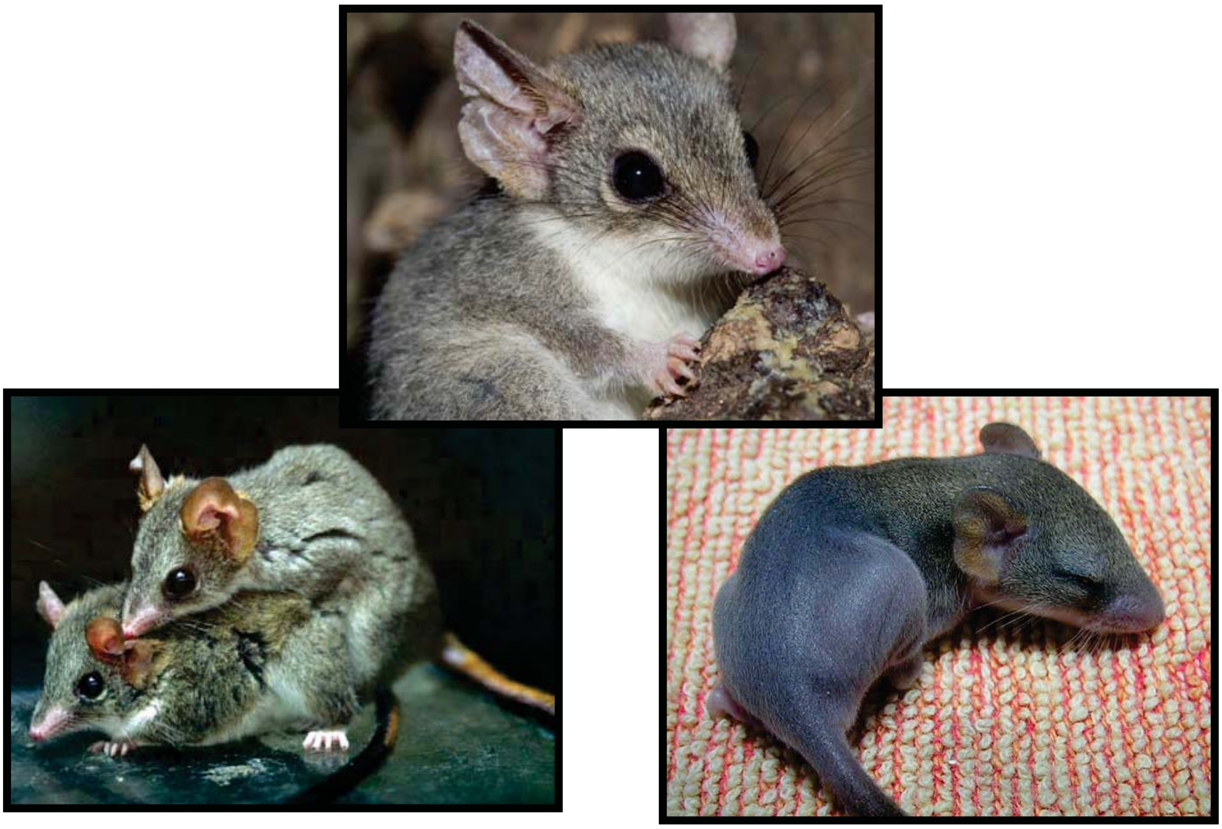
Reproductive strategies of the red-tailed phascogale (*Phascogale calura)*



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Who’s Your Daddy?

Over the last year, there has been some action happening quietly behind the doors of the Animal Health and Research Centre, and I’m not referring to the treatment of sick animals.

I’m talking about sex, and lots of it! This is the six-hour continuous lovemaking that has you hanging from the roof kind of sex. The new partner every day kind of sex. The make love til you drop kind of sex.

What! I hear you exclaim. That’s outrageous!

Well before you get too outraged maybe I should clarify a couple of things. The individuals involved in this rampant love-making are an endangered carnivorous marsupial known as the red-tailed phascogale. This species was once found over large regions of Australia, but is now only found in south-west WA, where they are rarely seen due to their small size and their tree-top antics occurring at night.

As I alluded to above, their sex-life is not for the fainthearted. They only breed once a year with mating occurring over a few weeks in winter – maybe close body contact makes cold days easier to bear. Both males and females have multiple partners, which can result in a litter of young having multiple fathers – so it really is a question of ‘who’s your daddy?’

At the end of those several weeks of frenzied sex, the males escape any parental duties by dying. Ah, the life of a male – live off mum for a quarter of your life, go out wandering the world for half your life while building up those muscles, then pick fights with any other bloke you meet, chase the girls and have sex as much as possible for the last quarter.

Meanwhile, mum is left with eight babies to raise without a single child support payment. Luckily the young are born smaller than a tic-tac, which makes giving birth easier. Of the fourteen or so young born, only eight manage to find a teat – for the others it is just bad luck. Mum carts the kids around continuously for about seven weeks, then decides they are big enough to stay in the nest while she goes ‘out on the tree’. Another seven weeks later and the kids are finally ready to leave home, having cleaned out mum’s pantry and strained her sanity. Finally if she’s survived all that she gets a bit of peace and quiet before jumping on the breeding roundabout again for a second year.

These sexy little creatures are the focus of my PhD project, which I’m doing with the assistance of Adelaide Zoo and Alice Springs Desert Park. By learning more about their sex-life, we hope to be able to improve the management of phascogales in captivity with the plan to release them back into some of their former range.

Though there are a lot of cute animals in the zoo, I happen to think the red-tailed phascogales are the zoo’s sexiest animals. What about you?

In 'South Australia's Zoo Times'. pp. 14.

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Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Wendy Foster
31st March 2008

Acknowledgements

It’s hard to know where to start as so many people have played a role in getting to this point. So I’ll start with my supervisors, Tags and Steve. Thanks for giving me the freedom to take this project wherever the fancy took me and for allowing me to do things in my own way at my own pace. Tags, thanks for your unwavering belief in me and your encouragement when I struggled to get through the day, let alone get much done on the PhD. Thanks also for sharing your knowledge, your stories and your passion for science and conservation. Steve, thanks for sharing your knowledge on a wide range of topics and for your thoughtful advice.

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Conference presentations

Foster WK, Taggart DA, Donnellan S (2005) Reproductive biology of the red-tailed phascogale (*Phascogale calura*). 'Australian Mammal Society Annual Meeting'. (Albany, Western Australia)

Foster WK, Taggart DA, Donnellan S (2006) Who's your daddy? Multiple paternity in the red-tailed phascogale. 'Australian Mammal Society Annual Meeting'. (University of Melbourne, Victoria)

Foster WK, Taggart DA, Donnellan S (2007) Captive breeding with multiple paternity: a case study using the red-tailed phascogale, *Phascogale calura*. 'Biodiversity Extinction Crisis Conference - a Pacific Response'. (Sydney University, New South Wales)

Foster WK, Taggart DA, Donnellan S (2007) How many babies, which sex and who's their daddy? Fecundity, prenatal sex ratio and paternity in the red-tailed phascogale. 'Australian Mammal Society Annual Meeting'. (University of New England, New South Wales)

Foster WK, Taggart DA, Donnellan S (2007) Mating strategies of the red-tailed phascogale. 'Parasites, Conservation, and Evolutionary Ecology; Connecting some Disparate Threads'. (Flinders University, Adelaide, South Australia)

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Additional manuscripts

Bradley AJ, Foster WK, Taggart DA (2008) Red-tailed phascogale. In 'Mammals of Australia'. (Eds S Van Dyck and R Strahan). New Holland: Sydney

Foster WK (2005) 'Who's your Daddy?" In 'South Australia's Zoo Times'. pp. 14.

Foster WK, Andrews L (2007) 'Proposal for translocation of red-tailed phascogales from Alice Springs Desert Park to Warraweena Conservation Park.' Royal Zoological Society of South Australia, Adelaide.

Awards

2008 Research Award from the Australasian Regional Association of Zoological Parks and Aquaria

Runner-up best overall presentation at the Australasian Section of the Society for Conservation Biology Meeting 2007, Biodiversity Extinction Crisis – a Pacific Response

Abstract

This thesis examines the reproductive biology of red-tailed phascogales, an obligate male semelparous dasyurid species, which is part of a captive breeding colony at Alice Springs Desert Park. The red-tailed phascogale belongs to a group of dasyurids that shows an unusual reproductive strategy amongst mammals, one which provides opportunity for understanding means by which individuals maximise their reproductive success and the role of sperm competition. The broad aim was to gain an understanding of the reproductive biology of red-tailed phascogales and explore means by which individuals can affect their reproductive success.

Examination of the red-tailed phascogale reproductive biology showed that females mated with multiple males and were capable of storing sperm in their oviducts for at least a five day period. Captive female red-tailed phascogales showed greater plasticity in their breeding season than has been observed in *Antechinus,* which exhibits the same life history strategy. Females were observed to invest heavily into the production of young, producing almost twice as many ova (15.1 ± 1.9) as young they can raise and 76% of females filling six to eight of the eight available teats in a breeding attempt. A 63% male bias was observed in young attaching to the teats, which could be produced through differential attachment of the sexes to teats at birth. Of the 846 young born in the captive breeding colony, 68% were weaned, with weaning occurring between 90-110 days of age and a 53% female bias observed in young being weaned.

By weaning, a litter of young weighed 380% of the mothers mass with male young tending to be heavier than females by weaning. No relationship was observed between maternal weight and either litter sex ratio or sex biased growth of young. A positive relationship between maternal body mass and body mass of offspring at weaning was observed, with the body mass of young at weaning correlated with its body mass at maturity. Multiple paternity was observed in more than half of the litters examined, with heavier males having increased siring success compared to lighter males. Genotyping showed that the effective population size for the captive colony was 1.9x that observed from the group managed studbook.

Male reproduction was also not as tightly constrained as in *Antechinus,* with spermatogenic failure not occurring in captive populations until after mating had occurred, meaning males are not reliant on epididymal stores alone for successful breeding. Scrotal diameter showed a positive relationship with testis and epididymal mass across male life, although this relationship was not evident when analysis was restricted to the time of peak sperm production. Captive males showed the opposite pattern of testosterone fluctuations to that observed in wild animals, with lowest levels occurring during the mating period. Captive animals were able to survive up to five years in captivity, in contrast to the obligate semelparity observed in wild males.

Although most captive females can survive to breed in a second year and females are known to breed in a second year in the wild, the reproductive strategy of females appears to be aimed at maximising the returns on their first breeding attempt. In males, the need to maximise the investment into the first breeding season is amplified through the complete absence of opportunity to breed again; either through post-mating mortality in the wild or spermatogenic failure in captivity.

The results of this study have implications for captive breeding of red-tailed phascogales, with their reproductive biology; spermatogenic failure, restricted breeding season, teat number limiting the number of young raised, high lactational investment into young, sex biases, the need to maintain genetic diversity and biases in siring success; providing challenges for the maintenance of a captive population. The results of this study also provide comparative information that contributes to understanding the unusual life history strategy of *Phascogale* and *Antechinus,* and contributes to the growing body of knowledge about mating strategies in marsupials.

Introduction

The paradox of sex

The fitness of an organism is not measured by how far they can run, how high they can jump, or how long they live for, but rather how many offspring survive and reproduce. Offspring can be produced by sexual or asexual means, but in comparison to asexual reproduction, sexual reproduction is very complex and has some distinct disadvantages. For example, the recombination of genes during sexual reproduction can break up allelic combinations that have proven successful by the fact the individuals have survived to reproduce (Maynard-Smith 1978; Williams 1975). There is also a two-fold cost of sex, where both males and females are needed to produce young, in contrast to the single individual required for asexual reproduction (Maynard-Smith 1978). In addition there is the time required for mate acquisition and sexual reproduction, the increased predation risk and the risk of sexually transmitted diseases (Otto and Lenormand 2002)

Based on reproductive effort alone, an individual that reproduces asexually within a sexual population would be expected to rapidly increase its numbers. Despite this, sexual reproduction is the dominant form of reproduction and this situation is known as the Paradox of Sex (Maynard-Smith 1998). Sexual reproduction has long-term benefits with an increased rate of adaptation to environmental variation. The variety of genotypes in sexual populations may result in individuals that have genetic combinations which enable them to survive the environmental change (Williams 1975). So although sexual populations may show a short-term decline as non-adapted individuals die out, sexual populations can potentially remain in situations where asexual populations are lost.

Sex has been proposed to be advantageous in situations where there are changes in a species environment or interacting species e.g. predators, prey, parasites, competitors (Maynard-Smith 1978). Sexual reproduction allows for widespread incorporation of new mutations into the gene pool, some of which may have advantages in new conditions. In asexual organisms, a beneficial mutation can only be incorporated into a line via the mutation occurring within the lineage. On the other hand, sexual reproduction allows for the genetic material from two lines to be combined, allowing the mutation to spread through multiple lines (Williams 1975). Asexual species also have no means for eliminating mutations, except by reversal mutations, so all offspring will have a mutation load that is at least the size of the parents (Muller 1932).

In contrast, sexual reproduction can dilute mutations as offspring only receive half of each parent's genetic material, thereby potentially only half of the line will carry the mutation into the future (Kondrashov 1988).

Changes that occur in the competitiveness of organisms as they evolve and adapt can impact on the survival of other organisms of the same or different species. The need for continual change in order to survive in a changing world has been called the Red Queen effect (Van Valen 1973), after Carroll's (1927) character in Alice in Wonderland. The Red Queen effect has been described between parasites and hosts, predators and prey, and between males and females (Ridley 1993). Parasites and pathogens have been proposed to be a driving force for sexual reproduction, with the short cycle of parasites enabling them to rapidly adapt to exploit their slow changing hosts. By utilising sexual reproduction, hosts can create new combinations of genes in their offspring that parasites are less adapted to attacking, improving the chances of those offspring surviving.

Reproductive investment

Despite the benefits available to offspring from sexual reproduction, male and female fitness is not necessarily maximised by the same mechanism (Parker 1979), with the optimal mechanism varying depending on ecological and physiological circumstances. Differences in the best outcome for the two sexes can lead to each sex evolving antagonistic adaptations that bias the outcome to their own interests while minimising the costs from the conflicting interests of the other sex (Chapman, Arnqvist *et al.* 2003). The unequal investment into gametes, whereby females produce large, relatively immobile gametes while males produce numerous, small motile gametes, is thought to be the basis for intersexual conflict (Bateman 1948). The chance of an individual young surviving can be improved by increased parental investment into protection and provisioning offspring. This increased investment takes resources from future reproductive endeavours, so individuals can try to manipulate their partner to increase their contribution thereby increasing the individual's fitness without increasing investment.

