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Submitted for the Degree of Doctor of Philosophy

*Sex Ratio Theory
Applied to a
Macropod Marsupial*

*Is reproduction by the tammar wallaby (*Macropus eugenii*) consistent
with sex ratio theory?*

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Abstract

Marsupial species provide an important research tool in attempts to unravel the complicated mechanisms of mammalian reproduction. Comparisons between metatherian and eutherian reproductive life history patterns used to achieve the same outcomes, illuminates processes that have been difficult to investigate in eutherian species alone. Adaptive sex allocation is one such area that has provided equivocal data that is much scrutinized and debated amongst scientists. The theory that has provided the most disparity in the literature, and lead to the most debate, is the Trivers-Willard Model (TWM) (Trivers & Willard, 1973). The TWM proposes that, in polygynous mammalian species where the male has increased variability in reproductive returns, mothers in 'good condition' should bias their offspring towards sons, to maximise their fitness. There are three main assumptions associated with the theory: 1) that sons will benefit more than daughters from increased maternal resource allocation, 2) that juvenile condition predicts adult condition and 3) that maternal quality is a predictor of offspring quality. Much of the previous support for the TWM has been based on research in eutherian species (*e.g.* ungulate species), which usually tests only one assumption at a time by *a posteriori* application of the theory to observed data. Macropod species fill similar niches to ungulate species in other ecosystems and provide easy access to pouch young at a very early stage of development, which facilitates the investigation of all the TWM assumptions on a single population. The assumptions were tested by 1) field collections on wild populations of Kangaroo Island *Macropus eugenii*, to see if the pattern occurred, 2) controlled experimental breeding, to find the true adaptive nature of any bias and to see if the effect could be produced by manipulation, 3) feeding experiments, to see if compensatory growth occurred, 4) further testing on females and males to assess the presence and timing of control mechanisms. The overall sex ratio of the sampled wild population was 1:1, but larger, older females showed a 74% chance of having daughters as opposed to sons, while smaller, younger mothers showed a 60% chance of having sons. The pattern was not repeated in the breeding experiments, as females showed an 81% chance of producing a son regardless of maternal condition. Juvenile females produced an overcompensation response to reduced resource availability, while males showed partial compensation, recovering body weight but not condition. There was no evidence of a post-conception maternally-controlled mechanism, as there was no discrepancy between genetic sex and phenotypic sex. However, adult males did show a reduced %Y sperm count, averaging 44-45% Y sperm, significantly lower than the expected 50%. Therefore, although some of the results showed confirmation of the TWM, when analysed separately, there was little support for the individual assumptions and it is more likely that there is a more complicated, stress induced, adaptive sex ratio process in this species, controlled by either (or both) sex prior to or at conception, which is influenced by external stimuli other than resource allocation or in conjunction with it.

Statement of Originality

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 to Aryn W. Perryman 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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Aryn W. Perryman

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CHAPTER 1

Introduction

1.1 Introduction

Mammals are a wide and varied group of animals, filling an eclectic array of ecological niches throughout the natural world. Over the past ~ 125 million years (Cifelli & Davis, 2003; Luo et al., 2003), three sub-classes have evolved, radiating throughout a wide variety of ecosystems, and dominating many of them (Tyndale-Biscoe & Renfree, 1987; Tyndale-Biscoe, 2001). The three main sub-classes are monotremes, marsupials (metatherians) and eutherians, which are mainly distinguished by their mode of reproduction. The most widespread of the three sub-classes is the eutherians (~4360 species), with marsupials less biogeographically distributed (~272 species) (Wilson & Reeder, 1993), ranging mostly in Australasia and South America, with one species *Didelphis virginiana* occurring in North America. Monotremes are comprised of only two families: echidnas (family *Tachyglossidae*), found in Australia and New Guinea, and the platypus (family *Ornithorhynchidae*), found only in Australia (Wilson & Reeder, 2005).

Although originally thought to be an early evolutionary step to the more advanced eutherians, recent evidence suggests that marsupials and eutherians have filled similar niches in their distinct habitats through co-evolution (Tyndale-Biscoe, 2001). This co-evolution most likely occurred because metatherian and eutherian animals have distinct physiological adaptations, which allow for separate manipulation of different environments, *i.e.* marsupials are better adapted to the fluctuating climate in Australia, while eutherians, in certain environments, are able to dominate metatherians, essentially out-competing them. With the introduction of European settlement in Australia two centuries ago (McKenzie et al., 2007), changed land use patterns, introduction of foreign predators and competition species, and increased encroachment on habitats have forced the extinction of a large portion of the extant marsupial species, a decline more severe than on any other continent (Baillie, 1996). This human impact has also permanently influenced those marsupial populations that have survived the invasion, effectively diminishing biodiversity from the Australian milieu. As Darwin once said:

So in the general economy of any land, the more widely and perfectly the animals and plants are diversified for different habits of life, so will a greater number of individuals be capable of their supporting themselves. A set of animals, with

*their organization but little diversified, could hardly compete
with a set more perfectly diversified in structure.*

(Darwin, 1859)

Thus, the stability of Australian ecosystems is currently under threat due to the systematic extinction forced by human colonization. For science, this is a detrimental, because the study of the similarities and differences between the three mammalian sub-classes illuminates the origins of mammalian reproduction, especially the adaptation of viviparity (Tyndale-Biscoe & Renfree, 1987), and comparisons help elucidate general principles of life history by disclosing the divergent methods undertaken by each to reach the same outcome.

1.2 History, Evolution and Taxonomy

Mammals first reached Australia in the Cretaceous, roughly 115 million years ago (mya), when it was still part of the southern super continent of Gondwana (Tyndale-Biscoe, 2001), with the earliest monotremes described in areas of New South Wales appearing at roughly 110mya (Archer et al., 1985). However, these early mammals were not members of the marsupial or eutherian lineages, which originated in what is now northern Europe roughly 125mya (Ji et al., 2002) for eutherians and ~100mya for metatherians (Cifelli, 1993), and came to dominate in the Tertiary (Tyndale-Biscoe, 1973, 2001), with peak diversification rates ~33mya driven by rodents, cetartiodactyla and marsupials (Stadler, 2011) (Table 1.2.1). There is some conjecture regarding the eutherian-metatherian split, as early discussions suggested that marsupials were an evolutionary stepping stone to the more advanced eutherian species, placing them on the same evolutionary branch (Tyndale-Biscoe, 1973), as an intermediate step to eutherian viviparity (Darwin, 1859; Huxley, 1880; Martin, 1902). However, more recent research has placed the common ancestor at least 125mya (Luo et al., 2003) in Laurasia, most likely between 148-159mya (Archibald, 2003), with some debate dating it even further back at 167-190mya (Woodburne et al., 2003). Despite the conjecture on the timing of the split, this evidence makes the two clades sister taxa evolving simultaneously, not one from the other.

Table 1.2.1 A synopsis of Australia's past (figure from Tyndale-Biscoe, 2001)

Years Before Present	Events
115 000 000	Earliest fossil mammal in Australia (not part of the modern mammal lineage)
110 000 000	Earliest fossil monotremes in Australia
55 000 000	Earliest fossil marsupials and eutherians in Australia
45 000 000	Separation of Australia from Antarctica
26 000 000	First known fossils of modern families of marsupials
14 000 000	Rise of kangaroos, possums, dasyurids; rodents enter Australia
6 000 000	Giantism in kangaroos and diprotodontids
60 000	Earliest human presence in Australasia
45 000	Extinction of giant marsupials
500	First European encounters with Australian fauna
200	European settlement
100	Decline/extinction of small-medium size marsupials

The evolution of the modern therian lineages occurred in South America, after migration from Laurasia through North America (Cifelli, 2004; Horovitz et al., 2009) and descent into the South American/Antarctic/Australian land mass (Cifelli & Davis, 2003; Sige et al., 2009). The North American marsupials died out, although this area was later recolonised by *Didelphis virginiana*, an opossum. The remaining marsupial species are commonly split into Ameridelphia (those living in South America) and Australidelphia (those living in Australia and adjacent islands), which is the most highly radiated group of extant marsupials (Szalay,

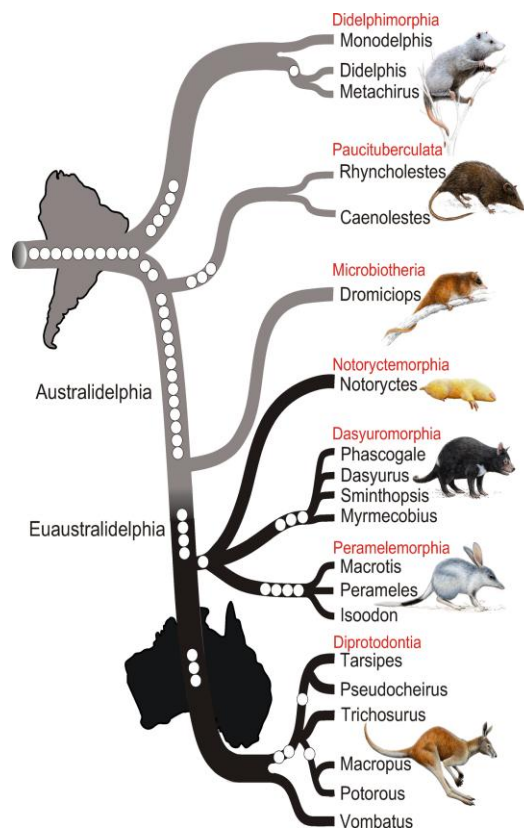


Figure 1.2.2 Phylogenetic tree of marsupials derived from retroposon data.

The names of the seven marsupial orders are shown in red, and the icons are representative of each of the orders: Didelphimorphia, Virginia opossum; Paucituberculata, shrew opossum; Microbiotheria, monito del monte; Notoryctemorphia, marsupial mole; Dasyuromorphia, Tasmanian devil; Peramelemorphia, bilby; Diprotodontia, kangaroo. Gray lines denote South American species distribution, and black lines Australasian marsupials. Figure obtained from Nilsson *et al.* (2010).

1982). There is evidence that some metatherian species that evolved in Australasia later recolonised South America (Kirsch *et al.*, 1991; Kirsch *et al.*, 1997; Nilsson *et al.*, 2010) and *vice versa* (Sige *et al.*, 2009) (Figure 1.2.2), but a definitive evolutionary split occurred when Australia separated from South America and Antarctica roughly 45mya (Nilsson *et al.*, 2004; Sige *et al.*, 2009). The most diverse order of Australidelphia is the Diprodontia with some 120 extant species (Asher *et al.*, 2004; Nilsson *et al.*, 2004), comprising koalas (*Phascolarctidae*), wombats (*Vombatidae*), possums (*Phalangeroidea* and *Petauroidea*), wallabies and kangaroos (*Macropodidae* and *Potoroidae*), with the Macropodoids representing the most diverse marsupial herbivores (Prideaux & Warburton, 2010).

