, individual males sired several of the young and were therefore more successful than other sires. If success was linked to intrinsic quality (eg. Simmons and Kotiaho 2002), then females may pass benefits onto both male and female offspring. The evidence for the sexy sperm hypothesis appears limited for other species (see above), but remains a possibility. In the honey possum, there is clear potential for a role in paternity biasing through differences in male sperm competition ability, although this may be mediated through some selection of sperm or conceptuses.
It is of interest that female honey possums show reproductive amortization (Chapter 6). Offspring that survive this process may do so because they are of greater genetic quality. Overproduction of young occurred prior to birth, with all females examined producing between 5 and 8 ova. This is despite having only four teats, to which the young must attach permanently for the first few weeks of life (Russell 1982). Clearly, the maximal post-partum litter size is four. Overproduction of ova cannot therefore, be explained by ‘resource tracking’ hypotheses (Temme and Charnov 1987; Kozlowski and Stearns 1989), in which the number of offspring before birth is facultatively reduced according to the prevailing conditions. Early reduction occurred between ovulation and development to the blastocyst stage, with an average loss of 13%. Attrition may occur soon after fertilization, since collapsed fertilized conceptuses were observed in the oviduct and in most cases this collapse was thought to be natural. Intrauterine variation in the diameters of blastocysts was also apparent. Loss also occurred between the blastocyst stage and the neonate stage, with an average loss of 34%. Total average loss of potential offspring between ovulation and the neonate stage was 42%.

Theoretically, selection for overproduction of conceptuses can occur when there are fitness differences between the offspring that can be identified by the mother in the early stages of development (Stearns 1987; Kozlowski and Stearns 1989). Such ‘selection arenas’ allow the mother to abandon those offspring that are likely to have a lower relative fitness. Differences in fitness, in both polyandrous and monandrous species, can arise due to chromosomal abnormalities, developmental instabilities, genetic incompatibilities, disease resistance or sex (Stearns 1987). Overproduction of ova occurs in several other species of small possum and in many species of dasyurid (see Section 6.4.3 and Ward 1998; Taggart et al. 2003). Supernumerary young have been noted in several species of small marsupial, and indeed may be common (Whitford et al. 1982; Mansberg and Broome 1994; Strahan 1995). Therefore, production of more conceptuses than can later attach to the teats, may not purely be explained by selection via multiple mating. Multiple mating, however, provides an opportunity to discriminate between a variety of paternal genotypes and
to choose the most superior. Indeed, Stockley (2003) found that amongst polytocos mammal species, polyandrous taxa had a lower rate of reproductive loss than monandrous taxa. However, litter size did not differ. Monandrous taxa produced a greater number of embryos, presumably to compensate for greater loss (Stockley 2003). In the honey possum, since females mate with multiple males, overproduction of ova or conceptuses followed by selective abortion, may provide a filter to ensure that young are fathered by intrinsically viable males, and/or genetically compatible males. This would increase the likelihood of giving birth to a complete litter of four young and decrease the risk of loss after birth.

At this stage it is only possible to speculate on the basis upon which conceptus fitness or paternal traits may be chosen in the honey possum. Possible mechanisms whereby genetic incompatibilities can arise seem abundant in mammals (see above and Zeh and Zeh 1996, 1997; Jennions and Petrie 2000; Tregenza and Wedell 2000; Zeh and Zeh 2001, 2003). Inbreeding was considered unlikely in the honey possum population given the high heterozygosity, low levels of relatedness (Section 5.4.3) and evidence that juvenile males disperse (Section 4.2.3). However, Tregenza and Wedell (2000) note that populations with a low risk of inbreeding are more likely to evolve mechanisms to avoid it. In addition to inbreeding avoidance, there is also an increasing number of studies showing paternity biasing based on genetic similarity (Stockley 1999; Foerster et al. 2003). Mechanisms of conceptus failure can occur through genomic imprinting or MHC incompatibility, and may represent selective abortion.