Limitations on female reproduction

The absolute upper limit of a female's reproductive potential is set by the number of ova ovulated. Rarely does every ova result in an offspring though as not all ova are fertilised, and meiotic errors and problems during fertilisation, such as genetic incompatibility, can result in embryo loss during early cell division (Zeh and Zeh 1996). Hence some degree of overproduction of ova compared to number of young raised will be seen. Where females increase their investment into young beyond ova production, through measures such as gestation, incubation or lactation, this limits the reproductive potential of females by the availability of resources to be invested into young (Fisher 1930). As such females are likely to be operating near their physiological limits of offspring production, with relatively low variance in reproductive potential (Daly and Wilson 1983). With compromises inevitably occurring between evolutionary potential and the constraints on that potential, e.g. through limited environmental resource availability, a range of life histories are seen across species, each adapting to particular conditions in their own way (Barnes and Partridge 2003; Stearns 1976).

Limitations on male reproduction

Males are also limited in their reproductive potential, although where males do not provide care for their young this is more so through the availability of fertile mates and the presence of competing males (Daly and Wilson 1983). Males must balance conflicting priorities in order to reproduce; the cost of finding and securing additional mates must be balanced against the siring potential of the current partner. Competition with other males can lead to increased mortality and males may have an increased predation risk due to showy sexual signals that make them more conspicuous to predators (Brooks 1999). Even testosterone, which can aid mate acquisition and mating behaviour, can result in decreased lifespan (Bradley, McDonald *et al.* 1980; Daly and Wilson 1983).

Males also need to balance the non-trivial costs of sperm production and mate acquisition against the likelihood of siring success. Sperm production must be balanced against the risk of running out of sperm, and declining fertility associated with the long-term epididymal storage of sperm (Taggart and Temple-Smith 1990; Wedell, Gage *et al.* 2002). Siring potential of the current partner may be affected by female age or size, the time required for sperm transfer, the number of sperm transferred, the timing of mating in relation to ovulation and whether the male provides any parental care (Wedell, Gage *et al.* 2002). Other males mating with that female, either previously or in the future, may also affect siring potential. This can lead to males guarding females and/or territories from other males, which impairs the female's ability to obtain other mates (Dewsbury 1982). Males' mating strategies must balance the likelihood of survival to future breeding opportunities with the likelihood of acquiring mates and siring young in the current season.

Investment in offspring

Fisher (1930) recognised that over their lifetime, parental investment into males and females must be equal, but that equal investment does not always equal the same number of males and females. Where the cost of producing one sex is greater, an uneven sex ratio would result, with more of the less costly sex produced. The ratio of sexually active males to females at a given time, or operational sex ratio, is a predictor of which sex competes for mates and how intense the competition is (Kvarnemo and Ahnesjö 1996). The operational sex ratio does not necessarily equal the number of males and females in the population though, as one sex may spend more time on a single reproductive event, while the other sex is free to pursue other mating opportunities.

Within polygynous species where there is large variation in male reproductive success, Trivers and Willard (1973) suggested that better ‘quality’ mothers would have a male bias in litters, while lower ‘quality’ mothers produce more females. Better quality mothers could potentially provide offspring with an advantage that is sustained through life, resulting in more successful competition for mates and an enhanced reproductive potential. Poor quality females may produce sons with a lowered competitive ability and hence they may not acquire mates, whereas the majority of females that survive would have the opportunity to reproduce. Different fitness returns from males and females is especially important when fecundity is low and there is extended parental investment (Godfray and Werren 1996).

Given the chromosomal sex determination of birds and mammals, a 50:50 sex ratio would be expected based on chromosome separation at meiosis. Various mechanisms have been proposed by which alterations to sex ratios may occur. Pre-natal biases in sex ratio may occur as the result of alterations in the proportion of sperm genotypes that reach ova. These biases may occur as a result of ejaculate biases, variations in female hormonal levels and changes in pH and mucus constitution within the female reproductive tract which can affect sperm motility (Krackow 1995). After fertilisation, hormonal variation has been proposed to have effects on sex specific embryonic mortality, with higher progesterone levels, which are affected by nutrition, favouring males (Krackow 1995). After birth, differential investment into young, can also alter the sex ratio (Cockburn 1990).

Polyandry

Promiscuity in males, or polygyny, is commonplace in the animal world and the benefits for males are relatively clear, as gamete production and investment into individual young generally are not limiting factors. For females promiscuity, or polyandry, has been found to be more common than once thought, although its advantages are less clear (Birkhead and Møller 1998; Gomendio and Roldan 1993b). Extra matings are energetically costly, take time away from other maintenance activities, may increase predation and injury risk, and also increase the risk of disease and parasite transmission (Stockley 1997). The widespread occurrence of polyandry suggests that females are offsetting these costs through acquiring advantages from multiple copulations beyond fertilisation assurance, since a single mating is generally sufficient to ensure fertilisation (Reynolds 1996; Wittenberger 1979; Zeh and Zeh 2001).

For females, both direct and genetic benefits have been proposed for multiple matings. Direct benefits may include nuptial gifts, paternal care from multiple males, stimulation of ovulation, ensuring adequate sperm supplies, decreased harassment from males when resisting is costly, and potential for mate assessment (Reynolds 1996). Genetic benefits may arise through a choice of paternity, mixed paternity, decreasing inbreeding, avoidance of genetic incompatibility, selection of more competitive sperm, avoiding defects from stored/old sperm or 'sexy sons' (Keller and Reeve 1995; Kempenaers, Verheyen *et al.* 1992; Loman, Madsen *et al.* 1988; Madsen, Shine *et al.* 1992; Stockley, Searle *et al.* 1993). The influence of each of these benefits is not clear and it is often difficult to disentangle their individual roles.

Although multiple matings may be costly, the cost of a second mating is small compared to the total cost of reproducing sexually rather than asexually. Multiple partners can potentially amplify the benefits of sexual reproduction with a cost that is small compared to the total reproductive cost (Yasui 1998). Lifetime monogamy could arise in situations where the benefits of a single partner are great, such as when both parents care for young, or when the cost of securing another mate is much higher. This view suggests that a promiscuous mating system is the norm, with other mating systems applying only when other factors affect the cost-benefit ratio of reproduction.

Sperm competition

Where females mate with more than one male during a receptive period, sperm from these males compete to fertilise the ova (Parker 1970). Sperm competition can be intense as males inseminate vast numbers of sperm, all of which are competing, both with other sperm within a single male's ejaculate and with sperm from other males, for fertilisation of a limited number of eggs. Despite large numbers of sperm being ejaculated in most mammals, often more than 10-100 million sperm for each ova, only a small proportion of sperm reach the site of fertilisation (Bedford, Rodger *et al.* 1984).

In species where sperm competition occurs, males tend to have relatively larger testes comprised of a greater proportion of spermatogenic tissue, suggesting a large investment into sperm production (Kenagy and Trombulak 1986; Rose, Nevison *et al.* 1996). Males may try to improve the likelihood of fertilising ova through increased sperm numbers, larger sperm size or differences in sperm morphological characteristics that improve the likelihood of fertilisation (Baker and Bellis 1988; Gomendio and Roldan 1991; Møller 1989). Males are likely to benefit from adjusting sperm numbers relative to the likelihood of successful fertilisation (Parker 1990a; Parker 1990b), with different methods utilised for monogamous and promiscuous species (Pound 1999).

Siring success may be affected by mating order, the delay between matings, the number and duration of matings and the timing of mating in relation to ovulation (Gomendio and Roldan 1993b). Variation in ejaculate size has been noted in relation to the presence of rival males, female mating status and female quality across a variety of species (for review see Wedell, Gage *et al.* 2002). From a female perspective, decreases in ejaculate volume and lowered sperm numbers due to previous matings may compromise the female's fertilisation rate due to sperm limitation (Warner, Shapiro *et al.* 1995).

Sperm choice

Mating with multiple males provides opportunity for females to influence paternity based on characteristics of both males and their ejaculates (Eberhard 1998). These characteristics may include male physical characteristics that affect mating opportunity and duration (Darwin 1859), major histocompatibility complex and pheromonal cues (Brown 1996; Neff and Pitcher 2004; Tregenza and Wedell 2000) and mating behaviour (Dewsbury 1972). Ejaculate characteristics include seminal products which affect female receptivity (Dziuk 1996;

Eberhard and Cordero 1995), sperm motility and their ability to transverse the female reproductive tract (Birkhead and Møller 1993), fertility and the numbers of sperm transferred (Gomendio and Roldan 1993a).

Which male sires offspring has consequences for female fitness, so females are unlikely to be passive receptacles for male gametes and those females that select better quality males will be favoured by natural selection. A wide range of responses are needed from females in order to produce offspring, from ovulating, refraining from digesting sperm and aborting embryos, to committing resources to offspring. This multiplicity of processes makes it unlikely that females will lose ultimate control of paternity of offspring (Eberhard 1998). As sperm from males of internally fertilising species have to negotiate the female tract, the opportunity arises for females to influence paternity to their advantage (Birkhead and Møller 1993).

The ability of females to affect paternity after the initiation of copulation has only been recognised recently (Birkhead and Møller 1993; Eberhard 1998). Female influences may include not allowing ejaculation, ejecting sperm, mating with another male, or altering ovulation to favour certain matings. Female sperm choice may arise incidentally due to physiological characteristics that originally had survival value also being exploited as a selective factor. For example, low pH of the vagina reduces microbial infections and males that produce semen that can survive in lower pH can favour a further reduction in female pH, with this then acting as a screening mechanism for males (Eberhard 1998). Disentangling female sperm choice and sperm competition can be difficult as the process is hard to observe and observations can be limited by a priori assumptions about competition and choice (Birkhead and Møller 1993; Birkhead 1998; Eberhard 1998; Kempenaers, Foerster *et al.* 2000; Pitnick and Brown 2000).

Multiple paternity

Where females have more than one young in a breeding attempt, sperm competition may be confirmed by the presence of more than one sire within a litter, although its absence does not exclude the possibility of sperm competition. Multiple paternity may arise as a side effect of sperm competition, or as a benefit that has driven females to mate with multiple partners (Loebel, Nurthen *et al.* 1992; Loman, Madsen *et al.* 1988; Madsen, Shine *et al.* 1992; Parker 1970).

Multiple paternity has been proposed to be associated with a strategy to reduce inbreeding (Stockley, Searle *et al.* 1993). Although meiosis and recombination within one individual can produce considerable variation, this is only a subset of the diversity available from multiple matings, as other males may have completely different alleles, allowing for access to a more diverse genetic pool (Yasui 1998). Genetic diversity alone isn't a benefit though unless it provides a fitness advantage to the female (Fisher, Double *et al.* 2006a; Newcomer, Zeh *et al.* 1999). Species that produce relatively few offspring throughout their lifespan, or only have a single reproductive attempt, have been suggested to improve their lifetime reproductive success with increased offspring genetic diversity (Wooller, Richardson *et al.* 2000).

Male semelparous dasyurids – strategy I life history

Amongst marsupials, one group of dasyurids or carnivorous marsupials, show a unique life history strategy amongst mammals. The reproductive strategy of these species is characterised by being monestrous, with a short, generally synchronised, annual mating period, at the end of which there is complete mortality of the males (Lee, Woolley *et al.* 1982). Just prior to die-off, males are found to have high cortisol levels (Bradley 1987; Bradley, McDonald *et al.* 1980), which although it provides short-term advantages through the mobilisation of energy, continued high levels lead to immune suppression and negative nitrogen balance (Bradley 2003). Male die-off has been associated with negative nitrogen balance, anaemia, gastro-intestinal haemorrhage and changes in renal function (Barker, Beveridge *et al.* 1978; Bradley 1987; Bradley 1990; Bradley, McDonald *et al.* 1980; Cheal, Lee *et al.* 1976; McAllan, Roberts *et al.* 1998; Woollard 1971).

This obligatory post-mating male die-off after a single, brief mating period, or strategy I life history, is described as *semelparity* by Braithwaite and Lee (1979) and contrasts with *iteroparity* where males participate in one long, or several breeding seasons. Semelparity can be seen as an extreme form of investment into a single reproductive attempt to the detriment of all future breeding attempts. This obligate semelparity is seen in plants, invertebrates and fish, but is rare in mammals (Braithwaite and Lee 1979), where it is only seen in *Antechinus* (Wood 1970; Woolley 1966), *Phascogale* (Bradley 1997; Cuttle 1982) and *Dasykaluta rosamondae* (Woolley 1991a).

Facultative semelparity has been observed in several marsupial species, with male die-off occurring variably across populations and years in dibblers *Parantechinus apicalis* and northern quolls *Dasyurus hallucatus* (Braithwaite and Griffiths 1994; Dickman and Braithwaite 1992; Mills and Bencini 2000; Oakwood, Bradley *et al.* 2001; Woolley 1991b). To account for this variability in die-off, Mills and Bencini (2000) proposed that die-off is dependent on a variety of factors which includes population density, habitat quality, prey availability and sperm competition.

Evolution of the strategy I life history

With testicular failure occurring at the end of the males first breeding season (Kerr and Hedger 1983), a strategy I life history is proposed to be an irreversible adaptive trap as any males that survive to a second season are unable to reproduce (Braithwaite 1979). As phylogenetic studies place the New Guinean *Murexia* as a sister group to *Antechinus* (Armstrong, Krajewski *et al.* 1998; Kirsch, Krajewski *et al.* 1990; Krajewski, Buckley *et al.* 1996; Krajewski, Torunsky *et al.* 2007), this suggests either independent acquisition of strategy I in *Antechinus* and *Phascogale,* or a reversion in *Murexia*. If strategy I is not ancestral, then three separate evolutionary paths are likely for the development of obligatory post-mating male mortality (Krajewski, Woolley *et al.* 2000). How male semelparity evolved is not known, although several different hypotheses have been put forward.