Modern eutherian and marsupial species have experienced very different levels of success, with marsupials comprising only 6% of mammalian species in the world (Lillegraven, 1975; Kelly & Sears, 2011a, b). Given the poor success of marsupial species in other parts of the world, the literature abounds with theories explaining the dominant radiation of marsupials in Australasia. Initially, Darwin, and many since him, assumed that eutherian mammals failed to cross into Australia from South America during the early Tertiary, and that this failure explains why metatherian species dominated Australia's therian assemblages due to lack of competition from better adapted eutherian species (Darwin, 1859). However, more recent evidence suggests a free flowing exchange of marsupial species across the South America/Antarctica/Australasia Gondwanan landmass in the early Tertiary (Szalay, 1982; Kirsch *et al.*, 1991; Kirsch *et al.*, 1997; Sige *et al.*, 2009), which occurred during the same time period as the development of South American eutherian mammalian species. In support of this idea, eutherian fossils were discovered alongside marsupial fossils in Queensland, both dating back to the Cainozoic period (roughly 55mya). This suggests that,

unlike in South America and possibly Antarctica, eutherians arrived in Australia and failed to persist for some reason (Godthelp et al., 1992). Hugh Tyndale-Biscoe (2001), one of the foremost experts on marsupial ecology, suggests that eutherian mammals failed to thrive in Australasia once the separation from Antarctica occurred (~45mya) and weather patterns on the continent changed forever with the development of the Antarctic current and the southern polar icecap. He suggests that metatherian species were pre-adapted to survive in the new Australian climate, with physiological adaptations, *i.e.* reproductive systems and metabolic rates, especially suited to arid environments, with low fertility soils and erratic climates.

1.3 Physiological Adaptations

Physiological adaptations have allowed marsupial species to diversify in the Australian environment and fill similar niches as their eutherian counterparts (Tyndale-Biscoe, 1973; Hume, 1999). The convergent evolution of the two groups (Archer et al., 2011) produced early comparisons between eutherian mammals on other continents, such as the woodchuck, antelope, mole, dog, wolverine, and otter with marsupial animals in Australasia and South America, *i.e.* the wombat, kangaroo, notoryctes, thylacine, Tasmanian devil and the water opossum, respectively (Main et al., 1959). Further examples are comparisons between the marsupial glider and the flying squirrel, or the ringtail possums and rabbits, both produce two types of faeces and eat the softer one for higher nutrition, or wombats and horses, which both digest grasses in the same manner, or lastly, numbats and eutherian anteaters, which have similar methods for feeding on termites (Hume, 1999). The strength of this co-evolution was so strong that many early taxonomists, after marsupials were discovered in the 1500's, placed them in eutherian families, and it was not until the 1800's, when a scientist named de Blainville (1816, 1834) started looking for more fundamental relationships, that marsupials were set apart (Tyndale-Biscoe, 1973).

Despite the similarities between eutherian and marsupial radiation in their distinct environments, the evidence for Tyndale-Biscoe's (2001) theory lies in the physiological differences between the two, which allow marsupials to thrive in extreme weather conditions, including prolonged periods of drought, and in areas of low fertility soils. The main physiological differences are found in the mode of reproduction and metabolic rate. Traditionally, the eutherian mode of reproduction and higher metabolic rate serve to increase the ability of eutherian mammals to competitively exclude equivalent marsupial species (McNab, 2005), as marsupials are less tolerant of cold environments and appear to be capable of coexistence only when fluctuations in local availability of food restricts reduced rates of reproduction in eutherians. For example, the arrival of dingos in Australia occurred at the same time as mainland extinction of thylacines, Tasmanian devils and eastern quolls, while carnivorous marsupials in South America were replaced by eutherian equivalents when the Panamanian bridge with North America was reconnected (Strahan, 1983; Corbett, 1995). However, the Australian continent represents a unique microcosm of environmental factors that allow marsupials to persist and thrive where eutherian species cannot.

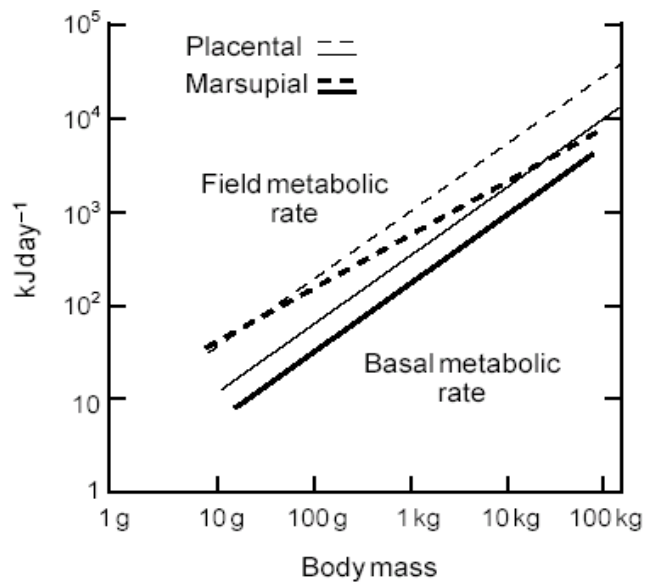


Figure 1.3.1.1 Comparison of basal and field metabolic rates in marsupials and eutherian mammals to show the greater metabolic scope of small marsupials than large marsupials and eutherians, and to show the lower metabolic rates of marsupials as a group to eutherians. Basal metabolic rates from Dawson and Hulbert (1970); field metabolic rates from Nagy (1987). (Figure cited from Tyndale-Biscoe, 2001).

1.3.1 Metabolic Rate

An animal's survival requires the utilisation of resources from its surrounding environment and one of the most crucial resources needed for survival is energy (Nagy, 2005), primarily in the form of chemical potential energy from digested food. Chemical potential energy is the 'common currency of life' that drives all biological processes, *i.e.*

thermoregulation, reproduction and foraging, for all organisms.

The primary goal of every

individual is the acquisition of enough energy to sustain life each day. In processing this energy, organisms exchange energy and materials with the environment and transform it into usable energy to 'power' their every movement and physiological process, otherwise known as *metabolism*. The rate at which this exchange and transformation occurs is known as the metabolic rate (MR) (Savage et al., 2004). There are three main measures of metabolism: basal metabolic rate (BMR), the minimum rate of metabolism compatible with endothermy (McNab, 1988), field metabolic rate (FMR), energy required for daily activity in the wild (Nagy, 1994), and maximum sustained metabolic rate, or maximal metabolic rate (MMR), the highest rate of sustainable energy expenditure without using fat reserves (Hammond et al., 1994).

Historically, the literature claimed that the average marsupial BMR is 30% lower than that of eutherians (Dawson & Hulbert, 1970), and many considered marsupials metabolically 'primitive' mammals in comparison (Dawson, 1989). However, more recent conjecture indicates parity between the two groups' maximum aerobic capacity, meaning metatherians have a greater aerobic scope (Dawson & Dawson, 1982; Hinds et al., 1993; Dawson et al., 2004) despite their low BMR (Figure 1.3.1.1). Although the residual variation in marsupials is slightly less than eutherians (Figure 1.3.1.2), the mean value of marsupial BMR remains ~30% below that of eutherians, but life history parameters are sometimes strong enough to mask phylogeny.

In the last 25 years, the development of data available on FMR in a plethora of marsupial species (Tyndale-Biscoe, 2001), has facilitated comparisons between metatherians and eutherians, as it is a much better indicator of a species' actual metabolic needs. Marsupial FMRs, unlike their BMRs, are much the same as eutherians, particularly in smaller marsupials (Nagy, 1987). Mammalian FMRs vary widely, mostly with variation in body mass (explains >90% of variation) and the FMR scaling of marsupials ($\text{kJ day}^{-1} = 10.83 \text{ g}^{0.582}$) is much higher than that of eutherians ($\text{kJ day}^{-1} = 4.63 \text{ g}^{0.762}$) (Nagy, 1994), sometimes having a scope of over three times their BMR, which is much greater than the scope for equivalent eutherian species (Nagy, 1987).

Marsupials have other physiological adaptations that contribute to their increased metabolic scope and energy conservation, allowing them to thrive in resource poor and climatically extreme environments. Metatherians have low conductance, or loss of heat, which, combined with low BMRs, facilitates energy conservation in species that have limited supplies of available energy (McNab, 2002). Marsupial species from low-productivity habitats due to their low BMRs also display less evaporative water loss (Withers et al., 2006), lower food/nutrient requirements (McNab, 1986), longer lasting fat reserves in harsh environments (Dawson & Olson, 1988) and lower voluntary food intake (Hume, 1999).

Despite the conservative metabolic profile of marsupial species, in most environments eutherian species have the competitive edge due to their higher BMR and body temperatures. Eutherians are more thermoregulative (Lillegraven, 1975; Lillegraven et

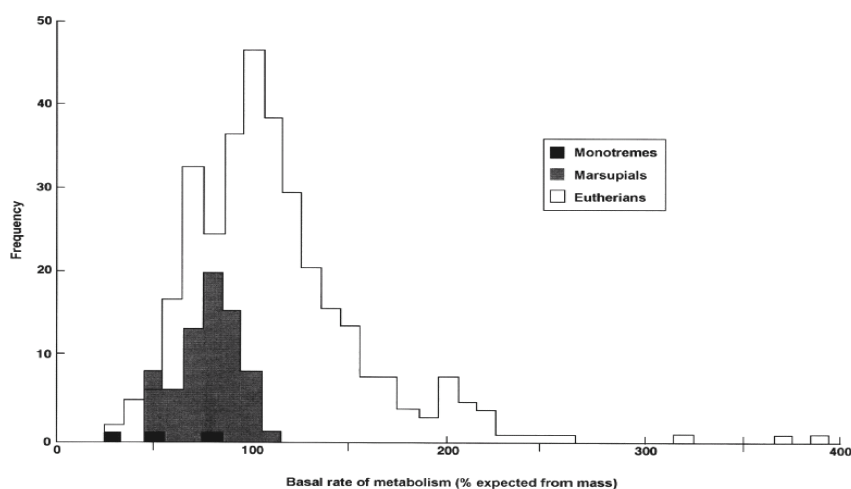


Figure 1.3.1.2 Frequency distribution of basal metabolic rate (BMR) in marsupials (from McNab, 2005) and eutherians (from McNab, 1988a), expressed as a percentage of the values expected from body mass in the general mammal curve derived from McNab (1988a) (figure cited from McNab, 2005).

al., 1987), with increased nerve conduction and growth rates (Russell, 1982b), which in environments without resource restrictions would competitively exclude their marsupial

contemporaries. However, McNab (1986) argues that metatherians can reach equivalent radiation with their eutherian counterparts in environments where resources are scarce and energy expenditure must be economical. Thus, the increased metabolic scope exhibited by marsupial species most likely affords them an advantage in extreme environments, as a low BMR conserves resources without limiting metabolism during activity (Tyndale-Biscoe, 2001). For example, comparisons between grazing stock and macropod marsupials in Australia show that macropodoids have water turnover rates of only 13% that of eutherian species and wild FMRs of 29% that of sheep, as a result macropod species are much better suited to the varying climate in central Australian rangelands (Munn et al., 2009). Nevertheless, this metabolic advantage has not resulted in wide marsupial success outside of Australasia, as the metatherian mode of reproduction prevents resource conversion into high rates of reproduction, and, although this might have been an advantage in environments of infertile soils and uncertain climates, it is a disadvantage in habitats of high resource availability (Tyndale-Biscoe, 2001).