Choice based on immunological dissimilarity may also lead to more viable offspring through parasite resistance, as discussed above. Also of note are studies showing the increased fitness of individuals with higher heterozygosity or genetic diversity (Coltman et al. 1998; Amos 2001; Hansson et al. 2001; Hansson and Westerberg 2002). This may explain the very high levels of heterozygosity (>90%) seen in the honey possum. In the FRNP, honey possums carry ticks, and occasionally some orange mites. However, immediately prior to the population crash in late spring
2000, the honey possums carried the highest loads of mites seen in the present study. Some individuals were carrying heavier loads than others, in some cases all over the cloaca, pouch opening or scrotum. This occurred at the end of a year when little food was available due to low rainfall. Individuals with increased parasite resistance may indeed be better able to tolerate poor conditions during food shortage, and to survive until flowering and food levels increase again. It is possible that conceptus selection arenas operate to choose an optimal level of parasite resistance.

Other mechanisms of female choice of conceptuses for overall viability, as opposed to just compatibility, are less apparent. Perhaps those conceptuses that can attain a greater share of maternal nutrition in the uterus ‘outcompete’ other conceptuses. This may be especially pertinent to the honey possum if the nutritional plane of the mother is compromised during times of little flowering. Sperm selection may occur on the basis of surface proteins and there is evidence that it does occur in animal taxa (Olsson et al. 1996, 1997; Zeh and Zeh 1997; Stockley 1999). Stearns (1987) suggested that, since sperm may be influenced by paternal characteristics, it may be more reliable to select conceptuses because once the sperm and ovum nuclei have fused, complementarity can be assessed in the diploid state of the offspring.

Interestingly, Zeh and Zeh (2001) point out that polyandry creates post-copulatory conditions that promote conflict between rival paternal genomes, and this may itself lead to a greater incidence of genetic incompatibilities and failed conceptuses. In a monogamous mating system, the reproductive interests of males and females coincide, whereas when females mate with more than one male, the reproductive interests of the sexes can be in conflict (Zeh and Zeh 2003). Male responses to female choice may lead to evolution of an aggressive paternal genome, and it has been argued that multiple mating is a prerequisite for the evolution of genomic imprinting (Zeh and Zeh 2001). Polyandry may evolve for other types of incompatibility avoidance and for promoting overall quality. Once evolved, polyandry may become a self-perpetuating process that fuels further incompatibility. Perhaps
sperm competition in the honey possum promotes fertilization by sperm conferring ‘good genes’ advantages, but overproduction of conceptuses and their subsequent reduction, allows screening for genetic incompatibility and ‘worst genes’.

Reproductive amortization in the honey possum occurred from ovulation to weaning, with an average of 62% loss. Although possibly only the largest females wean four young, some are definitely capable of doing so. Brood reduction from four young on four teats does not constitute overproduction at birth. Post-partum selection arenas, like that described for the common shrew (Stockley and Macdonald 1998; Hosken and Stockley 2003), could not solely explain brood reduction in the honey possum, but it is possible that those that do not survive to weaning are the least fit. Since females carry blastocysts and pouch-young concurrently, paternity assignment could be used to look for a difference in the distribution of paternity between the blastocysts in utero and the young in the pouch. Given that each set of young is a large proportion of a female’s lifetime reproductive effort, pre-partum amortization for quality/compatibility, followed by facultative brood reduction according to the available resources and a female’s condition, may provide an optimal strategy to ensure offspring quality and maximal numbers.

The fluctuating environment in which the honey possum lives, superficially lends itself to hypotheses of random mating for diversity per se, as a means of bet-hedging. This implies, however, that advantageous traits would change from year to year (Yasui 2001). One possibility is that if the favoured genotype for parasite resistance varied from year to year, multiple mating by females would increase diversity amongst the litter and improve the likelihood of some of the offspring having the preferred genotype. In addition, depending on the similarity of each of the males to the female, some offspring may be of greater heterozygosity, conferring greater resistance to a broader range of diseases. However, in a bet-hedging strategy, the advantage arises purely by chance. Deterministic mechanisms such as sperm competition, or female choice of compatible or dissimilar sperm or conceptuses, are explained by the sexy sperm, good genes or genetic incompatibility hypotheses.
(Yasui 2001). Given the testes size of male honey possums and the overproduction of ova that provides an opportunity for female choice, it is likely that such a deterministic mechanism does occur, and that bet-hedging alone does not maintain multiple mating in this species.