Environmental predictability

Braithwaite and Lee (1979) hypothesised that low and variable adult survival and a reliable breeding season only long enough for one litter to be raised, may produce intense reproductive effort that occurs to the detriment of survival. This is broadly confirmed by *Phascogale tapoatafa* and *Antechinus* occurring in highly predictable, seasonal environments (Lee, Woolley *et al.* 1982). In these localities, late lactation coincides with the abundance of arthropods in spring (Lee, Bradley *et al.* 1977). Two other strategy I species are not always restricted to these environments though, with *Phascogale calura* and *D. rosamondae* having ranges that included semi-arid and arid regions, although *P. calura* is now restricted to the more seasonal south-west region of its range (Friend and Friend 1992; Woolley 1991a). This semi-arid and arid distribution of semelparous species indicates that strategy I species can survive in a wider range of environments than expected from the predictable environment hypothesis.

Female mortality

Kraaijeveld *et al.* (2003) argued that high female mortality during pregnancy and lactation would select for male promiscuity, as males that mated with multiple females would be more likely to have some young survive to breed. Where female mortality is high, male mortality would also be expected to be high, so only a single breeding season would be likely for most males (Lee, Woolley *et al.* 1982). Where the likelihood of surviving to a second season is low, males should maximise their investment into their first season even if it reduces survival prospects. Limited information is available on population densities and survivorship though.

Sperm competition

Dickman (1993) hypothesised that sperm competition is a driving force behind semelparity. Each male tries to maximise their likelihood of paternity in a promiscuous mating system, which has detrimental effects on their long-term survival. Both sexes mate with multiple partners (Scott and Tan 1985; Shimmin, Taggart *et al.* 2002) for up to 18 hours per mating (Taggart, Shimmin *et al.* 2003) during the several week mating period (Dickman 1993; Tyndale-Biscoe and Renfree 1987). The extended matings are thought to restrict other male mating opportunities by tiring females, and mate guarding and female defence (Braithwaite 1979; Dickman 1993; Marlow 1961; Shimmin, Taggart *et al.* 2002). After mating, females can store sperm within the crypts of the oviduct for up to 16 days, providing an arena whereby sperm mixing and competition for position can occur (Selwood and McCallum 1987; Shimmin, Jones *et al.* 1999; Taggart and Temple-Smith 1991). Sperm competition has been confirmed in several species through the observation of multiple paternity occurring within litters (Holleley, Dickman *et al.* 2006; Millis 1995; Shimmin, Taggart *et al.* 2000a).

Reproductive investment in strategy I dasyurids

As marsupial young undergo a period of fixed lactation, during which young are permanently attached to a teat, the number of teats sets the maximum litter size and is thought to be linked to environmental variability (Beckman, Banks *et al.* 2007; Shimmin, Taggart *et al.* 2000b). Despite this, female dasyurids tend to produce more ova and embryos than teats available, which means female fitness is not compromised by the inability to fill all teats (Tyndale-Biscoe and Renfree 1987). The production of supernumerary young also provides another arena for selection to occur, as all young are normally born simultaneously (Taggart, pers. obs) and less competitive young do not secure a teat. Although there are costs of producing excess full-term embryos, these are limited in marsupials by the young being born at an early stage of development. Decisions to abandon young should not be made on avoidance of wasting parental investment so far, rather on the ability to supply the investment needed to bring offspring to maturity (Russell 1982).

Sex ratio

In strategy I dasyurids, males only survive for a single mating season, while females can potentially survive for multiple seasons, resulting in biases in the adult sex ratio. Biased litter sex-ratios have also been observed (Ward 2003), with the size and direction of the bias varying between species, populations and female breeding age (Cockburn 1990; Cockburn, Scott *et al.* 1985a; Cockburn, Scott *et al.* 1985b; Fisher 2005; Vestal, Lee *et al.* 1986). The stage at which preferential selection for one sex occurs is not clear, although prenatal effects appear likely in *A. agilis* (Davison and Ward 1998).

As the female makes the major investment into the production of young, any manipulation of sex ratio is likely to be female driven. Male roles in affecting sex ratios will be minimised through multiple males contributing sperm, reducing each individual male's effect (Ward 2003). Female strategy I dasyurids store sperm in crypts within the lower isthmus of the oviduct for extended periods prior to ovulation (Selwood 1982; Selwood and McCallum 1987; Shimmin, Jones *et al.* 1999; Taggart and Temple-Smith 1991). Embryonic sex ratio manipulation in eutherians tends to occur at a stage of development that coincides with marsupial post-natal development, suggesting the mechanisms operating in eutherians do not necessarily apply to marsupials (Ward 2003). Sex ratio may also be altered during lactation using selective brood reduction as a means of offspring selection (Cockburn 1994). Differential investment into the sexes may also be observed as size dimorphism during lactation (Soderquist 1993).

Size dimorphism

Adult strategy I dasyurids are sexually dimorphic with male to female weight ratios ranging between 1.2:1 and 1.8:1 compared to <1.3:1 for iteroparous dasyurid species (Lee, Woolley *et al.* 1982). The larger body size of strategy I males has been proposed to have a range of benefits; niche separation (Fox and Whitford 1982), greater somatic stores that can be allocated to mate acquisition (Woollard 1971), intrasexual dominance (Braithwaite 1979) and buffering of intersexual aggression (Cockburn and Johnson 1988).

The promiscuous mating system of these species can produce large variation in male reproductive success, with larger males, at least in *A. agilis*, fertilising more females and siring more offspring than smaller males (Kraaijeveld-Smit, Ward *et al.* 2003). This variation in reproductive success means males appear to benefit from extra investment by mothers (Marlow 1961; Soderquist 1995b), although mothers need to be in good condition for biased investment to occur (Dickman 1988). Growth has also been observed to be affected by the number of mates a female has and the timing of those matings (Fisher, Double *et al.* 2006b)

Polyandry in strategy I species

Multiple paternity within litters has been recorded in three of the strategy I dasyurids, *P. tapoatafa* (Millis 1995), *A. agilis* (Shimmin, Taggart *et al.* 2000a) and *A. stuartii* (Holleley, Dickman et al. 2006), with factors such as genetic similarity (Parrott, Ward *et al.* 2006), male genetic quality (Fisher, Double *et al.* 2006a), mating order (Kraaijeveld-Smit, Ward *et al.* 2002b; Shimmin, Taggart *et al.* 2000a) and male body size (Kraaijeveld-Smit, Ward *et al.* 2003) observed to affect siring success. In litters sired by a number of males, young are likely to have a wider variety of phenotypes, meaning some may be successful in adverse conditions as well as in good conditions. A consequence of this is a high level of genetic diversity in the population (Kraaijeveld-Smit, Lindenmayer *et al.* 2002; Lambert, Jones *et al.* 1997) and high genetic diversity has been found to decrease parasite load (Loman, Madsen *et al.* 1988), a consideration for immune-suppressed strategy I males (Bradley 1987; Bradley 1990).

Larger, dominant males may be able to obtain more matings, but in a variable environment, other factors such as degree of immune suppression, will affect survival to mating and hence reproductive success. Sub-dominant males have several methods by which they could improve their siring success. They may have more sperm available and inseminate more sperm with each mating, as they are not depleting their sperm stores as much as dominant males through multiple matings (Stockley and Purvis 1993). Sperm storage in the oviduct of the female provides an arena in which sperm competition may occur (Dickman 1993; Taggart and Temple-Smith 1991), potentially allowing for screening out of poor quality sperm, and also allowing sperm from multiple males to be available at ovulation (Møller 1998; Shimmin, Taggart *et al.* 2000a). Subdominant males may also displace previous males sperm in the isthmic crypts, and as they mate closer to ovulation less of their sperm may be shed from the crypts, increasing the proportion of sperm available for fertilisation (Shimmin, Taggart *et al.* 2000a).

Red-tailed phascogales

The red-tailed phascogale*, Phascogale calura,* is a species that was not included in the original description of dasyurid life history strategies by Lee *et al.* (1982). Field work conducted with populations in Western Australia found that males were semelparous, dying shortly after a ~ 3 week breeding period, although the females survived for multiple breeding seasons (Bradley 1997). The breeding season occurred during July and was followed by a gestation period of ~30 days (Bradley 1997).

Red-tailed phascogales are nocturnal and weigh between 38 and 68 g (Bradley 1997) which is substantially smaller than *P. tapoatafa* which weighs ~231 g (Soderquist 1995a). Red-tailed phascogales are opportunistic feeders, taking a variety of prey including spiders, insects, small birds and mammals, foraging extensively on both the ground and in the canopy (Kitchener 1981). Females have a type 1 pouch (Woolley 1974) meaning their eight teats are on an exposed circular patch of skin and not covered by a skin fold. The maximum litter size a female can raise is thus eight, although more young are born than can actually attach to a teat (Bradley, Foster *et al.* 2008). Multiple paternity and testicular failure have been observed, and their reproductive strategy appears similar to *Antechinus* although mating occurs over a longer time period in captivity (Foster 2005).

At the time of European settlement *P. calura* was found in south-western Western Australia and across arid and semi-arid regions of Australia, but has since undergone a large decrease in range (Friend and Friend 1992). Currently populations are restricted to south-western Western Australia, a region that has a predictable, seasonal environment. A captive breeding colony has been established at Alice Springs Desert Park since 2001, where breeding is being undertaken with the intent to learn more about the species biology and to trial methods for translocation.

Red-tailed phascogales as a model species

The unusual life history strategy of strategy I dasyurids make them useful subjects for studying reproductive investment. The single annual breeding season means generations are discrete and the obligate post-mating mortality of males in the wild means males are limited to a single breeding attempt. These factors mean investment into breeding attempts can be more easily disentangled than in continuous breeders, providing an opportunity to assess ways that lifetime reproductive success can be maximised. By analysing patterns of siring success under different conditions, information can be gained on how males maximise their reproductive potential.

The occurrence of sperm competition in a species that also exhibits spermatogenic failure with consequent sperm limitation is highly unusual amongst mammals. Studies of sperm competition in these unusual breeders can shed light onto how males can improve their siring success. From a female perspective, post-mating male mortality removes many direct benefits, such as paternal care and future mate assessment as explanations for polyandry. Instead genetic benefits are likely to play a major role in driving the females' mating strategy. Females have large litters into which they have a large resource investment, showing overproduction of young and biased sex ratios which make them useful subjects for exploring reproductive investment.

The strategy I life history may be part of a continuum, from obligate semelparity with spermatogenic failure, as exemplified by *Antechinus*, to facultative semelparity without spermatogenic failure, as seen in *Dasyurus hallucatus* and *Parantechinus apicalis*. *Phascogale tapoatafa* appears similar to *Antechinus* but with potentially greater plasticity in the breeding season (Millis, Taggart *et al.* 1999). Whether red-tailed phascogales are more similar to *Antechinus* or *P. tapoatafa* is not clear presently.

Studying the reproductive biology of the red-tailed phascogale will allow for comparison with *Antechinus*, aiding our understanding of this unique life history strategy, and how species can maximise their reproductive potential. It will also shed light on the reproductive biology of the red-tailed phascogale, a poorly studied, endangered marsupial, aiding captive breeding attempts.

Aims

The broad aim of my thesis is to examine the reproductive strategies used by male and female red-tailed phascogales to maximise their reproductive fitness. In order to achieve this aim, I firstly needed to determine the reproductive parameters of red-tailed phascogales to determine the constraints within which reproductive investment can be manipulated. Secondly having determined these constraints, I then examined several areas in which investment into young can be altered.

The thesis is separated into six chapters with the first three documenting the reproductive biology of red-tailed phascogales and the last three examining reproductive investment. The chapters are written as papers, with the formatting and style of each reflecting the journal in which the paper has been published or submitted to. The aims for each chapter are:

1. Characterise the breeding seasonality and reproductive success of captive female red-tailed phascogales.
2. Characterise sperm production and spermatogenic failure in captive male red-tailed phascogales.
3. Characterise the growth and development of red-tailed phascogale young and assess whether differences in growth occur across captive colonies.
4. Examine female fecundity and test for age related changes in fecundity, assessing whether sex ratio biases can be generated by differential attachment of the sexes to teats at birth.
5. Assess parental and gender influences on the growth of red-tailed phascogale young.
6. Assess whether multiple paternity occurs in captive red-tailed phascogale litters and how multi-male groups impact on the maintenance of genetic diversity in a captive breeding colony.

CHAPTER 1

Timing of births and reproductive success in captive red-tailed phascogales, *Phascogale calura*

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Eight young in the pouch of a female red-tailed phascogale on the day of birth

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CHAPTER 2

Changes in sperm production in a species exhibiting obligatory spermatogenic failure

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Hemi-castration of a male red-tailed phascogale

Abstract

In mammals, relative testes size is related to species mating system and the likelihood of sperm competition. In several dasyurid species though, spermatogenic failure occurs in males prior to mating, meaning they are reliant on epididymal sperm stores. Males of these species also show mortality after their first breeding attempt, or semelparity. A male semelparous dasyurid, the red-tailed phascogale *Phascogale calura,* was examined to determine the pattern of seasonal changes in sperm production in captivity, and the relationship between scrotal diameter, testes mass and epididymal mass and sperm availability. In contrast to *Antechinus,* another semelparous dasyurid, that ceases sperm production prior to mating, captive male red-tailed phascogales produced sperm throughout the mating period. Scrotal diameter showed a positive linear relationship with testes and epididymal mass across adult life, but no significant relationship was evident during the period of peak sperm production. Captive males had maximal scrotal diameters for 2 ½ months, which is longer than has been observed in wild animals, where scrotal diameter declines approximately one month earlier than we observed. Captive male red-tailed phascogales were able to survive beyond their first mating period, although they were no longer able to produce sperm. Testosterone levels of captive red-tailed phascogale were lowest during the mating period which contrasts to that observed in other studies of wild red-tailed phascogales, where captivity appeared to ameliorate factors that constrained both the duration of sperm production and survivorship.

Introduction

Amongst mammals, a reproductive strategy in which all males die after their first and only breeding attempt is uncommon. This obligate semelparity is only known to occur in three genera of carnivorous marsupials, *Antechinus*, *Dasykaluta* and *Phascogale* (Bradley 1987; Lee, Woolley *et al.* 1982; Woolley 1991a), with facultative semelparity, where some males survive in some populations or years, observed in dibblers *Parantechinus apicalis* and northern quolls, *Dasyurus hallucatus* (Dickman and Braithwaite 1992; Mills and Bencini 2000). Species showing obligate dasyurid semelparity, or strategy I life history (Lee, Woolley *et al.* 1982), also exhibit spermatogenic failure around the time of mating (Inns 1976; Kerr and Hedger 1983; Millis, Taggart *et al.* 1999; Taylor and Horner 1970; Woolley 1966), which is absent in facultative semelparous marsupials. Due to the occurrence of spermatogenic failure, a strategy I life history is proposed to be an irreversible adaptive trap as any males that survive to a second season are unable to reproduce (Braithwaite 1979).