1.3.2 *Reproduction*

Eutherians have a higher rate of reproduction, under conditions of high resource availability, than equivalent marsupial species (Russell, 1982b). The reproduction rate of eutherian mammals is correlated with their higher BMRs as a group, with an increase in BMR (corrected for body size) linked to a decrease in gestational period and an increase in post-natal growth rate, fecundity, frequency of population cycles (McNab, 1980, 1986; Stephenson & Racey, 1995), amplitude of lactation (Glazier, 1985; McLean & Speakman, 2000) and litter size (Genoud, 1988). Such an association between BMR and reproductive output in marsupials is not seen (McNab, 2005), as the rate of maternal resource and waste exchange cannot increase without risk of immunological rejection due to the type of tissues used for resource exchange in metatherian species. Therefore, an increase in BMR in eutherians equates to an increased reproductive yield, which occurs whenever resource availability permits.

There are physiological constraints on marsupial reproduction that restrict the maternal ability to convert abundant resources into high rates of reproduction. Marsupial young are born very small, usually between 0.001-0.01% of adult body mass (as small as 5mg in the honey possum (*Tarsipes rostratus*) and as large as 800mg in the red kangaroo (*Macropus rufus*) (Renfree & Shaw, 1996)), after short gestation periods, at which point they crawl to the maternal pouch, attach to a teat and finish development during a prolonged lactational period (Tyndale-Biscoe & Renfree, 1987) (Figure 1.3.2.1). Although the duration of pregnancy in metatherians is similar to that of small eutherians, the rate of resource transfer during pregnancy is much lower in marsupial species. For instance, the marsupial quokka, *Setonix brachyurus*, and the wild rabbit, *Oryctolagus cuniculus*, both have a gestation period of 27-28 days, but the rabbit gives birth to ~6 young each weighing 35g, while the quokka gives birth to 1 young weighing 0.34g (Tyndale-Biscoe, 1973). The difference in resource transfer between the two species represents a 600-fold increase in maternal investment by the rabbit. After parturition, the rabbit mother weans her young after 3 weeks of lactation, while the quokka's lactation lasts for 30 weeks. A female rabbit can produce three full litters to independence in the same amount of time, while a seasonal quiescence period in the quokka reproductive cycle means only 1 young is produced each year (Tyndale-Biscoe, 2001). Therefore, if these two species coexisted in an environment with no resource restrictions, the rabbit would soon overrun the quokka (McNab, 2005).

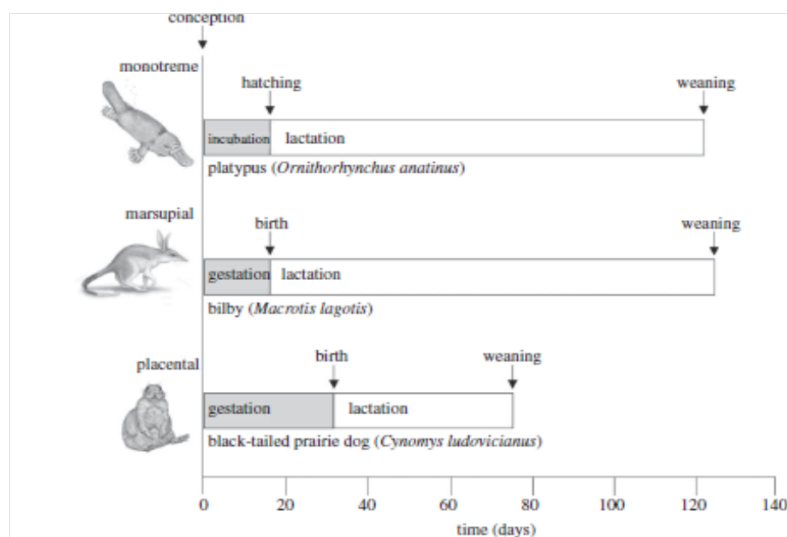


Figure 1.3.2.1 Schematic differences in timing of the life history in similar-sized monotremes, marsupials and placentals. Female body mass *ca* 1500g (Hamilton et al., 2011)

If a faster growing marsupial is used for comparison, *e.g.* the long-nosed bandicoot (*Perameles nasuta*), which is also one of the few marsupials to use a eutherian like placenta during gestation (Padykula & Taylor, 1976), marsupial maternal allocation is still far

below that of the eutherian. The bandicoot's lactation is much shorter than the quokka's, but it is still three times longer than the rabbit's. The increased rate of

resource transfer displayed by eutherian mothers is due to the utilization of the eutherian placenta during a longer gestation, while marsupial species are restricted by the resource transfer capabilities of lactation (Table 1.3.2.2). Even in the case of the bandicoot, the placenta employed by the eutherian species is far more complicated than the rudimentary placenta developed in the much shorter gestation period of the bandicoot.

Table 1.3.2.2 Comparison of maternal investment in 2 marsupials and a eutherian, the wild rabbit (figure from Tyndale-Biscoe, 2001)

Species	Mass (kg)	Gestation (days)	Neonate (mg)	Litter Size	Lactation (days)
Wild Rabbit <i>Oryctolagus cuniculus</i>	1.2	28	6000	6	21
Quokka <i>Setonix brachyurus</i>	1.2	27	340	1	200
Long-nosed Bandicoot <i>Perameles nasuta</i>	0.8	12.5	250	3	60

The eutherian placenta (chorioallantoic placenta) (Behringer et al., 2006) is derived from embryonic and maternal tissues that facilitate the maternal passage of resources to the embryo at an amplified rate without triggering an immunological rejection response (Lillegraven, 1975; Parker, 1977; Hayssen et al., 1985; Lillegraven et al., 1987). Alternatively, marsupial embryos develop inside a uterine shell membrane that isolates maternal tissues from fetal tissue, protecting the embryo from immunological rejection (Freyer et al., 2003).

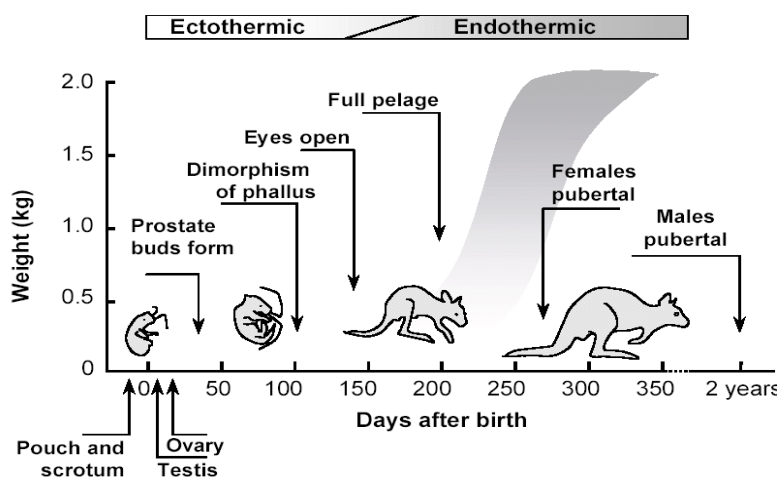


Figure 1.3.2.3 Stages of development in the tammar wallaby, development of the young in the pouch, occurring after parturition. Figure based on Tyndale-Biscoe & Janssens (1988), and cited from Renfree et al (2001).

Marsupial young, due to their prolonged development during lactation rather than gestation, are born ectothermic (Frappell, 2008) and in an altricial state, prior to most organogenesis (Tyndale-Biscoe & Renfree, 1987; Frigo & Woolley, 1997;

Miller et al., 2001; Behringer et al., 2006; Renfree et al., 2006; Shaw & Renfree, 2006), including sexual development, which transpires post-parturition except for the development of the pouch and scrotum, which is controlled separately and occurs prior to parturition (Shaw et al., 1988; Nurse & Renfree, 1994; Renfree et al., 2001b) (Figure

1.3.2.3). Conversely, eutherian species tend to give birth to neonates that vary widely between altricial and precocial, but all have undergone organogenesis in the embryonic environment prior to parturition (Behringer et al., 2006). For example, a 200 day old tammar wallaby (*Macropus eugenii*) is physiologically equivalent in size and development to an ungulate calf at birth (Janssens & Ternouth, 1987) or a newborn ruminant, making days 0-218 of lactation in the tammar equivalent to gestation and early lactation in some eutherian species (Cork, 1991).

Altricial eutherian neonates are also more highly developed than the altricial young of marsupials and monotremes and develop faster after birth (Ferner et al., 2009; Mess & Ferner, 2010) (Figure 1.3.2.4), which is a direct consequence of the higher eutherian BMR and increased energy transfer during *in utero* placental development. Placental progeny have a high metabolic rate at birth and attain adult metabolic rate and thermoregulatory capacity early in postnatal development, while marsupial neonates have a low metabolic rate and attain adult metabolic rate and thermoregulatory capacity later in pouch life (Munn & Dawson, 2001; Szdzyu et al., 2008; Szdzyu & Zeller, 2009). However, despite these metabolic differences, both metatherian and eutherian neonates follow the same development path, *i.e.* lung development in both groups follows the same mammalian pathway, but at different paces (Szdzyu et al., 2008).

In accordance with marsupial post-natal development, marsupial mothers have three distinct stages of lactation during pouch life, each with a different molecular composition (Green, 1984; Green et al., 1988; Young et al., 1997; McNeilly, 2001; Trott et al., 2003; Rose & Flowers, 2005).

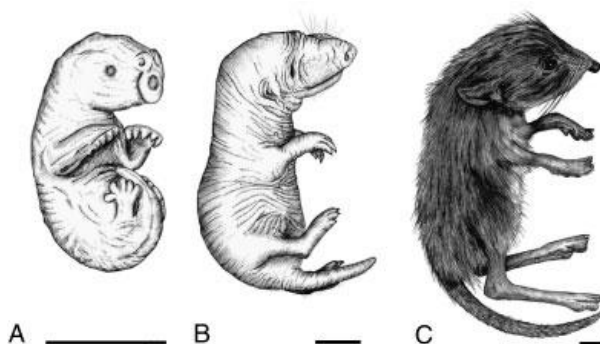


Figure 1.3.2.4 Original drawings of neonatal (A) *Monodelphis domestica* a newborn marsupial with an embryonic appearance, note the undifferentiated oro-nasal region and hind limb paddles, and the more advanced forearms; (B) *Suncus marinus* an altricial eutherian newborn with a significantly more advanced appearance, with well developed extremities including claws, but is naked with closed eyes; and (C) *Macroscelides proboscideus* a precocial eutherian newborn with a far more advanced appearance, open eyes and fully furred (Szdzyu & Zeller, 2009).

et al., 2003; Rose & Flowers, 2005). Marsupial mothers use these stages of lactation to drive offspring development, unlike in eutherians where lactation is controlled by the offspring (Haig, 1993, 1996) and only one formula is produced for the duration of lactation (slight changes in composition occur in eutherians, but not to the same extent), (Merchant & Sharman, 1966; Green, 1984; Oftedal, 1985; Trott et al., 2003), *e.g.* the lipid

content of milk in the rabbit doubles during lactation while the increase in *Macropus eugenii* increases by more than 400% (Green et al., 1983). As lactation in eutherian mammals is much shorter, but offspring development rate is higher, eutherian lactation has a higher transfer rate of energy and nutrients, while marsupial lactation is much longer, *i.e.* 18 months in the grey kangaroo (*M. major*) (Kirkpatrick, 1965). Marsupials have lower daily milk and energy outputs and a lower maximum lactation output than eutherian species, but the total energy output of metatherian lactation, to weaning of an independent young, is equivalent to that of eutherian species (Cork & Dove, 1989), and size of young at weaning is consistent across the lineages (Hamilton et al., 2011).