### 7.4 Genetic diversity and multiple paternity in the honey possum

The honey possum populations in the FRNP had a high allelic diversity and heterozygosity (Chapter 5). This genetic diversity may provide the basis for a multiple mating strategy, if it reflects variation in male quality, or it may be a consequence of it. Petrie et al. (1998) suggested that in birds that facultatively seek extra-pair matings, an increased tendency toward extra-pair matings would be expected under a good genes hypothesis, when variation in male quality is higher and opportunities exist for females to ‘trade up’. This hypothesis was supported by findings that amongst bird species there is a positive correlation between extra-pair paternities and levels of polymorphic allozyme and RAPD (randomly amplified polymorphic DNA) loci (Petrie et al. 1998).

However, genetic variation is also an outcome of multiple mating. Mating with several different males increases the allelic diversity within a litter and, if genetically compatible mates are more dissimilar, then individual heterozygosity of the offspring may also be higher. Heterozygosity would also be high in the population as a result of allelic diversity between mating individuals. Regardless of whether females mate multiply for good genes, inbreeding avoidance or genetic compatibility, the outcome of multiple paternity is increased genetic diversity. The agile antechinus has high levels of genetic diversity at microsatellite loci as well as high levels of multiple paternity (discussed in section 5.4.1; Kraaijeveld-Smit et al. 2002). Multiple mating in pseudoscorpions is also coincident with 95-99% heterozygosity at minisatellite loci (Zeh et al. 1998).
Multiple paternity increases the effective population size considerably, in some cases up to double that resulting from polygyny and monogamy (Sugg and Chesser 1994; Chesser and Baker 1996). In this way, multiple mating preserves greater genetic diversity and increases the potential for a species to adjust to environmental change and avoid inbreeding in natural populations (Sugg and Chesser 1994). This may be a significant factor in the survival of honey possum populations. This assumes that microsatellite variation, like that assessed in this study, reflects genome-wide variation, including that at loci for fitness traits, for which evidence is uncertain (Hansson et al. 2001; Hansson and Westerberg 2002; Sherwin et al. 2003). The ability of this species to breed its way out of low density times may also underlie the success of the honey possum in surviving in such a fluctuating environment, and is reassuring for its long-term survival.

7.5 Synthesis of the mating system

In populations of the tiny marsupial honey possum in the FRNP, breeding can occur throughout the year, and sexually active males are present all year round. Embryonic diapause, slow growth of young and asynchrony of female oestrus, would lead to a skewed operational sex ratio, with more males than oestrous females.

The incidence of female multiple mating was very high. Between 86 and 95% of litters exhibited multiple paternity, depending on the method used to determine it. The true value is likely to be close to 95%. The estimated number of sires within a litter was often three or four. The risk and intensity of sperm competition would therefore be high. Male honey possums have much larger testes than expected for their body weight (4.2% of body weight) and the longest sperm known for any mammal (356µm). Studies of a diverse range of species indicate that large testes evolve in response to selection through sperm competition, and long sperm is likely to be adaptive in sperm competition. Presumably these traits in the honey possum also reflect sexual selection for a large investment in spermatogenesis. Adult males
are significantly smaller than adult females, and possess no ornament or armament, and it is unlikely that they fight overtly for access to females. Since females undergo oestrus unpredictably over time, and are spatially dispersed, males are most likely to compete in their ability to locate females and monitor their reproductive status. The mating system is consistent with sperm-based scramble competition.

Offspring production by female honey possums appears limited by their unusual diet, consisting solely of nectar and pollen, and each set of 2-4 young represents at least 25% of lifetime reproductive output. A multiple mating strategy by females presumably allows the genetic quality of their offspring to be maximized. Males that succeed in the ensuing sperm competition may be of greater intrinsic quality. Overproduction of conceptuses by females presents the opportunity for them to select conceptuses fertilized by intrinsically viable males, or genetically compatible males. This may maximize the chances of giving birth to a full set of four young and minimize their loss after birth.

Honey possums live essentially solitary lives, but are tolerant of each other and the home ranges of both sexes overlap. Although there is no direct evidence that males mate with several females, their lack of provision of parental care and the asynchrony of female breeding, would allow them to do so. Females carrying young are intolerant of males and there is no social structure to suggest that females monopolize certain males. The mating system is thus likely to be promiscuous.

Changes in annual rainfall lead to changes in the flowering resources available, in turn leading to population density fluctuations. However, this did not affect the mating system of the honey possum. Promiscuous mating is probably maintained, during times of low population density, because the number of individuals present during such times was still sufficient that several mates of the opposite sex would be available. The behavioural dominance of females suggests that multiple mating is an active strategy pursued by females.
References