Sperm competition, which occurs when females mate with multiple males in a fertile period (Parker 1970), may drive the evolution of male semelparity (Dickman 1993). With promiscuous matings occurring, factors that improve male mating and siring success in the short-term can have deleterious effects on long-term survival. For example high levels of testosterone during the mating period in wild males may aid mate acquisition and territory defence (Scott 1987), but with a detrimental effect on long-term survival (Bradley 1990; Bradley, McDonald *et al.* 1980; Wilson and Bourne 1984). In rats, high levels of testosterone also suppress the early stages, up to stage VII pachytene, of sperm production, while still allowing for the later stages to be completed (McLachlan, Wreford *et al.* 1994; Sun, Irby *et al.* 1989). This was not observed in *A. stuartii* though which was suggested to indicate testosterone concentration has little impact on spermatogenic activity (McAllan 1998). Sperm competition has been observed in several strategy I dasyurid species (Holleley, Dickman *et al.* 2006; Kraaijeveld-Smit, Ward *et al.* 2002a; Shimmin, Taggart *et al.* 2000a).

In captivity, males are capable of surviving beyond their first breeding season (Woolley 1966), indicating that factors that drive semelparity have been removed. Factors that may play a role are; agonistic male-male interactions (Scott 1987), improved food availability decreasing the likelihood of animals reaching a negative nitrogen balance (Woollard 1971) and a reduction in the time and distance travelled when searching for mates (Friend and Friend 1992). Despite improved survival in captivity, the absence of germ cells in testes of post-breeding males means they are incapable of breeding after that first season (Kerr and Hedger 1983; Millis, Taggart *et al.* 1999), a factor that can lead to the collapse of captive populations without careful management.

Why spermatogenic failure would occur in a species that exhibits sperm competition is not clear when high sperm numbers, sperm densities per ejaculate or ejaculate volumes are associated with an increased probability of siring young (Birkhead and Hunter 1990; Parker 1990a). *Antechinus* ejaculate low numbers of sperm compared to other mammals (Taggart and Temple-Smith 1990), but sperm transport in the female reproductive tract is highly efficient, with more than 1 in 7 sperm reaching the isthmus (Taggart and Temple-Smith 1991). The spermatogenic failure shown by these species means males are reliant on the sperm stored in the epididymis during mating (Taggart and Temple-Smith 1990), potentially limiting the number of mates that can be fertilised and the number of sperm that can be allocated to any one mating (Taggart and Temple-Smith 1990).

In mammals, sperm production rates and sperm reserves are correlated with testes size (Møller 1989), with testes size also showing a relationship to mating system in eutherians (Harcourt, Harvey *et al.* 1981; Kenagy and Trombulak 1986; Møller 1988), marsupials (Rose, Nevison *et al.* 1996; Taggart, Breed *et al.* 1998), birds (Møller 1991) and butterflies (Gage 1994). Testis size is difficult to measure non-invasively and consequently scrotal diameter has been used as an indirect measure of testis size and sperm production, with varying degrees of success (Coulter and Foote 1979; Martínez, Limas *et al.* 1994). For the strategy I dasyurids, spermatogenic failure means scrotal diameter during mating may not reflect the maximal testis size or sperm numbers due to sperm only being present in the epididymis.

The unusual sperm production characteristics and life-history strategy of strategy I dasyurids make them interesting to study in relation to sperm competition. Our study aims to investigate seasonal changes in sperm production in the red-tailed phascogale *Phascogale calura,* and their relationship with testis mass, epididymal mass and scrotal diameter. Knowledge of these relationships will provide information on the utility of scrotal diameter as a non-invasive measure of sperm production in species showing spermatogenic failure. As high testosterone levels are associated with post-mating mortality in wild animals, we also aim to examine the testosterone levels in captive males to determine whether the same pattern of changes occurs as is observed in the wild (Bradley 1987).

Methods

Animals

Red-tailed phascogales were maintained at Alice Springs Desert Park (ASDP) and Adelaide Zoo and their housing and feeding details are given in Foster *et al* (2006). From weaning in November 2004, eleven males were maintained in 12L:12D lighting until April 2005 when they were returned to natural lighting (Foster, Caton *et al.* In Press). Animal ethics approval was obtained from the University of Adelaide (S-09-2004(A) and S-038-2005) and the Department for Environment and Heritage (4/2004 and 23/2005).

Changes in scrotal diameter and testis and epididymal mass

In 2004, fortnightly measures of scrotal diameter were obtained, to the nearest 0.1mm, from eight males in the Adelaide colony, between 183 and 430 days of age. In 2006, scrotal diameters were collected approximately fortnightly from nine males at Adelaide between 217 and 366 days of age. In 2004 and 2005, testis and epididymal samples were collected from males at both ASDP and Adelaide. Males were anaesthetised using 5 % isofluorane in oxygen (1 L/min), then maintained on 2 % isofluorane and a testis and epididymis removed (hemi-castrated as per Taggart and Temple-Smith 1990). Testes and epididymides were collected from five males at 306 days of age, prior to the breeding season, with 24 additional samples collected following mating, between 368 and 472 days. In 2005, 14 samples were collected from males aged between 198 and 400 days of age. Scrotal diameter, body mass, testis mass and epididymal mass are expressed as average ± standard deviation.

Spermiogenesis

Testes and epididymides were fixed in 10% formaldehyde, wax embedded, sectioned and stained with haematoxylin and eosin. The timing of emergence and disappearance of mature spermatids and presence of germ cells in testes was recorded, as was the timing of sperm movement through the epididymides.

Post-mating epididymal sperm counts

Six males were castrated at 370 days of age (2006), when breeding females were pregnant or had given birth. One epididymis from each male was placed in Dulbecco's Modified Eagle Medium (DMEM) and divided into six segments, with segments 1 and 2 from the caput, segments 3-4 from the corpus and segments 5-6 from the cauda. Sperm were removed from the epididymis using the cut and squeeze method of Taggart and Temple-Smith (1990). Sperm were diluted in 500 µl of DMEM for segment 1 and 1 ml for segments 2-6, and counted using a haemocytometer with sperm numbers given as average ± standard deviation.

Testosterone

Animals were anaesthetised as above and blood collected fortnightly, from 222 days of age til 370 days of age, from the lateral tail vein using a heparinsed 26G needle. Plasma samples were removed and frozen until analysis. Testosterone levels were determined using the method of Bradley *et al* (1980), which has been previously used for red-tailed phascogales (Bradley 1987), and values were expressed as average ± standard deviation.

Results

Seasonal changes in scrotal diameter and testis and epididymal mass

Animals in 2004 and 2006 showed a very similar pattern of change in scrotal diameter. Immature males at 198 days of age had a scrotal diameter of 10 ± 1.3 mm, which increased to 15 ± 1.3 mm between 260 and 339 days of age (Figure 1). After 339 days of age, scrotal diameter started to decline, reaching 13 ± 1.2 mm when animals in the wild would be dying, but continuing to decline to 11 ± 0.5 mm by 415 days of age.

At 198 days of age, the testes of immature males weighed 16% of the mass of testes at 306 days of age, prior to mating, while the immature epididymis represented 12% of the mass of a mature epididymis. Animals that were maintained in natural lighting showed a 75% decline in testis mass between 306 and 374 days of age, while epididymal mass remained between 0.07 and 0.10g during the same time period (Figure 1). In animals that were housed in 12L:12D lighting for five months after weaning, changes in testes and epididymal mass were accelerated, occurring ~100 days earlier than they had been observed in animals maintained in natural lighting (Figure 1). At 306 days of age when testes were at maximal size, the testes of red-tailed phascogales constitute 0.8% of body mass, with this declining to 0.2% post mating. The percent of combined testes and epididymal mass that the testes contributed to was highest in males at 183 days of age, 79 ± 7%, declining to 50 ± 5% at 370 days of age.

Spermatogenesis

Animals housed in natural lighting had several layers of germ cells present in the seminiferous epithelium, with numerous mature spermatids in the tubule lumen in the three weeks prior to mating (Figure 2a). By the time females had given birth, males only had occasional spermatocytes present and mature spermatids were still present in the tubules of individuals (Figure 2b). Ten weeks after ovulation occurred in females, 9/11 females giving birth on the same day, spermatids were absent from the tubule lumen of all males. Sixteen weeks after ovulation, only Sertoli cells were present in the epithelium, the lumen was completely occluded (Figure 2c) and the testis was in the same condition in second year animals.

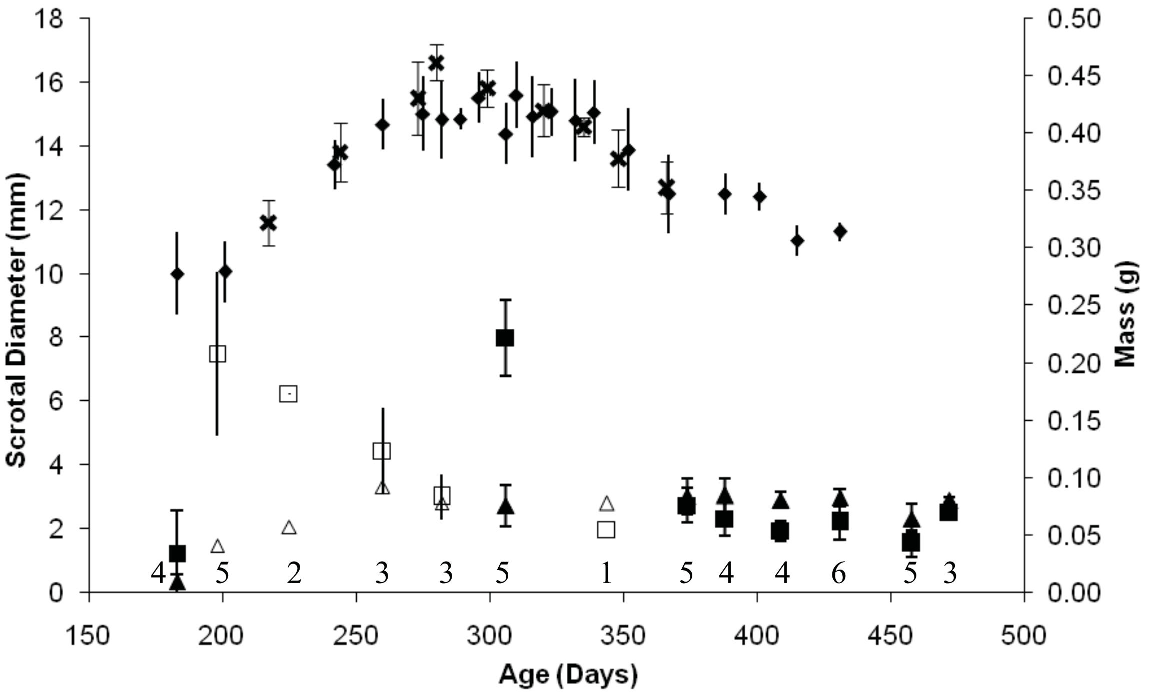

Figure 1 – Scrotal diameter and testis and epididymal weights for male red-tailed phascogales.

Weights from animals held in natural lighting in 2004 are indicated by solid shapes, while weights for animals maintained in 12L:12D from weaning to March 2005 are indicated by outlined shapes; Testis (■ and □), epididymis (▲ and △). Sample sizes for testes and epididymal weights are given below each timepoint at which measures were collected. Measures of scrotal diameter obtained in 2004 are indicated by ◆ (n=8) and 2006 by **x** (n=9).

Animals housed in 12L:12D lighting for five months following weaning, showed an earlier onset of sperm production compared to animals housed in natural lighting. Spermatids were observed in 198 day old animals from 12L:12D lighting, while a sample collected from 183 day old animals housed in natural lighting, had no spermatogenic activity. Samples collected at day 225 from animals housed in 12L:12D lighting, showed a similar level of spermatogenic activity to that observed in animals housed in natural lighting at 306 days. At 286 days, the spermatogenic activity of animals housed in 12L:12D lighting was similar to that observed in animals in natural lighting aged 370 days.