Despite these physiological differences, the common thread of all mammalian reproduction is that maternal resources supply all the nutrition to rear offspring to independence (Charnov, 1993). So, the maternal metabolic rate drives the production of biomass, which limits the ways in which life-history traits can trade-off to meet metabolic and nutritional requirements of progeny at different stages of development (Hamilton et al., 2011). The rate of offspring production depends on the rate at which maternal biomass can be produced and circulated (West et al., 1997). Despite the physiological life-history differences between the three mammalian groups, they all expend a similar amount of energy on reproduction (when corrected for body size) (Hamilton et al., 2011). In addition, pre-weaning survival is equivalent between the two groups, as pouch-rearing in metatherians increases survival, but decreases litter size, while in eutherians, survival rate is reduced due to exposure to additional external sources of mortality, but this is offset by larger litter sizes (Hamilton et al., 2011), thus the rate of production is consistent across lineages. In fact, despite the slower development times and ages at first reproduction of marsupial mammals, the maximum lifespan scales similarly with that of eutherians, when corrected for body size (Hamilton et al., 2011). This shows that eutherian reproduction is not superior to metatherian reproduction, rather the different reproductive physiologies and ecologies between the two clades are each adapted to particular environments (Russell, 1982a; Dickman & Woodford Ganf, 2006).

Australian metatherian species have physiological adaptations that are better suited to the extreme climate and resource fluctuations of the continent. Because the maternal marsupial investment in gestation is small, and most development takes place in the pouch during a prolonged lactation phase, female marsupials can terminate offspring in unfavourable conditions without large losses in resource investment (Tyndale-Biscoe,

2001), as evidenced by red kangaroos (*M. rufus*) who lose their young during lactation failure caused by adverse conditions (Frith & Sharman, 1964). These early stages of reproduction can then be repeated quickly with a reduced fitness cost. This is in contrast to eutherian species, as once a eutherian mother is committed to pregnancy, termination is only possible with a serious loss of investment and at high risk to the mother. In habitats where resources are predictably, seasonally abundant, the rapid transfer of resources to eutherian neonates prohibits any competitive advantage afforded by the marsupial mode of reproduction. However, the lengthy metatherian reproductive cycle, with reduced daily maintenance costs, is highly suited to unreliable environments.

The reduced maintenance costs associated with the lower BMR and increased metabolic scope of marsupials also means they have lower water turnover and reduced rehydration requirements (Kinneer et al., 1968; Lentle et al., 1999), need less daily dry matter and lose less weight on poor forages than eutherian species (Hume, 1974, 1999), have lower heart rates, when adjusted for body mass, (Kinneer & Brown, 1967) and lower nutrient requirements (Hume, 1999) than eutherian mammals. These adaptations afford a competitive advantage to marsupials, for example, when attempts to graze sheep on Rottneest Island occurred in the 1960's, the sheep all suffered deficiencies in essential elements (*i.e.* copper and cobalt) (Barker, 1961b, a) but the indigenous macropod, the quokka (*Setonix brachyurus*), suffered no deficiencies as requirement for these elements were 50% less than in the sheep. In addition, macropod marsupials, although conforming to the same predictive model for heat balance, energy requirements and exercise/locomotion as eutherians and with similar muscle make-up, density and aerobic capacity (Dawson et al., 2004), exhibit independence between metabolic rate and locomotion speed not seen in eutherian mammals, this saves up to 50% of the energy that would be used for locomotion by eutherian counterparts (Baudinette, 1994). Thus, when these adaptations are considered in conjunction with the similarity in reproductive production across the groups, it becomes clear that these divergent reproductive strategies are metabolically equivalent ways of being a mammal (Hamilton et al., 2011).

1.4 Population Pressures, Conservation and Research Value

Although marsupial species are well adapted to the extreme climate changes in the Australian landscape and have radiated more widely in Australasia than anywhere else on earth, recent changes to weather patterns due to human use, development, agriculture and global warming, coupled with hunting and introduced species, are causing mass fauna extinctions (Tyndale-Biscoe, 2001). When humans first migrated to the Australian continent a mass extinction of large mammalian species occurred, roughly 45,000-50,000 years ago, due to over hunting, at rates that were unsustainable by the slow reproductive cycle of metatherians, and changed patterns of fire occurrence (Bradstock et al., 2005), which altered the landscape from a drought adapted mosaic to fire-adapted desert scrub (Miller et al., 2005). However, recent research in Tasmania found no link between the megafaunal extinction during this period and human overhunting (Trueman et al., 2005; Webb, 2008; Cosgrove et al., 2010), suggesting that causal factors on the island were increasing aridity and drought during the period and indicating a juxtaposition of several factors may have led to the extinction event on the mainland continent.

After this mass extinction event, 306 terrestrial mammalian species proliferated until European colonization 200 years ago. Since European settlement, 27 of those species have become extinct (EPBCA, 1999; McKenzie & Burbidge, 2002), which represents 28% of the world's mammalian extinctions since 1600AD, a rate far above any other continent (Baillie, 1996). Australia has the worst record of any continent or country for the extinction of mammal species within historic times (Short, 1998), a rate of change that is equal to that occurring during the last glacial maximum (~21,000 years ago) (Johnson et al., 2005). At present, there are 4 critically endangered metatherian species, 35 are endangered and 55 are vulnerable (EPBCA, 1999). Unlike in the historic megafaunal extinction, where the largest mammalian species succumbed, intermediate species, between the weights of 35g to 5500g, appear to be the most vulnerable in modern times due to the increased vulnerability to introduced pests at this size, unless the species is arboreal (Burbidge & McKenzie, 1989; Chisholm & Taylor, 2010).

There is a direct relationship between the presence of introduced species and the local extinction of native mammals (Burbidge et al., 1997; Burbidge & Manly, 2002). Research on some of the Australian continental islands emphasizes this relationship by representing a miniature view of the mainland picture. There are 10 mainland marsupial species that no longer inhabit the mainland and are found only on offshore islands, with 9 species close to

extinction on the mainland that have island populations of conservation significance (Burbidge et al., 1997), in total there are 35 islands protecting 18 taxa of Australian threatened species. When predators have been introduced to these islands, local extinctions occur rapidly, with this single dynamic the most important in reduced faunal numbers, more important than land clearance or human development. The presence of introduced predators, like the fox (*Vulpes vulpes*) and feral cat (*Felis catus*), impacts on the social behaviour of marsupial herbivores, especially during feeding (Banks, 2001), reducing their family group sizes and distributions. The pressure of these introduced predators, combined with drought and/or human population pressures, is directly responsible for local metatherian extinctions (Short, 1998). For example, three of the five species of rat-kangaroos (*Potoroidae* spp.) in New South Wales are now extinct, with a fourth threatened, due to the introduction of predators (mammalian fauna make up at least 22% of a cat's diet in some areas (Kutt, 2011)), despite these species surviving through human settlement, the introduction of the rabbit (*Oryctolagus cuniculus*), and severe drought episodes.

Rainfall underpins population dynamics for many herbivorous species around the world and there is increasing evidence that changing rainfall patterns due to global warming are having a detrimental impact on their population survival (Dunham et al., 2004; Ogotu et al., 2007, 2008). In Australia, the greatest proportion of extinctions have occurred in arid or semi-arid regions (McKenzie et al., 2007). However, Australian herbivores are well adapted to episodes of fluctuating rainfall, as climate is a key factor in the evolution of social organization of metatherian species, unlike in eutherian species where evolution of social organization is linked to body-size and distribution (Fisher & Owens, 2000). For example, macropod numbers increase exponentially with good rainfall and decrease heavily during drought periods. Such fluctuations are intrinsic to the ecology of these species and occur locally almost annually and regionally about every 10 years, counterbalancing the rapid increase during high rainfall (Caughley et al., 1985). It is not unusual for these populations to decrease by 40-45% during drought years and rebound by 35% in non-drought years (Caughley et al., 1984; Bayliss, 1985; Caughley et al., 1985). Interestingly, some macropod species have increased with domestic ruminant intrusion into their lands, most likely due to feed and water supplementation associated with husbandry (Jonzen et al., 2005), and the change to pasture and increased water points (Landsberg et al., 2003), e.g. the eastern grey kangaroo (*Macropus giganteus*) has increased its range into the more arid habitat of the red kangaroo (*M. rufus*), due to increased water availability coincidental with ranching of domestic species (Dawson et al., 2006).

Despite marsupial adaptations to prolonged weather conditions that lead to reduced resource availability, the combination of two or more population regulating influences, can have severe impacts on grazing animal numbers, *i.e.* high grazing pressure followed by a drought or fire, or fire conducted under drought conditions (Scholte et al., 2007; Yarnell et al., 2007), in conjunction with the added pressure of introduced predators or competition species, is having detrimental effects on population numbers (Woinarski et al., 2011). In addition, European agriculture has had a significant and most likely permanent (Bickford & Mackey, 2004) impact on the vegetative landscape and environmental systems of Australia (Dodson & Mooney, 2002; Bickford & Gell, 2005; Hobbs, 2005), including soil properties (Baker, 1998), which is changing the ecology of native species, decreasing endemic species' ability to recover from severe climate events.

The consequence of anthropomorphic changes to climate patterns and global temperatures is having a direct affect on the Australian environment, increasing and prolonging drought episodes and altering fire and flooding events (Gibson et al., 2010; Lindenmayer et al., 2010), which has carry on effects on native metatherians. Naturally, as metatherian life-history is more closely impacted by climate factors, recent research on endemic marsupial species', *e.g.* quokka (*S. brachyurus*), ability to cope with the changing climate patterns, indicate an increasingly severe population and distribution contraction with increasingly severe climate events (Gibson et al., 2010) (Fisher et al., 2001a). Furthermore, the timing of drought episodes is important (Parrott et al., 2007; Sims et al., 2007), as reduced resources during the reproductive cycle is associated with decreased growth rates and size of young, and increased maternal mortality under the increased load of lactation, as in the *Phascogale tapoatafa* (Rhind & Bradley, 2002), which can impact population density for several successive years. The populations of Australian mammals are decreasing under the pressure of a combination of anthropomorphic influences. This decline in mammal populations has a follow on effect on other Australian species (Meserve et al., 2001), and a direct impact on Australian biodiversity.

As metatherian species become increasingly limited in numbers, their conservation and importance to mammalian research becomes more critical. Modern understanding of mammalian diversity and life histories is based almost exclusively on eutherian species (Fisher et al., 2001b), the comparative study of metatherian biology sheds light on the development of our own life histories. The differences between the three mammalian clades help to elucidate the origins of mammalian life history traits, especially reproduction and viviparity (Tyndale-Biscoe & Renfree, 1987), through disclosing the divergent means used

by each to achieve the same ends. The parallel evolutionary paths taken by each group provides interesting insights into the degree of physiological divergence accumulated over time (Baudinette, 1994) and the mechanisms behind co-evolution (Coulson et al., 2006; Selwood & Coulson, 2006), and reconstructing the biology of all mammalian groups provides critical information for the understanding of mammalian, and thus human, evolution (Cardillo & Lister, 2002).

At the turn of the 20th century, the study of marsupial reproduction was a major contributor to the understanding of mammalian reproductive processes (Renfree, 1995), and in the past several decades research on marsupial species has regained this position (Hinds et al., 1990a; Bedford, 1996; Hinds et al., 1996; Renfree et al., 2001b; Behringer et al., 2006). In an age when conservation is becoming more and more important for the survival of mammalian species, the study of reproduction in marsupials, where access to the neonate occurs early in development, facilitates the advancement of captive breeding protocols that profoundly change current and future conservation attempts (Rodger, 1990; Robertson et al., 2006). During the prolonged development in an easily accessible pouch, *e.g.* research on hormones is facilitated due to ease of direct access to the young without disruption to the maternal-fetal resource/waste connection, processes like organogenesis, sexual development and the development of the nervous system and brain are all easily accessible. In addition, the young can be removed from the teat and replaced without loss, allowing surgical procedures and other scientific methods, such as cross fostering, to occur without surgery (Renfree et al., 2001b; Robert & Schwanz, 2011). Similar procedures are much more difficult in eutherian species, as the physiological systems of the young are normally fully developed by the time of, or soon after, birth. In addition, marsupials have no complicated embryo implantation or placenta (Renfree, 1995), have fewer chromosomes and experience embryonic diapause.

There are intrinsic benefits to the study of marsupial species, as it may have profound bearing on conservation efforts, especially in Australia. As an increasing number of mammalian species decline towards extinction, the understanding of the ecology of these species becomes important for conservation and sustainability purposes. Evidence suggests that extended European land use practices in Australia are proving to be untenable, and, as indicated by the growth of kangaroo harvesting and deer farming on other continents, it is possible that humans will increasingly turn to native species as sustainable replacements for domesticated ruminants (Fletcher, 2001; Martin et al., 2004; Choquenot & Warburton, 2006; Martin & Kadokawa, 2006; Munn et al., 2009).

In 150 years, introduced species have caused acute and possibly irreparable alterations to the Australian environment (Tiver & Andrew, 1997; Baker, 1998; Dodson & Mooney, 2002; Bickford & Gell, 2005; Johnson et al., 2005), coupled with changing climatic variables, which may be detrimental to long term marsupial survival. As Australian marsupials are at an increased risk of extinction, are the major mammalian group on the continent, hold a unique position in mammalian evolution and their study makes important contributions towards illuminating our own natural history, they should be conserved and studied for their own intrinsic value to biodiversity. Such research is necessary to stem the tide of extinction and preserve history, as conservation complacency could have irreversible effects (Tyndale-Biscoe, 2001; Selwood & Coulson, 2006; Lindenmayer et al., 2011).

1.5 Sex Ratio Theory

Over the last several decades, the parity in numbers of sexes amongst progeny produced by sexual reproduction has been examined in numerous species from many different animal families, but the majority of the literature concerns eutherian species to date. Different offspring sex ratio patterns have been observed across the animal kingdom, but attempts to pinpoint a mechanism, or an overarching pattern, in mammals remains controversial at best. However, such investigation and discussion is necessary, as this is a fundamental demographic parameter crucial to the viability of populations (Stelkens & Wedekind, 2010). A better understanding of any environmental influences on the adaptive skewing of sex ratios is integral to the understanding of causal mechanisms and the biological significance of a species' ability to control the sex ratio of its offspring. Understanding exactly how this phenomenon works, and being able to predict its occurrence, would facilitate the estimation of population characteristics that are difficult, or too expansive to measure conventionally (West et al., 2000). Such understanding may provide significant benefits for attempts to control diseases or conserve populations.

Although intrigued by the idea that parents will split their reproductive effort between the sexes by producing approximately equal numbers of male and female progeny, Darwin found it so intricate a conundrum that he preferred leaving its solution to future generations (Darwin, 1871). Early theory on sex ratio suggests that equal contribution of male and female parental resources, namely genetic resources, to their offspring results in an equal population sex ratio of 1:1, with an equal chance of obtaining either sex at any reproductive event (Fisher, 1930). Fisher's explanation was further developed in the 1960's, when several exceptions to his theory of equal sex ratio were noted and the discussion regarding unequal resource allocation by parents began (Hamilton, 1967). In the 1970's, the interest in the population dynamics of such skewing increased exponentially, as new models were suggested (Hamilton, 1972; Trivers & Willard, 1973; Williams, 1979; Charnov, 1982), improving the understanding of existing theories and producing fresh insight on the topic.

The Trivers-Willard Model (TWM) (Trivers & Willard, 1973) is one such theory. The TWM contends that high quality mothers, in populations with high variance in paternal fitness due to male competition, and where females are usually monogamous, are more likely to "invest" in male offspring, as this will provide more grandchildren than investing in a daughter. Although daughters are more reliable breeders than sons in such breeding systems, the reliability of the daughter is little influenced by any quality transmitted from her

mother, whereas the probability that a son will breed is strongly influenced by the qualities transmitted from his mother. He may sire the output of many females, while the reproductively more reliable female is limited to 1 or 2 offspring per year (Trivers & Willard, 1973). Thus, low quality females would benefit more from producing daughters, who will produce offspring irrespective of adult size, rather than by producing a small, unsuccessful male that may never breed due to subordination in the male hierarchy.

However, this theory requires that several assumptions are met:

- 1) Male offspring benefit more than female progeny from increased resource allocation from mothers by increased adult reproductive rates;
- 2) There is no compensatory growth in the young, meaning offspring condition is an indicator of adult condition; and
- 3) The quality of the mother is associated with the quality of the progeny.

Thus, mothers within local resource competition should be able to adaptively allocate their resources to young that will increase their fitness and that of their successive generations at the time of reproduction (Charnov, 1982; Clutton-Brock et al., 1984). Clutton-Brock, in refining the TWM, suggests that the allocation of resource investment in one sex or the other is more heavily dependent on the effect of that investment on the reproductive triumph of that male or female progeny, rather than on the variance of reproductive success (Clutton-Brock & Albon, 1982).

Since its publication, the TWM, due to its logical and intuitive appeal, has sparked a great many investigations into the adaptive allocation of sex in offspring, and to date the article has been cited over 1,568 times. Interestingly, despite much of the early literature in this canon applying Fisher (1930) and TWM (1973) concurrently, by nature, they are mutually exclusive and the assumptions of the TWM at the family level are diametrically opposed to Fisher's theory of equal sex ratio variance at the population level (Frank, 1987; Frank, 1990; Wade et al., 2003).

Trivers and Willard (1973) originally discussed their hypothesis with regards to a hypothetical caribou (*Rangifer tarandus*) population, as ungulate species proved ideal for testing their assumptions, although supporting empirical data for their theory was nonexistent at the time (Hewison & Gaillard, 1999). Most ungulate species are polygynous, sexually dimorphic species, where males compete vigorously for females and the dominant males have increased access to receptive females. This contravenes many of the assumptions of Fisher's theory of equal sex ratio. For example, sex ratio equality in a population assumes that the costs of rearing either sex is uniform, however this is not true for many ungulate

species, where sons are often larger than females at weaning (Kojola, 1998) and the costs of producing sons are higher than those of daughters (Clutton-Brock et al., 1981; Clutton-Brock & Albon, 1982; Rutkowska et al., 2011). Fisher's theory also assumes that populations mate randomly, a phenomenon rarely seen in natural environments (Cockburn, 1990).

Much of the research published on the consistency of sex ratio bias and the TWM provides an inconsistent picture, with some authors finding support for the theory while others found no support. However, overall, the data from ungulate species supports the model, although the relationship is strongest when preconception parameters connected with female dominance are used, when sexual size dimorphism is more male biased and when gestation times are longer (Sheldon & West, 2004). Currently, the TWM is increasingly applied and tested in other mammalian species, also with varying results. Studies on other mammals are less supportive than in ungulates, showing around 34% are in accordance with TWM (Cameron, 2004), with indices measuring maternal condition at conception the most predictive of sex ratio.

The equivocal nature of these new data (Hewison & Gaillard, 1999) is usually due to inconsistent experimental parameters, lack of understanding of species ecology and the strict conditions necessary to satisfy the TWM (Clutton-Brock & Iason, 1986; Frank, 1990; Kojola, 1998). Much of the research applies the TWM *post hoc*, assuming that the observed bias is adaptive (Stuart-Dick & Higginbottom, 1989). Since the new catalogue of sex ratio bias research investigates the phenomenon in a wide variety of animals, *i.e.* porcine species (Mendl et al., 1995; Meikle et al., 1996; Servanty et al., 2007), primates (Bercovitch et al., 2000), ungulates (Clutton-Brock et al., 1984; Kojola & Eloranta, 1989; Kojola, 1998; Hewison & Gaillard, 1999; Enright et al., 2001; Saltz, 2001; Hewison et al., 2002; Garroway & Broders, 2007), reptiles and frogs (Dorizzi et al., 1991; Wibbels et al., 1994; Alho et al., 2010; Stelkens & Wedekind, 2010; Wedekind, 2010), domestic ruminants (Rorie, 1999; Rorie et al., 1999; Roche et al., 2006; Green et al., 2008; Rizos et al., 2008), birds (Burley, 1981; Ellegren et al., 1996; Komdeur et al., 1997; Griffin et al., 2005; Pryke & Griffith, 2009), mice (Danforth, 1926; Krackow, 1992; Krackow, 1997; Rosenfeld et al., 2003; Rosenfeld & Roberts, 2004; Shibata & Kawamichi, 2009), hippopotamus and elephants (Zschokke, 2002; Visscher et al., 2004), and humans (Guerrero, 1974; Harlap, 1979; James, 1990; Grech et al., 2000; Mathews et al., 2008; Cameron & Dalerum, 2009), the reason for the elusiveness of hard evidence in support of the TWM is highly likely due to the vagrancies in individual species' ecology, and a lack of coherence with the assumptions of the TWM.

In accordance with the plethora of species being tested for sex ratio bias, large varieties of environmental and physiological influences are linked to the phenomenon as causal factors. Many of these causal factors are species-specific, ranging from litter size (Servanty et al., 2007) to illegal hunting (Marealle et al., 2010), attractiveness of a selected mate (Burley, 1981; Ellegren et al., 1996; Cameron & Dalerum, 2009; Pryke & Griffith, 2009) and timing of insemination (Guerrero, 1974; Harlap, 1979; Rorie, 1999; Rorie et al., 1999) to alcohol consumption (Danforth, 1926; Chaudhuri, 1927) and stress (Schuster & Schuster, 1972; Zorn et al., 2002; Bruckner et al., 2010; Marealle et al., 2010; Ohlsson & Shah, 2010).