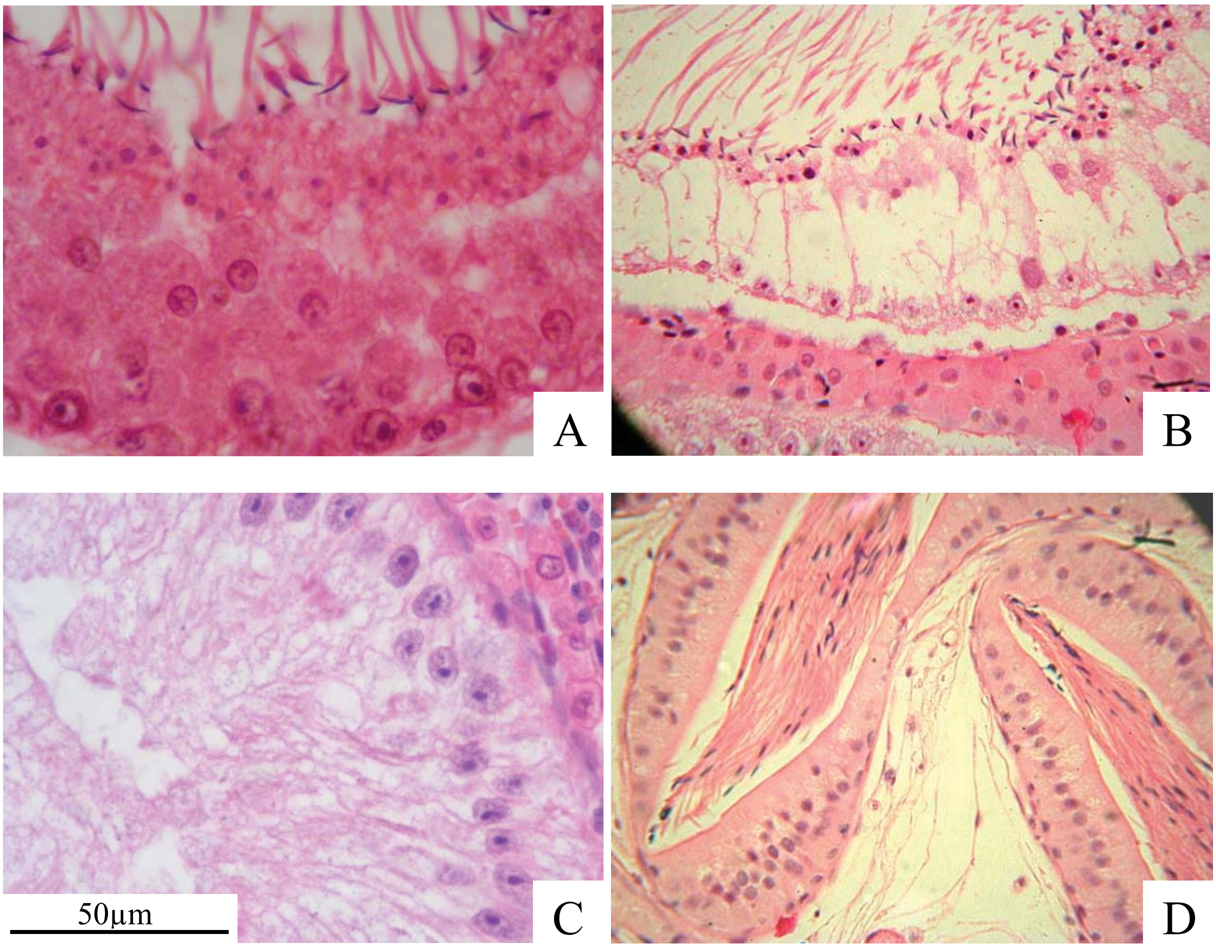

Figure 2. Sperm production in red-tailed phascogales.
Approximately three weeks prior to mating there are several layers of germ cells present in the seminiferous tubules, with the lumen containing mature spermatids (A). A month after mating, when females are giving birth, germ cells are depleted but mature spermatids are still present in the lumen of the tubule (B). Three months post mating, no spermatids remain in the lumen of the seminiferous tubule, and the lumen is narrowed with sertoli cell cytoplasm filling most of the lumen (C). One month after mating, the slit-like lumen of the cauda epididymis is still filled with sperm (D). The scale for all panels in the figure is given in C.

Epididymal sperm storage

Animals housed in natural lighting had no spermatozoa present in the epididymis at 225 days of age, but sperm were present throughout the length of the epididymis by 259 days of age. By the time all females had given birth, spermatozoa were still present throughout the epididymis. Ten weeks after females had ovulated, no spermatozoa were evident within the epididymis, but deposits were evident within the ductules.

Variation in lumen shape was evident along the length of the epididymis, with that of the caput and corpus being circular in cross-section, while that of the cauda was narrow and slit-like (Figure 2d). The head of the spermatozoa in the caput epididymis was situated at right angles to the tail and a cytoplasmic droplet was also present beneath the sperm nucleus of all spermatozoa in this region of the duct. As spermatozoa progressed from caput to cauda, the nucleus realigned until it lay parallel to the tail in the cauda. The cytoplasmic droplet was also lost from each spermatozoan during passage through the corpus region of the ductus epididymis. In the cauda epididymis, free cytoplasmic droplets could be seen in large numbers within the lumen of the duct.

Relationship between testis mass, epididymal mass and scrotal diameter

Scrotal diameter showed significant relationships with epididymal mass (f=23.3, $p<0.0001$, $r^2 = 0.412$), testis mass (f=32.9, $p<0.0001$, $r^2 = 0.465$) and combined testis and epididymal mass (f=51.61, $p<0.0001$, $r^2 = 0.610$) when all records were combined (Figure 3). When records were restricted to the period of sperm production (n=10), no significant relationship was evident for testis mass (f=1.78, p=0.224, $r^2 = 0.203$), epididymal mass (f=1.02, p=0.342, $r^2 = 0.113$) and combined testis and epididymal mass (f=2.78, p=0.139, $r^2 = 0.285$).

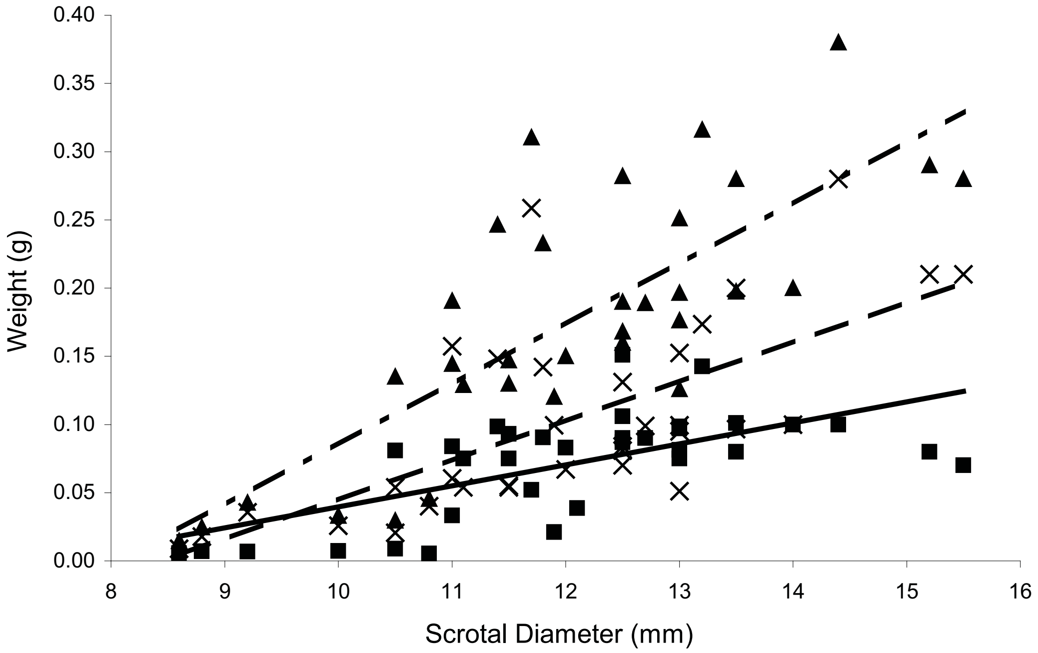

Figure 3. Relationship between scrotal diameter and epididymal and testis mass in red-tailed phascogales.

Testis mass (**x** and ----), epididymal mass (■ and ——) and combined testes and epididymal mass (▲ and — - — -).

Post-mating epididymal sperm counts

When all females were either pregnant or lactating, males still had 1.5 x 10^6 ± 5.9 x 10^5 sperm/epididymis. The histology of the testis and cauda epididymis at this time can be seen in figures 2b and 2d. Sperm were present throughout the length of the epididymis, with 57% of sperm found within the last third of the epididymis (Figure 4). The number of sperm recovered from epididymides post mating had no significant relationship with epididymal mass (f=0.256, p=0.639, r^2 = 0.060), testis mass (f=7.30, p=0.054, r^2 = 0.646), combined testis and epididymal mass (f=2.725, p=0.174, r^2 = 0.405) or scrotal diameter (f=2.289, p=0.205, r^2 = 0.364).

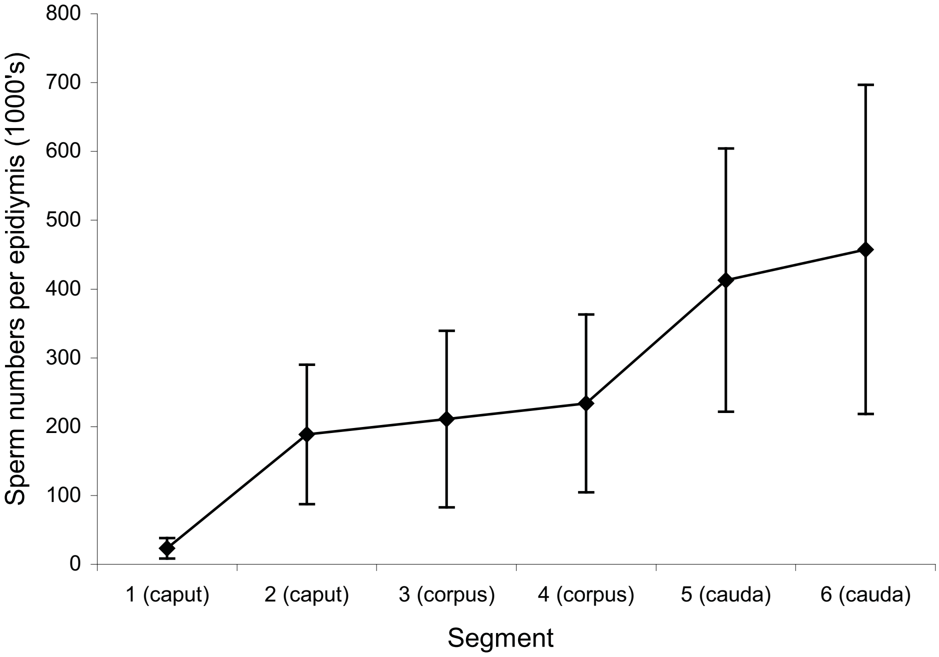

Figure 4. Average ± standard deviation epididymal sperm counts from six male red-tailed phascogales one month post mating.

Testosterone levels and male body mass

Testosterone levels were highest in mid-February 11.3 ± 3.7 ng/ml, declining to 2.8 ± 1.1 ng/ml by the middle of the mating period at 326 days (Figure 5). Males had achieved an average body mass of 59 ± 6 g by 250 days of age with only a slight increase in body mass occurring over the following months (Figure 5). At the start of the mating period, males had an average mass of 64 ± 7 g, decreasing slightly to 61 ± 8 g during mating, before increasing to 67 ± 11 g by the time the females had given birth.

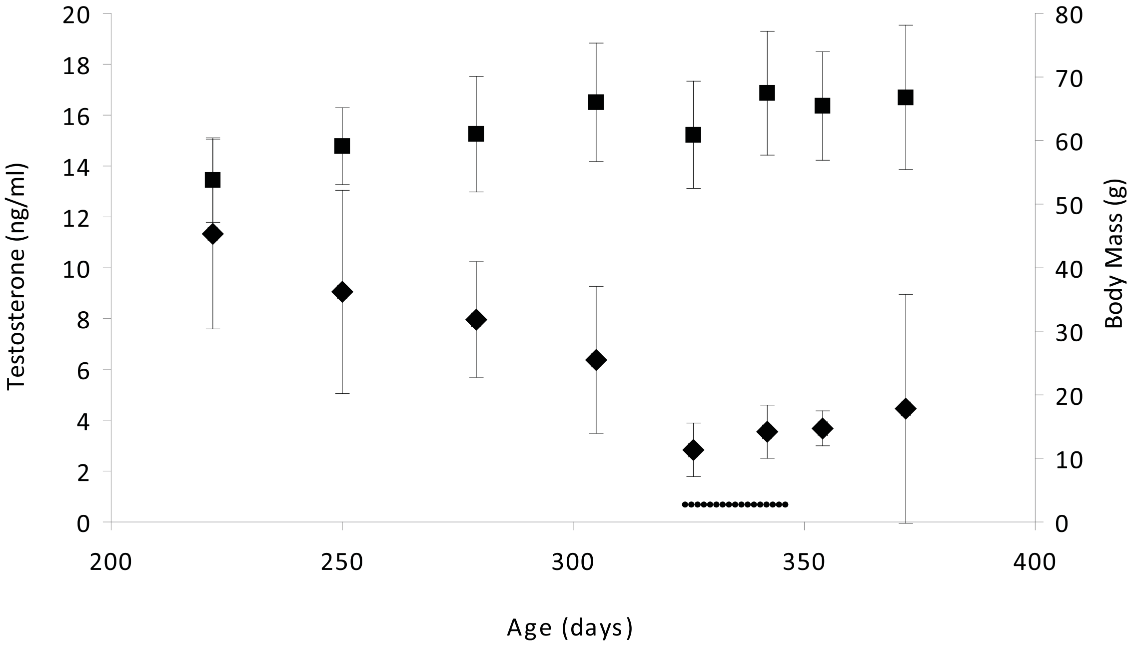

Figure 5. Testosterone levels (◆) and body mass (■) of captive male red-tailed phascogales.
Mating period is shown by the horizontal dotted line.

Discussion

This study confirms that red-tailed phascogales exhibit spermatogenic failure and the pattern of sperm production is similar to that observed in *Antechinus agilis* (Kerr and Hedger 1983), although with differences in timing of spermatogenic failure. In *Antechinus* the seminiferous tubules are depleted of sperm by the start of the mating period (Kerr and Hedger 1983), while red-tailed phascogales are still producing sperm until mating has been completed. Within populations of *Antechinus* the mating period is highly predictable from one year to the next (McAllan, Dickman *et al.* 2006). A similar predictable pattern was seen in wild red-tailed phascogale populations studied for five years (Bradley pers. obs), but the red-tailed phascogale mating period in captivity appears more variable, with potential to occur over several months (Foster, Caton *et al.* In Press). This variable mating period has resulted in the early stages of spermatogenesis still occurring in the mating period of animals at ASDP in 2004, while in the Adelaide colony that mated 6-8 weeks later, only sperm maturation was occurring.

The absence of germ cells in the testes of males after mating means that even though captive red-tailed phascogale males can survive for up to five years (Foster and Bradley, pers obs), they are incapable of breeding after the first breeding season. This reproductive senescence, which in *Antechinus* is highly synchronised and closely aligned with the mating period (Inns 1976; Kerr and Hedger 1983; Taylor and Horner 1970; Wilson and Bourne 1984; Woolley 1966), is less so in *Phascogale tapoatafa* (Cuttle 1982). This reproductive senescence has implications for captive breeding programs as young must be produced every year to stop the population becoming functionally extinct due to the lack of fertile males.