However, as the predominant parameter of the TWM, maternal condition is the most widely studied and most attributable cause of sex ratio skewing in most of the literature. Maternal physiology such as age (Saltz, 2001; Hewison et al., 2002; Saltz & Kotler, 2003; Rosenfeld & Roberts, 2004), dominance ranking (Clutton-Brock et al., 1984; Huck et al., 1988; Mendl et al., 1995; Meikle et al., 1996; Nevison, 1997; Brown & Silk, 2002), nutrition (Enright et al., 2001; Rosenfeld et al., 2003; Rosenfeld & Roberts, 2004; Green et al., 2008; Mathews et al., 2008) and weight or body condition (Kojola & Eloranta, 1989; Sunnucks & Taylor, 1997; Roche et al., 2006) have all been used as measures of maternal quality as per the TWM.

The aim of this thesis is to provide some clarity to this data on progeny sex ratio bias by establishing the existence of the TWM in a marsupial species, and then provide some comparison to the available eutherian literature. A better understanding of phenomenon like sex ratio bias in conservation species facilitates breeding programs, as in ignorance of such phenomenon it is possible to inadvertently bias the sex of young during breeding and produce an abundance of an undesired sex or more of one sex, and the understanding of how anthropomorphic changes to the environment impact on population dynamics.

CHAPTER 2 *Evidence of Sex Ratio Skew in a Wild Population of *Macropus eugenii* and the application of the Trivers-Willard Model*

2.1 Introduction

2.1.1 *Marsupials and Sex Ratio*

To better elucidate the role of the TWM in mammalian adaptive sex allocation, marsupials provide a unique opportunity to observe young much earlier in development than most eutherian mammals, sometimes prior to 90% of maternal investment (Hayssen et al., 1985). In fact, marsupial species appear to produce skewed sex ratios more frequently than eutherian mammals (Clutton-Brock & Iason, 1986; Cockburn, 1990), with at least partial support for the TWM in most instances of marsupial sex ratio skewing, though not always as an exclusive explanation (Austad & Sunquist, 1986; Johnson, 1989; Cockburn, 1990; Higginbottom, 1991; Ashworth, 1995; Sunnucks & Taylor, 1997; Fisher, 1999).

Interest in the sex allocation patterns of metatherians began in the 1960's (Main et al., 1959; Shield, 1962; Caughley & Kean, 1964) and proliferated in the 1980's, with discoveries that the common brushtail possum (*Trichosurus vulpecular*) (Austad & Sunquist, 1986; Isaac et al., 2005) and several *Antechinus* species (Dasyuridae: Marsupialia) (Cockburn et al., 1985a) show forms of sex ratio skewing at birth, although in *Antechinus stuartii* the manipulation of sex allocation occurs at weaning rather than at birth (Cockburn, 1994). Similarly, some semelparous dasyurid species also show a sex allocation strategy that occurs after parturition, overproducing progeny and then selecting against females at the point the young are attaching to the limited available teats (Foster & Taggart, 2008). However, these early conclusions on sex allocation tactics in dasyurid species do not concur with mechanisms employed by other marsupial species, as there appears to be a difference in strategy between polytocous and monotocous mammals (Grant & Chamley, 2010).

The most robust support for the TWM comes from species that are monotocous and polygynous in nature, making each breeding episode an all-or-nothing choice that will last an entire reproductive cycle, e.g. ungulates. Macropod marsupials (Macropodidae) fill a similar ecological niche to ungulates and are prime candidates to exhibit support for the TWM (Hume, 1982; Jarman, 1984), living in sexually dimorphic family groups (though some are only weakly dimorphic), which are polygynous or promiscuous in nature (Andrewartha & Barker, 1969; Inns, 1980; Clutton-Brock, 1989; Grigg et al., 1989). The variance of male reproductive success is usually higher than females, as males physically

compete, males will fight for mating access, with the larger male usually victorious, to win increased access to receptive mating partners and tend to disperse over wider territories (Russell, 1982b; Stuart-Dick & Higginbottom, 1989). Thus, the reproductive success of sons, compared to daughters, is dependent on adult body size.

As with the literature on cervid species, early investigations of adaptive sex ratio bias in macropod species also produced ambiguous results. A review by Cockburn (1990) revealed that just over half of all early sex allocation studies in macropods exhibited significant sex ratio skewing (Table 2.1.1). If the information in Table 2.1.1 is analysed as a whole data set (this is possible because studies with low sample sizes ($n \geq 100$) or poor experimental design have been excluded from the list), the discrepancies in the occurrence of bias in sex ratio amongst species, some of which are very closely related phylogenetically, might be due to different environmental cues in their separate ecosystems. Therefore, the adaptive ecology of each separate population within its distinct environment might give rise to a variation in sex allocation strategies across the family (Stuart-Dick & Higginbottom, 1989), e.g. *Setonix brachyurus* exhibiting a female biased ratio (Shield, 1962) while *Petrogale xanthopus* exhibits an extreme male biased ratio (Poole et al., 1985).

This interspecific adaptation to environment might also extend to intraspecific adaptations causing divergent strategies amongst populations of the same species inhabiting different ecosystems. The continental climate in Australia is highly diverse across latitudinal, longitudinal and altitudinal gradients (Tyndale-Biscoe, 2001), making it much more likely that geographically isolated populations of the same species evolve variations in some life history traits. A similar physiological example is the Abrolhos Island *M. eugenii* adapting the ability to drink sea water (Kinneer et al., 1968), as no fresh water is available for many months of the year, while the Garden Island and Kangaroo Island tammars are incapable of such a feat (Hume, 1999). A comparable geographical adaptation in gender selection might explain why Maynes (1977) and Johnson (1989) found no sex ratio skewing in populations of *M. parma* and *M. eugenii* in New Zealand and Australia, yet more recent examinations of these species in New Zealand found sex ratio bias in alignment with the TWM (Sunnucks & Taylor, 1997), while Ealey (1963) and Poole & Marchant (1987) found no bias in *M. robustus*, whereas later research found a significant 58% male bias (Ashworth, 1995).

Table 2.1.1 Compilation of early investigations into sex ratio trends in Macropodidae. Significance refers to departures from an equal sex ratio of 1:1 (F probability <0.05; one-group X^2 test), only larger sample sizes are included (partially taken from Johnson (1989)).

Species	Males %	Significance found	Reference
<i>Bettongia penicillata</i>	60%	Yes	(Christensen, 1980)
<i>Macropus kangaroo</i>	58%	Yes	(Caughley & Kean, 1964)
<i>M. agilis</i>	50%	No	(Kirkpatrick & Johnson, 1969; Merchant, 1976; Bolton et al., 1982)
<i>M. eugenii</i>	54%	No	(Johnson, 1989)
<i>M. giganteus</i>	54%	Yes	(Johnson & Jarman, 1983; Johnson, 1989; Stuart-Dick & Higginbottom, 1989)
<i>M. parma</i>	53%	No	(Maynes, 1977)
<i>M. robustus</i>	48%	No	(Ealey, 1963; Poole & Merchant, 1987)
<i>M. rufogriseus</i>	55%	Yes	(Fleming et al., 1983; Johnson, 1989; Stuart-Dick & Higginbottom, 1989)
<i>M. rufus</i>	52%	Yes	(Johnson & Jarman, 1983; Johnson, 1989)
<i>Petrogale xanthopus</i>	66%	Yes	(Poole et al., 1985; Robinson et al., 1994)
<i>Setonix brachyurus</i>	44%	Yes	(Main et al., 1959; Shield, 1962)

2.1.2 Rainfall and Marsupial Sex Ratio

For metatherians in Australia, the evidence is that maternal strategies and environmental changes are working in conjunction on the sex ratio bias. The marsupial's extraordinary physiological flexibility and the ease with which a reproductive attempt may be abandoned (Russell, 1982b) means that environmental factors are more important in the development of marsupial ecological behaviour than in eutherians (Fisher & Owens, 2000; Fisher et al., 2001b). In fact, the ability to abort a reproductive attempt and replace it with a diapause at small cost to the metatherian mother, is cited by some leading researchers as the very reason that primitive marsupial radiation occurred in the unpredictable Australian climate, allowing the group to prevail over early Australasian eutherian species (Russell, 1982b; Tyndale-Biscoe, 2001).

As the dominant large herbivores endemic to so many ecosystems in Australia, macropod species are regularly subjected to varying amounts of rainfall and long drought periods (Caughley et al., 1984; Caughley et al., 1985; Cairns et al., 2000). Rainfall, or lack thereof, has a direct influence on the quality and quantity of food available (Bredenkamp et al., 2002; Ogutu et al., 2007), causing spatial variation, which stimulates or impedes the reproductive success (Gaughwin et al., 1984; Caughley et al., 1985; Higginbottom, 2000; Rhind & Bradley, 2002; Southgate & Carthew, 2006). These drought periods cause reduced body size and condition, increased mortality amongst juveniles, population decline or dispersal and reduced fecundity (Caughley et al., 1984; Caughley et al., 1985; Clancy & Croft, 1992; Fisher et al., 2001a; Rhind & Bradley, 2002; Parrott et al., 2007; Pople et al., 2007).

Research on the impact of the resulting maternal stress caused by the reduction in available resources has provided support for the TWM in macropods, and a link between rainfall and sex ratio bias (Fisher & Owens, 2000; Delean et al., 2009). For example, Fisher's (1999) study on *Onychogalea fraenata* substantiated the model, as mothers in good condition, which was based on body size, produced a greater number of sons; and even the larger better conditioned mothers, after dramatic drought induced weight losses, showed sex ratio skewing. In some cases, the degree of change to elicit a response in a population is minimal: 60-100mm alteration in mean annual rainfall (Caughley et al., 1984).

Despite the evidence that changes in rainfall affect maternal body condition, which in turn influences sex ratio, proof that this sex ratio bias categorically follows the presumptions of the TWM is still evasive. In *M. eugenii* the research is inconsistent, with Johnson (1989) and Inns (1980) finding no sex ratio bias, while Sunnucks & Taylor (1997) and Wright & Stott (1999) have both found male biased sex ratio in accordance with the TWM, interestingly this data encompasses several different populations. However, the greatest support for the TWM comes from these monotocous macropod species (Robert & Schwanz, 2011). As the literature suggests that offspring sex ratio is adaptable, with facultative changes depending on varying conditions, it is likely that sex ratio bias relies on the annual fitness of the local landscape and its environmental or demographic cues (Cockburn, 1994; Fisher, 1999; Isaac et al., 2005; Robert & Schwanz, 2011). This translates into interplay of sex ratio theory, like the TWM, and external resource cues, rather than a non-adaptive derivative of male exposure to maternal resources (Clutton-Brock et al., 1981; Clutton-Brock & Albon, 1982).

The unique reproductive method of metatherian species may help to unravel the relationship between sex ratio, maternal condition and environmental cues. Previous sex ratio theory testing has centred around three approaches, though they are rarely tested concurrently (Clutton-Brock & Iason, 1986). First, individual sex ratio bias at parturition, which should see high quality mothers bias their offspring to the more beneficial sex, *i.e.* high quality mothers should have more sons and low quality mothers should have more daughters, as sons will benefit a mother's overall fitness by securing dominance in the paternal hierarchy (Frank, 1990). Secondly, maternal resource transfer inequality during development, as high quality mothers should channel more resources per capita into the sex that will benefit the most from increased maternal care, so sons should have higher growth rates and body size than daughters (Clutton-Brock et al., 1981; Clutton-Brock, 1991; Kojola, 1998). Lastly, reproductive costs over time, as there should be *per capita* differences in the cost of

production between the sexes, experienced more rigorously by poor quality mothers. Therefore, mothers with a greater number of sons than daughters should have higher fitness costs, and subsequently, shorter lifespans or lower reproductive outputs, due to the higher maternal reproductive costs (Rutkowska et al., 2011). Monotocous metatherian species, such as *M. eugenii*, allow researchers to investigate all three of these threads of inquiry simultaneously.

The tammar wallaby is particularly useful as a test case as the species is monotocous (Tyndale-Biscoe & Renfree, 1987), sexually dimorphic, polygynous in nature, and appears to fulfil the other requirements for species that authenticate the TWM. Monotocous species, in manipulating the sex of pouch young, must make an optimal decision that maximizes the ratio between benefit gained and maternal cost, as the maximum investment for a species like the tammar may reach 60% of maternal body weight (Murphy & Smith, 1970). However, unlike its eutherian counterparts, *M. eugenii* has a very short gestation period, as the largest phase of offspring growth and maternal cost occurs during the pouch life of the young (Russell, 1982b; Renfree, 1993, 1995; Renfree & Shaw, 1996). Consequently, if a pouch young (PY) is aborted or lost, the cost of replacement is minimal.

The ability to replace young quickly and at low cost, coupled with high fecundity rates (Inns, 1980) and the extreme synchronous nature of breeding in this species (Andrewartha & Barker, 1969; Inns, 1980; Tyndale-Biscoe & Renfree, 1987; Renfree, 1992) allows individual sex allocation comparisons under the same environmental conditions across a population. The species is also a highly researched model, both ecologically and reproductively (e.g. (Andrewartha & Barker, 1969; Berger, 1970; Tyndale-Biscoe et al., 1974; Inns, 1980; Tyndale-Biscoe & Renfree, 1987; Hinds et al., 1990a; Renfree & Shaw, 1996; Wright & Stott, 1999), with a wide breadth of literature available to assist in any attempt to pinpoint causes and mechanisms of an adaptive sex allocation trend.

The current study aims to resolve some of the ambiguity surrounding the true nature of sex allocation in macropod marsupials. Most of the previous examinations have applied the TWM *a posteriori* to explain already observed patterns (Stuart-Dick & Higginbottom, 1989). Here, variation in rainfall, including drought and abundant periods, will be used to assess the impact of environmental cues on sex ratio allocation in the tammar wallaby (*Macropus eugenii*). If TWM is present in the sampled population, then maternal body condition parameters, such as weight and kidney fat index, should be positively correlated with the proportion of sons produced. Thus, heavier rainfall during the breeding season should

produce larger, better conditioned mothers who produce more sons, while drought affected mothers should be in poorer condition and produce more daughters. The adaptable nature of the phenomenon will be tested for predictability by discussing the observed data in relation to four main questions:

1. Does rainfall impact on maternal condition, and is maternal condition an indicator of pouch young condition?
2. Does maternal investment vary between sons and daughters?
3. Does maternal condition alone bias sex ratio?
4. Is the TWM present in this population? If not, then what?

2.2 Methods and Materials

Data were obtained from 110 adult tammar wallabies (*Macropus eugenii decres*) shot by local licensed farmers on Kangaroo Island during the month of June over the years 2005 to 2007, representing several years of varied rainfall. The animals were shot under Department of Environment and Heritage (SA) destruction and scientific permit number Y24988 and University of Adelaide Animal Ethics permit S-079-2005B on several farms on the Dudley Peninsula, Kangaroo Island in the area of Penneshaw (35° 43'06"S, 137° 56'25"E). These farms ranged from moderately culled to non-culled private farms, but not all contained livestock. Culling was carried out over a three to four day period each June, although the same property was not visited on consecutive nights. Prior to dissections, carcasses were weighed to obtain Maternal Full Weight to the nearest .01kg on a 10 kg live weight balance and the length of the foot was measured linearly with callipers to the nearest mm from the proximal end of the calcaneus to the base of the toenail at the distal end of the fourth digit, excluding the toenail (Inns, 1980) (Figure 2.2.2). The age of each individual was estimated by measuring the molar progression from the upper jaw and teeth using a reference line, measured perpendicularly across the skull at the anterior zygomatic arch, thus giving the Molar Index (MI) (Kirkpatrick, 1964) (Figure 2.2.1). The MI was then used to estimate the log age of each individual, by using the following equation: $\log_{10}(\text{age, in days}) = 2.0939 + 0.4067(\text{MI})$ (Inns, 1982).

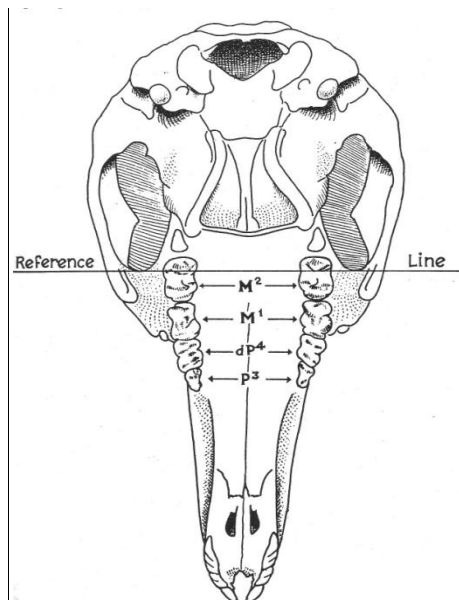


Figure 2.2.1

Molar progression and macropod age, a line is drawn between the rear of the anterior zygomatic arch, and the MI is calculated by assessing the number of molars that have migrated anterior to that line (Kirkpatrick, 1964).



Figure 2.2.2

Pes measurements of adults and pouch young were taken with callipers to the nearest mm, from the proximal end of the calcaneus to the base of the toenail at the distal end of the fourth digit, excluding the toenail; here marked with yellow.

The carcasses were then dissected to obtain measurements of body condition. The measurements used were gut weight (all digestive organs were removed and weighed separately on the same balance to 1g), total left peri-renal fat and left kidney weight were taken on a 100g balance to the nearest .01g (Monson et al., 1974; Van Vuren & Coblenz, 1985) (Figure 2.2.3). The Kidney Fat Index (KFI) was then obtained by using the following equation: $\text{Kidney fat index} = \text{peri-renal fat} / \text{kidney weight} \times 100$ (Riney, 1955). Maternal Body Condition (MBC) was also calculated by dividing the full weight by the foot length³ (Krebs & Singleton, 1993; Green, 2001; McGowan et al., 2008).

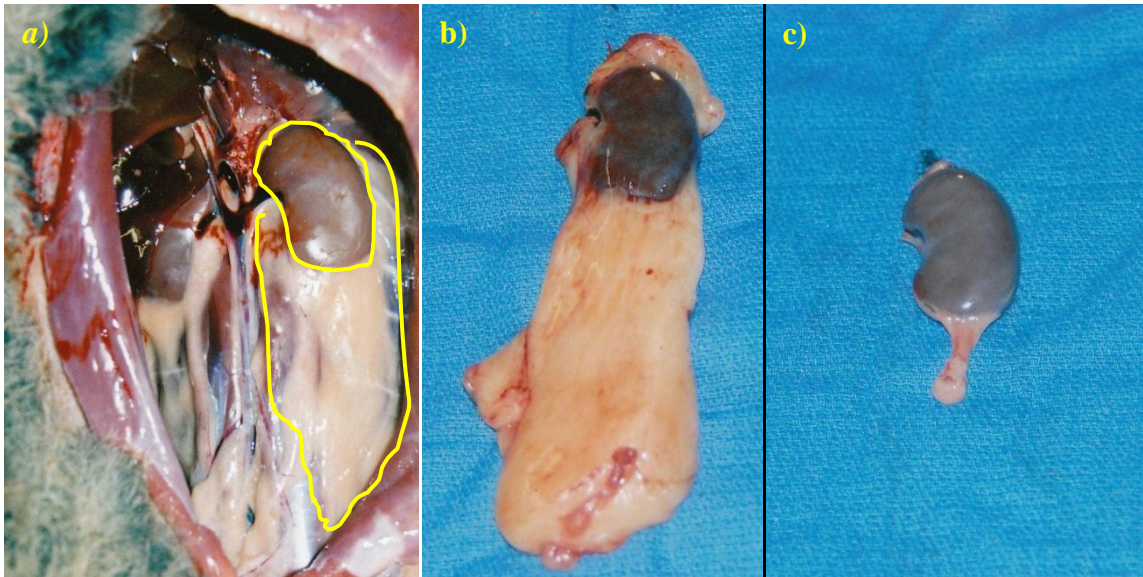


Figure 2.2.3

- a.) Undisseminated left kidney and left peri-renal body fat, fat included in measurement is marked in yellow
- b.) A dissected left kidney, with adipose tissue still attached, this is an example of a large deposit of fat, the kidney and the fat were weighted separately
- c.) A dissected left kidney, with adipose tissue still attached, this is an example of a small deposit of fat, the kidney and the fat were weighted separately

Pouch Young (PY) were removed from the maternal pouch and sexed (Figure 2.2.4), and then body condition measurements were obtained. The PY were weighed on a 100g balance to the nearest .01g, and the tibia (measured from the tibial crest to the plantar aspect of the tarsus with the tarsal joint held at a right angle) length was taken with callipers to the nearest .01mm, head (measured from the rostrum to posterior of the skull) size was taken with callipers to the nearest .01mm, and left foot length (measured as with the adults) was recorded with callipers to the nearest .01mm (as per Murphy & Smith, 1970) (Figure 2.2.5). Later PY age was estimated using growth curves for head, tibia and foot length, estimates were standardized to remove any annual variation in data collection analysis (Murphy & Smith, 1970; Inns, 1982). This measurement, as outlined by Murphy & Smith (1970), is an estimate of PY skeletal development that predicts PY age prior to the age of 320 days. As development of individual body parts occurs in different stages of pouch life, it is beneficial to use such an estimate to assess the overall growth of the PY, as this will avoid any skewing

based on a single factor. The pouch young body condition (PYBC) was also calculated, see MBC calculation for adults (weight/foot length³) (Krebs & Singleton, 1993; Green, 2001; McGowan et al., 2008).