The timing of spermatogenic failure is crucial to male reproductive success because sperm stores are depleted by both mating and by sperm loss through the urine, spermatorrhoea (Taggart and Temple-Smith 1990). At the end of the breeding season, we found around 1.5 x 10^6 sperm in an epididymis, whereas in *Antechinus* the epididymal sperm numbers declined from 3.5 x10^6 per epididymis prior to breeding, to 0.3 x 10^6 per epididymis post breeding (Taggart and Temple-Smith 1990). In *Antechinus,* the low sperm availability has been related to the slit-like lumen in the cauda epididymis, which is similar to that observed in red-tailed phascogales. The slit-like lumen has been proposed to limit the numbers of sperm ejaculated, with two to nine matings resulting in depletion of the cauda epididymides of sperm (Taggart and Temple-Smith 1989; Taggart and Temple-Smith 1990). With red-tailed phascogales still producing sperm during mating, males are potentially able to mate with more partners due to the continued replenishment of caudal sperm stores.

Captive red-tailed phascogales achieved a similar maximum scrotal diameter and pattern of scrotal diameter decline as has been observed in wild animals, although the timing of changes in scrotal diameter differed (Bradley 1997; Friend and Friend 1992). Captive animals had maximal scrotal diameters for more than twice as long as wild animals, with the decline in wild animals occurring a month before mating commenced. Scrotal diameter reflects some of the changes in testis and epididymal mass in male red-tailed phascogales across their adult life, although no relationship was evident when the analysis was restricted to the period of maximal sperm production when information on sperm numbers is most valuable. Factors such as scrotal fat deposits and fluid accumulation are also likely to vary seasonally, limiting the strength of the relationship between scrotal diameter and sperm production. From the limited number of epididymal sperm counts, no relationship between sperm numbers and epididymal mass, testis mass or scrotal diameter was evident. In contrast, *A. agilis* sperm numbers showed a significant, positive linear relationship with testis and epididymal mass ($r^2 = 0.726$, $p<0.0001$ and $r^2= 0.407$, $p<0.0001$ respectively, Taggart, pers. obs.) when examined at mating ± two weeks, but the relationship with scrotal diameter was not assessed.

The reason for the occurrence of spermatogenic failure is not clear, although testes regression to 5-15% of maximal size (Taggart and Temple-Smith 1992; Wilson and Bourne 1984) may allow for energy that would otherwise be diverted into sperm production, to be used for inter-male competition and mate acquisition (Kenagy and Trombulak 1986; Taggart, Selwood *et al.* 1997). With high testosterone levels associated with mate acquisition (Scott 1987), the increase in testosterone levels in the wild at the start of the breeding season in *Antechinus* and *Phascogale* (Bradley 1987; Bradley 1990; Bradley, McDonald *et al.* 1980; McDonald, Lee *et al.* 1981) may improve reproductive success. Wild populations of red-tailed phascogales show relatively low testosterone levels until the start of territorial behaviour, with levels at mating being 10-15x higher than pre-breeding levels (Bradley 1987). This is in contrast to what was observed in the captive population where testosterone levels were lowest during the mating period. In our study multi-male and female groups were established prior to the breeding season though and maintained together until females were due to give birth, with no aggression observed between males. This was in contrast to the aggressive behaviour observed when males were first housed together in the presence of oestrous females (Foster pers obs).

Increases in testosterone levels are associated with increased plasma cortisol levels (Bradley 2003), with high levels of cortisol being implicated in the suppression of the immune and inflammatory responses and post-mating mortality (Bradley 1987; Bradley 1990; Bradley, McDonald *et al.* 1980; McDonald, Lee *et al.* 1981). No significant increase in cortisol was observed in captive *P. tapoatafa* (Schmidt, Taggart *et al.* 2006), where testosterone levels were again lower than those observed in the wild (Millis, Taggart *et al.* 1999). This suggests that although cortisol levels weren’t examined in our study, they are unlikely to have been as high as those observed in wild animals (Bradley 1987). Intermale social interactions have been implicated in elevated cortisol levels in *Antechinus* (Scott 1987) and olfactory stimuli from other males may also contribute to male die-off (Toftegaard, Moore *et al.* 1999) but both are likely to be ameliorated in captivity where interactions between males are restricted.

Compared to *Antechinus*, male red-tailed phascogales appear capable of mating for an extended period, which is in line with the greater plasticity in breeding observed in red-tailed phascogale females (Foster, Caton *et al.* In Press). The results from our study indicate obligate spermatogenic failure does occur in red-tailed phascogales, but factors associated with housing in captivity can result in both an extended period of maximal scrotal diameter and survival of males beyond the first mating period. Further investigation of the role of social factors may shed further light into the mechanisms that produce spermatogenic failure and post-mating mortality.

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CHAPTER 3

Comparison of growth and development of the red-tailed phascogale (*Phascogale calura*) in three captive colonies

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55 day old red-tailed phascogale young

61 day old red-tailed phascogale young

Foster, W.K., Bradley, A.J., Caton. W. & Taggart, D.A. (2006). Comparison of growth and development of the red-tailed phascogale (*Phascogale calura*) in three captive colonies.
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CHAPTER 4

Generation of sex ratio biases in the red-tailed phascogale (*Phascogale calura*)

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Red-tailed phascogale embryo

One day old red-tailed phascogale young

Foster, W.K. & Taggart, D.A. (2008). Generation of sex ratio biases in the red-tailed phascogale (*Phascogale calura*).
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CHAPTER 5

Gender and parental influences on the growth of a sexually dimorphic carnivorous marsupial

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Collection of head length from an adult red-tailed phascogale

Litter of red-tailed phascogale young at 41 days of age

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CHAPTER 6

Captive breeding with multiple paternity: an example in the semelparous red-tailed phascogale, *Phascogale calura*

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Mating red-tailed phascogales

Litter of eight red-tailed phascogale young

Abstract

When several males have breeding access to females and those females produce more than one young at a time, there is potential for multiple paternity within litters. This has implications for the genetic management of captive populations due to paternity being unknown. Red-tailed phascogales, *Phascogale calura,* are sexually dimorphic, carnivorous marsupials that are thought to exhibit multiple paternity. The endangered red-tailed phascogales have been part of a group managed captive breeding program since 2001 with two or three males given breeding access to female groups. In 2004, young were genotyped to gain a better understanding of how genetic diversity is maintained. Five additional litters were genotyped in 2005 to allow comparison of paternity spread with different numbers of males. Seven litters were found to have a single sire, eight litters had two sires and one litter had three sires. Paternity assignment allowed for the ratio of effective population to census population (N_e/N) for 2004 to be calculated and this was 0.51, which was 1.9 times larger than that calculated from the group managed studbook. If the genotyped census and family sizes are calculated without taking into account the effect of culling, the Ne/N ratio decreased to 65% of that observed when non-breeders were culled. No difference in the siring success of the dominant animals was observed between two and three male rotations, with only 1/58 young from the three male rotations being sired by the third male. A bias in recruitment success was observed in relation to male body mass with more offspring being recruited from heavier males.

Introduction

With growing numbers of species facing threats to their survival in the wild, the ability to maintain animals in captivity is sometimes seen as a species last hope of survival. Often only a limited number of founders are available to establish captive populations, and the genetic diversity represents a small subset of the diversity of the wild population, especially if founders are obtained from a small subsection of the species range (Mace 1986; Ralls and Ballou 1986). Over successive generations, genetic diversity is gradually lost through genetic drift, and an absence of migration means no new genetic material is introduced. This can lead to the population becoming increasingly inbred, showing declines in fertility and increased incidence of hereditary disorders and diseases and loss of adaptive potential (Laikre 1999).

To maximize the maintenance of genetic diversity, ideal populations would have an equal number of sexually mature males and females, each producing the same number of young, with no overlapping generations and no fluctuation in population size over generations (Soulé 1980). The conditions of an ideal population are rarely met in practice, especially for species that show biases in male siring success, which results in larger populations needed to minimise inbreeding (Soulé 1980), and the ethical issue of how to manage non-breeding males (Foose and Ballou 1988). Effective population size (N_e) can be used to indicate the size of an ideal population that has the same rate of genetic drift and inbreeding as observed in the actual population (N: Wright 1931), allowing measures to be compared across populations and species. Fluctuation in population size and variance in family size are important factors affecting effective population size (Frankham 1995b). Thus how the total population size is calculated, i.e. combination of breeding adults, senescent adults, juveniles (Frankham 1995b), and the life history stage at which young are counted, commonly sexual maturity (Fisher 1939), need to be specified.

With captive breeding prior to release becoming more widely used for endangered species, consideration of how genetic diversity is maintained is important to give animals maximal opportunity to survive, and to allow for potential evolutionary change (Crnokrak and Roff 1999; Frankham 1995a; Franklin 1980; Ralls, Ballou *et al.* 1988). One management tool that has not been explored extensively for captive breeding is multiple paternity, when a single female has young sired by multiple males in a single breeding attempt. Multiple paternity within litters can potentially act in the opposite manner to polygyny, and its role in the maintenance of genetic diversity within captive populations should be considered.

For species that have large litter sizes and promiscuous mating systems, multiple paternity may prove to be useful for maintaining genetic diversity in captivity. One group that this may apply to are the male semelparous dasyurid marsupial species (Lee, Woolley *et al.* 1982). These species exhibit characteristics that suggest that multiple paternity may be common (Taggart, Shimmin *et al.* 2003), with some characteristics providing challenges for the maintenance of a long-term, genetically diverse, captive population. Without careful management of these species, the breeding colony may collapse due to spermatogenic failure after the males first breeding season (Kerr and Hedger 1983), or the colony may grow rapidly due to the high fertility of females. Multiple paternity has been observed in several of these species, with factors such as genetic similarity (Parrott, Ward *et al.* 2006), male genetic quality (Fisher, Double *et al.* 2006a), mating order (Kraaijeveld-Smit, Ward *et al.* 2002b; Shimmin, Taggart *et al.* 2000a) and male body size (Kraaijeveld-Smit, Ward *et al.* 2003) influencing siring success.

The red-tailed phascogale, *Phascogale calura,* is an endangered male semelparous dasyurid species whose range has contracted significantly since European settlement, and is now restricted to south-west Australia (Baillie and Groombridge 1996; Kitchener 1981). In 2001 a captive breeding program commenced at Alice Springs Desert Park (ASDP), with six females and 12 males brought into captivity, and an additional three female and three male wild caught animals added to the program in 2004. Female red-tailed phascogales are group housed, tend to breed synchronously, care for others young and potentially utilise multiple paternity, so molecular genetic techniques that enable identification of parentage can aid population management.

We aimed to determine the spread of paternity within the captive red-tailed phascogale population, how the number of males given mating access affects this spread, and whether multiple paternity occurs. We then examined the relationship between male body mass and recruitment success, since male body mass affects siring success in the closely related *Antechinus* (Kraaijeveld-Smit, Ward *et al.* 2003). The genotyping results were then used to calculate the effective population size, which was compared to that estimated from the group-managed studbook and from a theoretical scenario in which variance in male siring success was minimised through pairing animals. The effect of measuring population and family size prior to and after culling was also assessed. The information gained from our study has been made available to the managers of the captive red-tailed phascogale population.

Methods

Captive breeding

In 2004, six breeding groups of red-tailed phascogales were established at ASDP, and the groups comprised (male:females); A) 3:4, B) 3:3, C) 3:3, D) 3:3, E) 2:4 and F) 2:3. Animals from groups A-E were captive born, and one male from each group was hemi-castrated (one testis and epididymis removed as per Taggart & Temple-Smith (1990)) prior to the breeding season for a separate study. Breeding groups were established to minimise inbreeding and where possible half or full sisters were mated with unrelated half or full brothers, and were designed as part of the captive breeding program, rather than as part of an experiment. Group

F contained wild born animals, assumed to be unrelated, and no males from this group were hemi-castrated. In 2005, eight breeding groups, each with 3-4 females and 2 males were maintained, and a single litter from five of these groups were genotyped.

Males were rotated through the female groups from early May until the females had given birth. Each of the males were placed with the females in 2 x 2 x 2 m outside enclosures, for three to four days on a rotating basis. When males were not with the females, they were housed individually in a 600 x 700 x 900 mm plastic box with a mesh front, while the other males were given access to the females (Figure 1). Rotations were chosen to allow multiple males to mate with the females, while not allowing male-male interactions that can result in injuries. As females have an extended oestrus, accepting mates over a minimum of five days (Foster, unpublished data), rotating males allowed multiple males to access females during their receptive period. This interval was also used as it was the most practical from a staffing perspective and limited the frequency of disturbances to animals. The order in which males actually mated was unknown though as matings can occur in the weeks prior to ovulation and animals were not actually observed mating. Details of feeding are given in (Foster, Bradley *et al.* 2006). Ethics approval was obtained from the University of Adelaide (S-09-2004(A) and S-038-2005) and the Department of Environment and Heritage (4/2004 and 23/2005).

Day	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
2 Male Rotation	A	A	A	B	B	B	B	A	A	A	A	B	B	B	A	A	A	B	B	B
3 Male Rotation	A	A	A	B	B	B	B	C	C	C	A	A	A	A	B	B	B	C	C	C

Figure 1. **Example rotations of male red-tailed phascogales through female breeding groups for groups with two and three males.**
Rotations commenced at least a month before mating and continued until females were known to be pregnant.

Microsatellite genotyping

Hair samples, comprising at least 20 hairs were collected for genotyping, from females, potential fathers and furred pouch young close to weaning. Ten microsatellite loci isolated from *Antechinus agilis* (designed as per Banks, Finlayson et al. 2005; Paetkau 1999) and three loci isolated from *P. tapoatafa* were screened for polymorphism in *P. calura* (Table 1). Clones for *P. tapoatafa* were constructed by P. Spencer (Murdoch University) as per Spencer & Bryant (2000) with primers developed by B. Hansen (Monash University).

Table 1 – Primer sequences for the microsatellite loci screened in *P. calura*.

Locus	Forward primer (5’-3’)[A]	Reverse primer (5’-3’)	Genotyping
Aa1A[B]	AGCTCCTTTTGTATCCTAAC	TCAGCCTCGATATTTTTCTAATG	2 alleles
Aa7A[C]	GTGAAGAGTCACTTAAAATGT	TTACCAGCCACACTTCAAGA	No product
Aa7M[B]	TGCTTTGTTCTTGCTAAGTA	ACAATCATATGTTTATGTAGCC	Difficult to read
Aa2H[B]	GGAGAGTTTATGATTGTTGTC	GTTATCAGGTCAAAAAGCTCAC	Weak product
Aa4F[C]	AGGGAGGGGCTAGATACT	GTTGCTCCTTCATCTCCATC	Null alleles
Aa7K[B]	TTTCTGGATGAACAGTTTGA	GAGATGTGAGCAGTTAGTGGAC	Single allele
BTP8[D]	CCACATTGTGACATGGGCAGCGC	GCATGCCTGCAGGTCGACTCTAGA	No product
Aa2B[B]	TTCACAGCCTAATGTCCCTA	GTACCACAAGATGCACCTAC	> 4 alleles
Aa4D[B]	TGATTAGGTGCCCACACTA	TCCACTCCCAATACTGACT	> 4 alleles
Aa4A[B]	CCAAATCTACGTAAAATATCC	TTTGATCCTCAGAGACTTGAT	> 4 alleles
Aa4J[B]	AGAACACTCTAACAACATCCT	TCTTCAGTCTCTCAATGAGTT	> 4 alleles
BTP7[D]	GCCCCAATGGTTAGTATTCTCC	GAAAGGCAGACACTTCCCCATTC	> 4 alleles
BTP9[D]	CTCACGGAGTCTTGCCCTCCAG	GGCCTTGGGACCTTCAGTTTGC	> 4 alleles

[A] Forward primers have a 5’ M13 extension (5’ TGTAAAACGACGGCCAGT 3’)

[B] primers obtained from Banks *et al.* (2005)

[C] primers designed for *A. agilis* as per Paetkau (1999)

[D] primers designed by Birgita Hansen and Peter Spencer for *P. tapoatafa*

Six loci that were found to be polymorphic in *P. calura* were chosen for paternity assignment, with all potential parents screened at these loci. The four loci that provided the greatest resolution between sires were selected for initial screening of the offspring. DNA was extracted from hair samples using Chelex (Walsh, Metzger *et al.* 1991). Loci were amplified in a two-step PCR using a hexachloro-6-carboxy-fluorescine (HEX) labelled M13 primer (HEX- 5'TGTAAAACGACGGCCAGT 3' Schuelke 2000). A two-step PCR was used to improve the amplification of samples due to the low DNA content of some extracts. The first 10 µl PCR comprised 1 µl of 10x buffer with $MgCl_2$ (25mM; Eppendorf, Hamburg Germany), 0.04 µl of HotMasterTaq (Eppendorf), 800 µM dNTP, 4 pmol forward primer, 4 pmol reverse primer and 2 µl of DNA extract. The second PCR was as above except the forward primer was replaced with 4 pmol HEX-labelled M13 primer and 1 µl of the previous PCR was used as the template. Both PCRs were conducted in an Eppendorf Mastercycler with an initial 2 min denaturation at 94^{o}C, followed by either 15 (first PCR) or 35 cycles (second PCR) of 20 sec at 94 ^{o}C, 20 sec at 55 ^{o}C and 30 sec at 70 ^{o}C, and then a final extension for 2 min at 70 ^{o}C. PCR products were visualised on a denaturing acrylamide gel using a Gel-scan 2000 (Corbett Research). Allelic states were determined by visual examination of the resultant banding pattern. After accounting for the allelic contribution of the mother, males were excluded as sires based on an absence of shared alleles with the offspring. A further two loci were tested if paternity could not be resolved from the first four loci. The ability of the system to exclude sires was assessed by the tester being blind to the inclusion of an additional 'offspring', which was actually a duplicate of the maternal sample, in three litters.

Recruitment success and male body mass

The proportion of litters that showed multiple paternity was determined, as was the number of young sired by each male. The genotyped litters were separated into those groups that had mating access to two or three males, and these were examined for biases in recruitment success, based on individual success and rank body mass of males at mating. Recruitment percentages for the male that sired the most young in the two and three male rotations were used as an indicator of the spread of paternity, and differences between the rotations were tested for using a Students t-test.

Significant variation from 50% recruitment success within the two-male mating groups were tested for by normal approximations of the binomial distribution, $B(_{n,p})$ (Binomial $(_{\text{sample size, probability}})$). The same methods were applied to three-male rotations except the recruitment success was changed to 33% and all three categories of each factor was tested. As such an element of pseudo-replication was involved, but it provided an indication of the directional change in each category that would be absent if only a single factor was tested.

2004 effective population size

The reproductive success of animals from the 2004 breeding season, as determined by genotyping, was used to calculate the effective population size (N_e), as a proportion of the census population (N; adults alive at mating). Calculations were as per Lande & Barrowclough (1990), with effective population sizes for males and females calculated separately as:

Males: $N_{em} = (N_m m_m - 1)/[m_m + (V_m/m_m) - 1]$

Females: $N_{ef} = (N_f m_f - 1)/[m_f + (V_f/m_f) - 1]$,

where N_m and N_f is the number of adult males and females alive at mating, m_m and m_f is the mean number of individuals reaching sexual maturity for adult males and females, and V_m and V_f is the variance in adult male and female contribution to the next generation.

These were then combined as:

$$N_e = 4/[(1/N_{em})+(1/N_{ef})]$$

The effective population size was also calculated based on studbook management, where a single male and female are allocated as the parents of all young in a group regardless of actual parentage, and a theoretical situation in which female reproductive success was the same as that observed in this study, but variance in male siring success was minimised through pairing animals.

The effect of culling on the genotyped 2004 effective population size was determined by; A) calculating the contribution to the next generation based on animals alive at weaning, i.e. prior to culling; and B) expanding the census population to include all animals weaned in 2003 i.e. no animals were culled, and calculating the contribution to the next generation based on animals alive at weaning.

Results

Primer selection

Thirteen primer pairs were screened and four were excluded due to non-amplification (Aa7A, BTP8) or lack of variation (Aa7K, Aa1A; Table 1). Three other primer pairs were polymorphic but were excluded due to the presence of null alleles (Aa4F), inconsistent amplification (Aa2H) or difficultly in typing due to narrow allele size range and stutter bands (Aa7M). Null alleles were detected during early screening when the paternity exclusion obtained at Aa4F for animals with a single allele did not match that generated at other loci. When null alleles were factored in, the results at all loci were consistent. The remaining six loci were selected for genotyping as each amplified well, had a minimum of 4 alleles and alleles were clearly resolved. The duplicate maternal sample that was included in three test litters was resolved as not being sired by any of the males present in all instances.

Breeding and genotyping success

Of the 20 females and 16 males mated in 2004, seven females did not give birth, and five males did not sire young. Four of the females that showed no evidence of cycling and did not give birth, belonged to a single breeding group (Group E). Thirteen litters (n=87 young) were genotyped, but the sire could not be identified for five of these young from two litters. These unidentified offspring were split between the two potential sires for N_e calculations. Paternity was assigned to 32 young from the five litters in 2005. Multiple paternity was evident in five of the 11 litters genotyped in 2004, and four of the five litters genotyped in 2005, with a single litter having young sired by all three males in 2004.

Recruitment success and male body mass

When recruitment success was assessed within two and three male rotations, one male within each group had a higher recruitment success than predicted (85%; $B(_{48,0.5})$, $p<0.001$ and 79%; $B(_{58,0.33})$, $p<0.001$ respectively). No difference in the siring success of the most successful male was observed between the two and three male rotations ($t=0.384$, $df=7$, $p=0.71$), with only a single young of the 58 born from the three male rotations sired by the third male. No reduction in recruitment success was evident with hemi-castration, with hemi-castrated males siring 60% of young in three male rotations.

When only the five litters in which multiple paternity occurred from the two male rotation were considered, a significant difference in recruitment success was still observed with one male recruiting significantly more young within a litter (75%; $B(_{28,0.5})$, $p=0.004$). The four litters with multiple paternity from the three male rotation also showed a bias in recruitment success, with one male siring significantly more young within a litter (60%; $B(_{30,0.33})$, $p=0.001$).

Within the breeding groups the heaviest males weighed 62 ± 8g (average ± standard deviation), while the lightest males weighed 52 ± 8g, for an average difference of 10 ± 5g (Figure 2). Within the three male rotations, the heaviest male had a higher recruitment rate than expected (62%; $B(_{58,0.33})$, $p<0.001$), with the middle weight male recruiting less young than expected (16%; $B(_{58,0.33})$, $p=0.002$). Weight was associated with biases in recruitment success in the two male rotations as well, with the heaviest male (71%; $B(_{48,0.5})$, $p=0.002$) again recruiting more young. When only the litters in which multiple paternity occurred were considered, no significant difference in recruitment success was observed for male body mass (50%; $B(_{28,0.5})$, $p=0.149$) in the two male rotation, but a bias was observed in the three male rotation with the heaviest male recruiting significantly more young (53%; $B(_{30,0.33})$, $p=0.010$). Across all litters the heaviest male sired at least one young in 15/16 litters and sired the most, or equal most, young in 75% of litters.

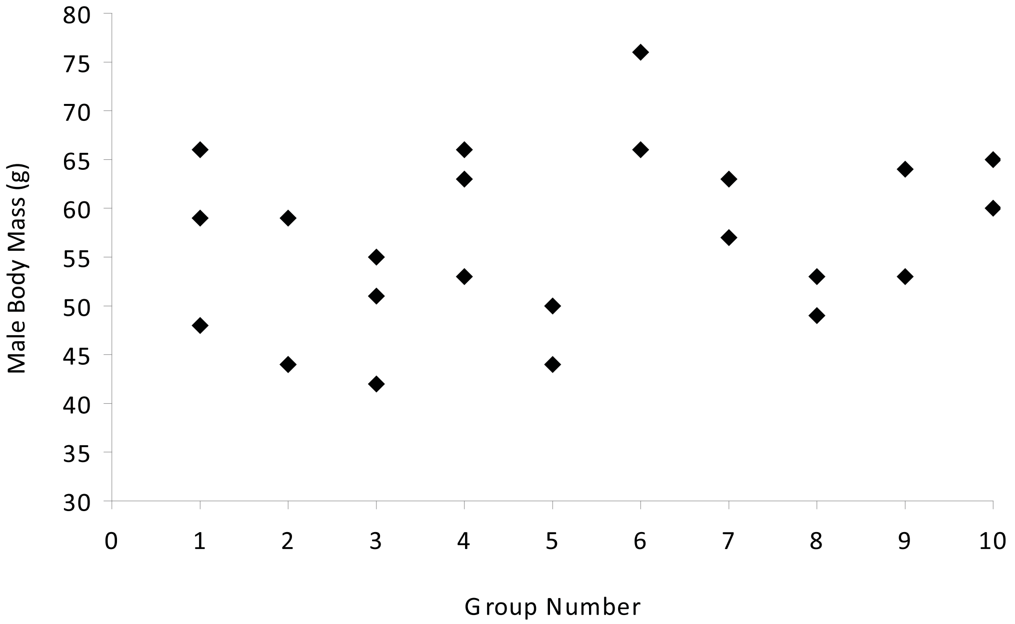

Figure 2. Body mass ranges of male red-tailed phascogale within breeding groups. Groups 1-4 represent 2004 three male breeding groups A-D and group 5 represents the two male group F. Groups 6-10 represent 2005 two male breeding groups.

Effective population size

Based on the genotyping results, the 2004 breeding colony had a N_e/N ratio of 0.51, which is 1.9x that generated from the group managed studbook (Figure 3A). If the recruitment success had been generated from monogamous pairings of animals, rather than from group housing animals, the N_e/N ratio would have increased to 0.6. When family size was calculated based on genotyping all animals alive at weaning, i.e. prior to culling, the N_e/N ratio increased to 0.58 (Figure 3B). Altering the census population to also remove the effect of culling i.e. including all animals weaned in 2003, and retaining the family size at that measured at weaning, saw the N_e/N ratio decrease to 0.33 (Figure 3B).

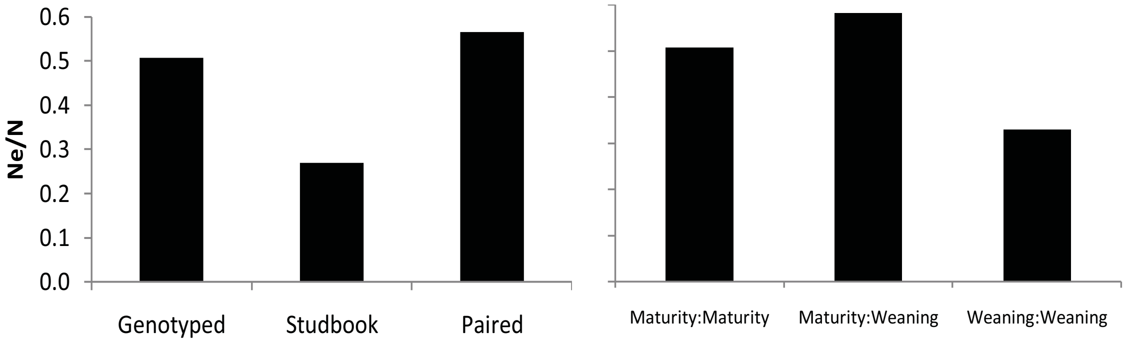

Figure 3. Effective population size as a proportion of the census size (N_e/N) for the red-tailed phascogale colony in 2004.

A) Comparison of N_e/N based on genotyping, the group managed studbook and a theoretical situation in which animals were paired. B) Comparison of N_e/N showing the effects of altering census and family sizes with culling (Census:Family). Maturity represents only animals alive at mating i.e. non-culled, while weaning represents animals likely to be alive if culling did not occur.

Discussion

Our study confirms that multiple paternity occurs among captive red-tailed phascogales and provides information on effective population size and how this varies with group and individual management, and the culling of excess animals. As the studbook assigned all young in a breeding group to two of the five to seven adults per group, their litters were assigned sizes up to four times larger than they actually were. Consequently, the studbook assignment, over-estimates the loss of genetic diversity, with the genotyping N_e/N ratio being 1.9 x greater than that generated from the studbook.

Variance in reproductive success depends on the stage that family size is considered, and as such the stage should be designated (Fisher 1939). Phascogales give birth to more young than they can raise (Foster and Taggart 2008), with litter size constrained to a maximum of eight by teat availability, but sex and paternity can not be determined non-destructively at birth. In our study, paternity was determined just prior to weaning, with 87% of young born reaching weaning, compared to the overall success rate of 68% between 2001 and 2007 (Foster, unpublished data). Due to the loss of young between birth and weaning, results from our study reflect recruitment rather than siring success, and a separate study has been undertaken to examine paternity of embryos in order to understand siring success.

Survival from weaning to maturity is quite high, 94% across years (Foster, unpublished data), if culling is not considered. Culling is an important part of the captive management of red-tailed phascogales though, and so N_e/N calculations were based on those animals alive at breeding. The N_e/N ratio calculated for the 2004 breeding season of 0.51 is higher than the average of 0.11 achieved in unmanaged wildlife populations (Frankham 1995b), and the average of 0.2-0.4 generally observed in captive populations (Frankham 1994; Mace 1986), but does not take into account fluctuation in population size across generations.

Consideration of genetic issues within captive populations are important as small founding populations and limited opportunities to introduce new genetic diversity can lead to declines in fertility and survivorship as individuals become increasingly inbred (Laikre 1999). To maximise effective population size, managers try to equalise family size, sex ratio, generation size and maximise the generation length (Frankham, Ballou *et al.* 2003). These genetic issues need to be balanced against practical and sociobiological factors though (Kleiman 1980), with issues like behavioural incompatibility and aggression restricting the breeding of 'good genetic matches' (Roberts and Gosling 2004). For red-tailed phascogales, some of the issues associated with their captive management and the relationship to maximising effective population size are given in table 2. These factors include limitations of the species life history strategy, e.g. spermatogenic failure of males after the first breeding season, housing constraints that limit population and group sizes, and limitations in the composition of founders.

There was an excess of males among the red-tailed phascogale founders and as such two males were given breeding access to each female. In this situation multiple paternity may have arisen from a directed breeding program aimed at providing an opportunity for retaining the genetics of males that would otherwise have been lost from the program. Multiple paternity may also occur in captive breeding programs as a consequence of housing constraints, from measures aimed at guarding against infertility or from a program aimed at reflecting some degree of the natural interactions between animals and providing some degree of behavioural mate choice.

Phascogale breeding is constrained by availability of holding space and group composition on reproductive success and synchrony (Foster, pers obs), which means that three or four females are housed together. The design of male rotations used in our study arose from observations made in previous breeding seasons of the extended oestrus period of the species, injuries from male-male fighting and availability of staff to undertake regular animal movement. Further work is needed to assess the impact of different mating rotations and group sizes on the spread of paternity, and these results balanced against space and husbandry requirements.

It has been proposed that free mate choice can promote offspring health, allowing for reaction to coevolving pathogens (Wedekind 2002), and the utilisation of multiple paternity allows a limited form of free mate choice to occur. Multiple paternity can potentially act in the opposite manner to polygyny, which is known to reduce effective population size, increase inbreeding and decrease genetic variation (Frankham 1995a). The benefits of multiple paternity may only apply for certain female groups sizes, above which the polygyny benefit obtained by the most successful male outweighs the benefits of polyandry.

Due to the absence of a significant difference in the distribution of paternity when two or three males were used, there appears to be minimal benefit to maintaining a third male per group unless every individual is genotyped. If all individuals are genotyped, the small proportion of young sired by the third male in a group can be detected and selected for breeding, otherwise their contribution may be lost during the selection of new breeders. If the expense and time limitations of genotyping can be met, utilising multiple paternity can be used where factors such as behaviour, life history characteristics and housing constraints don’t allow for the optimal genetic management to occur.

Knowledge of the factors that affect recruitment success can also be important for managing a colony to obtain the best spread of paternity. Our study found that male body mass significantly affected the spread of paternity in red-tailed phascogales, with heavier males having greater recruitment success. Braithwaite (1979) suggested that in male semelparous dasyurid species, male size could be beneficial for determining intrasexual dominance, and has been observed in *A. agilis* (Shimmin, Taggart *et al.* 2002) and *A. stuartii* (Fisher and Cockburn 2006) but the rotations used in our study meant that there were no direct interactions between males. Intersexual interactions, whereby females alter their behaviour to bias fertilisation towards larger males, may occur through measures such as longer matings, matings at most fertile times or by cryptic female choice (Eberhard 1998). A mating bias has been observed against smaller *Antechinus* males that lost male-male interactions (Fisher and

Cockburn 2006). The bias observed in our study could be explained by intersexual interactions, although the mechanism is unknown and requires further investigation.

Table 2. Practical constraints in the management of the red-tailed phascogale colony that affect N_e/N ratio

NOTE:
This table is included on page 97
of the print copy of the thesis held in
the University of Adelaide Library.

[*] Kerr and Hedger (1983)

The high siring success of hemi-castrated males suggests sperm numbers alone may not explain the difference in recruitment success. Parker (1990a) indicated that males could alter sperm allocation to particular females, thereby loading the 'raffle' and improving their chances of siring, but whether this extends to more than doubling the ejaculate from a single epididymis is unknown. Sperm transport is extremely efficient in the carnivorous marsupials

(Breed, Leigh *et al.* 1989; Taggart and Temple-Smith 1991) who have 100-1000 fold fewer sperm in their ejaculates compared to most other marsupials and eutherians (Taggart 1994; Taggart, Shimmin *et al.* 2003), making them interesting models for understanding sperm transport and sperm competition. Using hemi-castrated animals in experimental matings may provide a method for assessing the impact of sperm numbers on siring success.

Further work exploring how genetic similarity, the timing of matings, male body size and sperm numbers affect siring success can provide opportunities for adjusting the spread of paternity to maximise the maintenance of genetic diversity. With appropriate genetic monitoring, multiple paternity can act to offset some of the compromises made in genetic management of captive populations due to limitations imposed by husbandry and social constraints.

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Concluding statement

With red-tailed phascogales showing an unusual life history amongst mammals, they are useful subjects for exploring aspects of reproductive investment. The considerable maternal investment of red-tailed phascogales into the production of young, with losses and alterations in investment occurring at various stages of development, has shed light into the means by which females can attempt to maximise their reproductive investment. With females mating with multiple males and storing sperm within their reproductive tracts for several days prior to ovulation, there is a potential arena for generating biases in siring success, either through sperm competition or sperm choice. By producing more ova and embryos than they are able to raise, females are increasing the likelihood of starting lactation with the maximum litter size, but also allowing for biases in siring success or sex to be generated through differential attachment to teats.

Although there is investment into additional young that are lost at birth, this investment is small due to the young representing less than 0.05% of maternal weight and showing limited development at birth. With the amount of maternal investment increasing with the duration of lactation, alterations in investment that occur at later stages of development are likely to be more significant. Sex biased weight gain was evident during suckling and the weight of young at weaning was correlated with weight at maturity. With biases in siring success also observed, whereby larger males showed an improved siring success, the conditions for Trivers and Willard's (1973) sex biased maternal investment hypothesis were met. These findings provide a basis for comparative studies across the continuum of male semelparous dasyurids and contributes to our understanding of reproductive investment and the strategy I life history.

With red-tailed phascogales having gone extinct over much of their former range and currently being listed as an endangered species, knowledge of species biology is important for its management. The information gained from my study has practical implications for the management of this endangered species. My research was conducted in conjunction with the staff at ASDP where the captive breeding colony of red-tailed phascogales is maintained. This cooperative arrangement has enabled information to be incorporated into the management of the breeding colony, as well as observations made during the maintenance of the captive colony to be included into my research. The life history strategy of red-tailed phascogale provides challenges for the maintenance of a captive population and these factors have to be managed in the context of the practical restrictions of managing captive species, i.e. cost, space, husbandry needs, etc.

One set of factors that provides challenges for captive population management is the restricted breeding season and spermatogenic failure of males. Births among red-tailed phascogale were tightly synchronised on occasion, with many females at ASDP giving birth within a couple of weeks, but variation in breeding season was observed across facilities and across years. Although restricted to only part of the year, captive females showed greater plasticity in their breeding season than has been observed in *Antechinus* (McAllan, Dickman *et al.* 2006). Male reproduction was also not as tightly constrained as *Antechinus* with spermatogenic failure occurring in captive populations only after mating had occurred, meaning males are not solely reliant on epididymal stores for fertilisation success (Taggart and Temple-Smith 1990). This greater flexibility in breeding may reflect a different evolutionary pathway for generating a strategy I life history than occurred in *Antechinus* (Krajewski, Woolley *et al.* 2000).

The high level of investment by females into young can have impacts also on colony maintenance with the potential for rapid growth of the captive population. With females producing more ova and embryos than they can raise, there is a high incidence of females having six or more teats filled at birth and in captivity more than two thirds of young reach weaning. This can mean that a captive population could rapidly exceed the carrying capacity of holding facilities without culling non-breeding and post-reproductive animals.

Colony structure can also be affected by differential investment into sexes. Although there was a male bias in litters at birth, a female bias was evident in the captive colony at weaning. These sex biases are not a major problem in this captive population though due to its high productivity. The differential investment into the growth of young during lactation, whereby males are heavier than females at weaning, may have potential impacts on the genetic diversity of the captive colony due to the finding of a siring bias towards heavier males. The mating rotations used in the captive breeding colony at ASDP minimised the impact of weight compromising the genetic diversity of the population though by the use of related males within mating groups.

Confirmation of multiple paternity within litters meant that the practice of allowing multiple males access to females has been justified, with the limited contribution of the third male has lead to only two males per group being utilised in subsequent years. The genotyping results have been fed back to ASDP to be incorporated into their animal records, helping to provide more detailed information on the colony. The occurrence of multiple paternity within litters meant that genetic diversity within the population was better maintained than appeared from the group managed studbook. The ability to compare genotyping results with a group managed studbook has implications for the captive breeding of other species that utilise multiple paternity, not just red-tailed phascogales.

On a broader level, my study also contributes to the growing body of knowledge about mating strategies, especially sperm competition, generation of sex ratio biases and maternal investment into offspring. The reproductive strategy of both sexes appears to be aimed at maximising the returns on their first, and potentially only, breeding attempt. This is clearly apparent in males where the need to maximise the investment into the first breeding season is amplified through the complete absence of opportunity to breed again; either due to post-mating mortality in the wild, or spermatogenic failure in captivity.

Problems encountered

Working with an endangered species that is part of an active captive breeding colony placed some limitations on the project. Practical aspects such as availability of holding space limited animal numbers, as well as the housing configurations that could be utilised. Due to ASDP's colony being an active captive breeding colony, manipulations were limited and in some instances individuals were used in several studies which sometimes led to conflict e.g. hemi-castrating males for sperm production studies and then looking at the siring success of these males in a competitive mating situation. Sample collection at ASDP also had to occur around normal husbandry and staff having time to collect samples, which they generously did whenever the requests could be accommodated. With the cooperation of ASDP a large amount of new information has been gained that can aid captive management and the conservation of this species.

As the basic biology of the red-tailed phascogale was not well known, there was little information available on which to base husbandry and experiments. ASDP staff shared knowledge about husbandry, but different housing arrangement between Adelaide and ASDP meant appropriate husbandry had to be determined during the project. For example, multiple methods were utilised to settle animals in at Adelaide after transfer from ASDP. The most effective method was found to be placing animals in a large enclosure upon arrival rather than housing individually in smaller enclosures that enabled monitoring of individuals, but which resulted in some animals refusing to eat.

Assessing growth and development of young across multiple institutions is likely to have introduced some variability to the growth measures. In addition to the growth rate effects due to the different origin of WA animals, some of the lower growth rate observed in the WA colony may be related to differences in food and housing conditions. Lighting was observed to effect reproductive synchrony in both males and females, but the effects of temperature and lighting on growth are unknown and can not be determined from this study. Having different people collecting the measurements can also introduce error due to variation in measurement technique.

Some additional methods investigating aspects of the species reproductive biology were tried but were not successful, such as trials looking at mating behaviour not occurring due to the subject males not successfully mating. Genotyping of offspring was complicated by several systems having to be tested before consistent amplification could be achieved. The use of brothers in some mating groups meant that I could not establish paternity for several young with the markers available. This could be aided by the utilisation of further loci, and although a further eleven primer pairs were screened, only a single additional polymorphic locus was found, which was not sufficient to resolve paternity. Despite the problems encountered, a large amount of new information has been learnt about this species.

Future directions

In addition to the research that I have presented in this thesis, studies investigating siring success prior to the loss of supernumerary young, mating behaviour, vitamin levels and follicular development have been undertaken. ASDP have also been continually updating their husbandry and have undertaken several trial translocations, which have provided much useful information for future interventions with this species and other dasyurids. Together, the work presented in this thesis and the other studies listed above, have greatly increased our knowledge about red-tailed phascogales, but have also opened up areas for future investigation.

Further investigation into the genetic diversity of the captive red-tailed phascogale population would be useful for the captive management of this species, as well of other dasyurids and species that exhibit multiple paternity. Genotyping the founders, animals breeding in 2004 (examined as part of this thesis) and animals breeding several generations on, would allow for founder contributions to be determined. This would provide opportunity for tracking rates of allele loss and directly comparing the loss of genetic diversity based on studbook records and genotyping.

Further investigation into the role of reproductive strategies in species survival and evolution is needed, with comparison to determine the validity of using data collected from captive colonies to extrapolate to what is occurring in wild populations. Although baseline studies on wild phascogales have been conducted, further investigation into non-breeding and breeding home ranges, nest sharing during breeding, the timing of births in the wild, dispersal and changes in sex ratio throughout the year would provide a better understanding of the factors driving population dynamics.

With obligate male semelparity, like that seen in the red-tailed phascogale and other strategy I dasyurids being unusual amongst mammals, comparison with other species, especially the little red kaluta *Dasykaluta rosamondae,* would shed further light on factors affecting this life history strategy. Comparison of reproductive characteristics across the three evolutionary pathways that resulted in obligate male semelparity, and with two facultative male semelparous species, northern quoll and southern dibbler, would also provide opportunities to further elucidate the factors driving the evolution of semelparity.

The discrete population structure and limited numbers of breeding attempts of strategy I dasyurids make them useful subjects for investigating reproductive investment and sperm competition. With females mating with multiple males and storing sperm in their reproductive tracts for extended periods, opportunity is available to investigate sperm characteristics during storage. For example, in *Sminthopsis crassicaudata* along the length of the oviduct there was differential degrees of sperm occupation in the crypts with differences also observed in the morphology of sperm in relation to position within the oviduct (Bedford and Breed 1994). Development of *in-situ* PCR would allow for testing if there was differential occupation of crypts by sperm from different males or by X or Y bearing sperm.

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