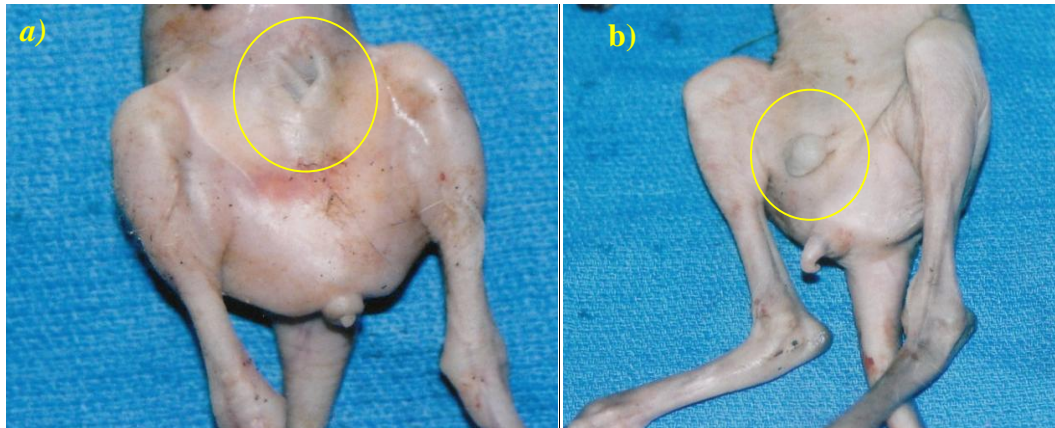


Figure 2.2.4

- a.) Genitalia of a female tammar pouch young, pouch is clearly visible
- b.) Genitalia of a male tammar pouch young, scrotum is clearly visible



Figure 2.2.5

- a.) Head measurement is marked in red on each pouch young
- b.) Tibia measurement is marked in yellow on each young
- c.) Foot measurement is marked in white

Rainfall data were obtained from the Australian Government Bureau of Meteorology station number 022841 (Kingscote Aerodrome), 47 kilometres from the study site, for each sample year, which were compared against other sample years and against the average using ANOVA and the Tukey-Kramer *Post Hoc* test. Once the body condition parameters had been assessed for normality using the Shapiro-Wilk test of normality, and any transformations enacted as necessary, the maternal body condition parameters were analysed against rainfall year as the explanatory variable using ANOVA, those parameters tested were Maternal Full Weight, KFI, MBC, proportion of Gut Weight (g) to Full Weight (g) and the Estimated Age, obtained from the Molar Index analysis. In addition, to make increase the rigour of the analysis, a Bonferroni Correction was done to assess the independence of multiple ANOVAs performed on the same data set. General Multiple Regression analysis

was also used to assess the relationships between Estimated Age and the other maternal body condition factors.

Pouch Young condition was assessed using PY weight, PY estimated age (as estimated age is an overall growth parameter estimated from the head, tibia and foot lengths) and PYBC. These factors were analysed against rainfall year using ANOVA (once the normality of each parameter had been established using the Shapiro-Wilk test and enacting any necessary transformations of the data) and the Tukey-Kramer *Post Hoc* test, and against the maternal body condition parameters using General Multiple Regression. The body condition and size data for each sex were compared using ANOVA, with PY sex as the treatment factor. Pouch Young sex ratio was evaluated for significance of rainfall year using ANOVA, and assessed for deviation from the expected 1:1 ratio using Chi² tests. Sex ratio is expressed as male:female, with male PY counting as successes. Log Regression of a Binomial and ANOVA with the Tukey-Kramer *Post Hoc* test were used to find relationships between sex ratio and maternal body condition factors. A Bonferroni Correction was also used to assess independence when multiple ANOVAs were performed on the same data set.

2.3 Results

2.3.1 Rainfall

Using entire year (2005, 2006 and 2007) and average as treatments for an ANOVA, there was no significant deviation in rainfall amongst years (test statistic=0.96, p value=0.421, df of 3) or from the average (Figure 2.3.1.1). This was most likely due to the mediating effects of above average rainfall during certain periods, which compensated for below average rainfall during other periods. When 2004 was included as a treatment, as *M. eugenii* have diapause that are conceived during the breeding period that may not reactivate until the following breeding period (Shaw & Renfree, 1986; Renfree, 1993; Spindler et al., 1998), there was still no significant difference between years (test statistic=0.71, p value=0.588, df of 4) or from the average.

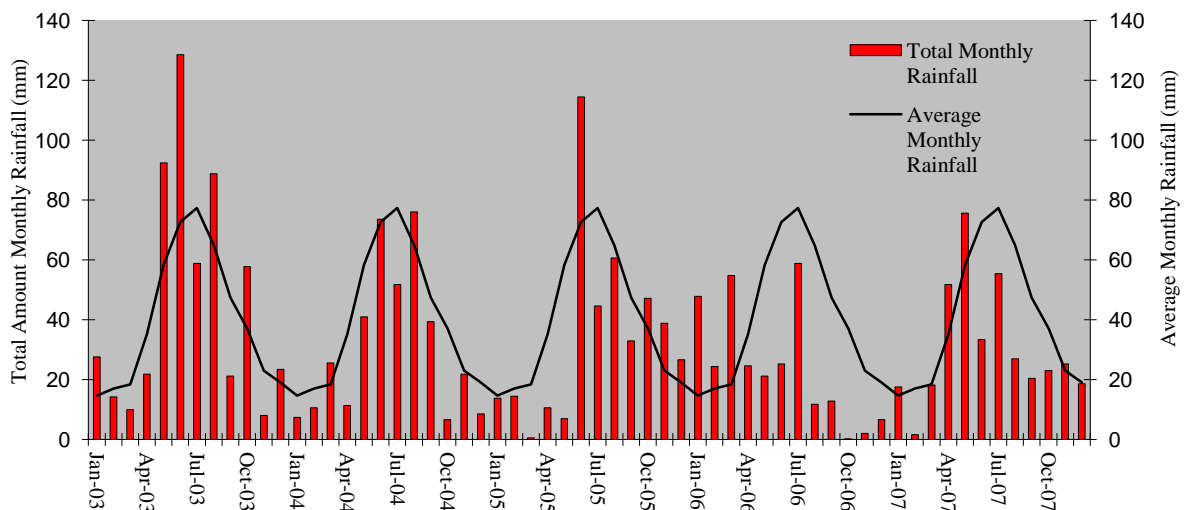


Figure 2.3.1.1 Total rainfall for the years 2003 through to 2007. Data was obtained from the Kingscote Weather Station, Australian Bureau of Meteorology (<http://www.bom.gov.au/>).

However, factors that influence sex ratio at emergence from the pouch are likely to act during the breeding season (January to early June each year (Tyndale-Biscoe, 1973; Inns, 1980), namely during mating (January), pregnancy and parturition (February) and early pouch life (March). When rainfall amounts during these periods were considered, significant discrepancies between years become apparent (Figure 2.3.1.2).

An ANOVA of the January-March (2005-2007) interval showed a significant deviation in rainfall during the study years and from the average (test statistic= 6.64, p value= 0.015, df 3). Using the Tukey-Kramer *Post Hoc* test (Kramer, 1956; Hayter, 1984), the exact relationships were visible. The rainfall in 2006 was significantly higher than the average ($q=-4.399$, $P<0.05$) and that which fell in 2005 ($q=-5.619$, $P<0.01$) and 2007 ($q=5.12$, $P<0.05$), while there was no deviation in 2005 and 2007 from the average rainfall for this time of year. If 2004 was included in the analysis, the ANOVA was still significant for these

months (test statistic=5.25, p value=0.015, df of 4), with 2004 much lower than 2006 rainfall ($q=-4.810$, $P<0.05$).

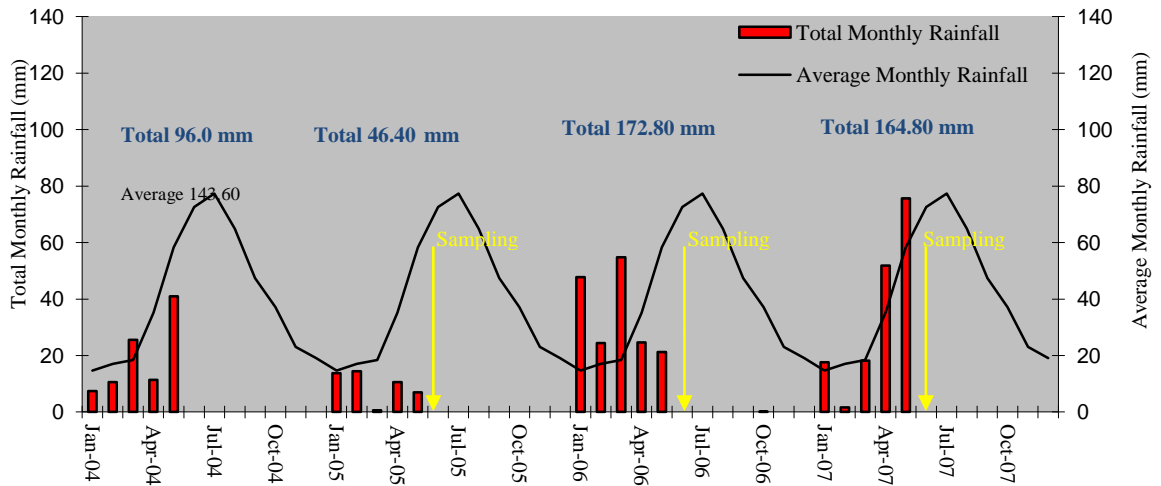


Figure 2.3.1.2 Total rainfall for the years 2004 through to 2007, during the breeding season of *M.eugenii*. Sampling indicated by the yellow arrows. Data was obtained from the Kingscote Weather Station, Australian Bureau of Meteorology (<http://www.bom.gov.au/>).

A similar examination of the April-May interval also showed significant divergence (test statistic= 8.51, p value=0.033, df of 3). The rainfall in 2005 during this period was significantly lower than average (TK Post Hoc: $q=4.532$, $P<0.05$), while precipitation in 2007 during this period was significantly higher than both 2005 ($q=-6.547$, $P<0.01$) and 2006 ($q=-4.866$, $P<0.05$). There was no need to include 2004 in this analysis, as the months of April and May are predominantly periods of PY growth with very little mating occurring. If a PY is lost during these months, a diapause will reactivate and post partum mating may occur, but the occurrence is rare in this species. If conditions during these months are poor enough to cause widespread abortion or resorption it is likely that females will enter anoestrous and not activate the blastocyst (Tyndale-Biscoe & Renfree, 1987).

It is also interesting to note that the entire summer period, from October to February, was significantly different in the sample years from the average (ANOVA: test statistic=12.68, p value= <0.001, df of 3) (Figure 2.3.1). The summer of 2005-2006 was significantly higher in the total amount of rainfall than norm (p value=0.038, t statistic=-3.06 on 4 df) and the summer of 2004-2005 (p value=0.014, t statistic=-4.17 on 4 df) and 2006-2007 (p value=0.003, t statistic=-6.42 on 4 df). The total rainfall in 2006-2007 was almost significantly different from the average (p value=0.062, t statistic=2.57 on 4 df), although not from 2004-2005.

2.3.2 Maternal Condition

Overall maternal body condition average statistics are given in Table 2.3.2.1, Shapiro-Wilk testing shows that maternal full weight and MBC showed a normal distribution, while the KFI data was skewed to the left, with $\sqrt{\text{KFI}}$ used as best fit and maternal age was skewed to the right, with $\sqrt{\text{Age}}$ used as best fit. Considering sample year, rather than monthly rainfall, as the treatment for ANOVA analysis, there were significant differences between the sample years for many of the maternal body condition parameters. The $\sqrt{\text{KFI}}$ (controlled for age) (test statistic=3.67, p value=0.030, df of 2) in 2007 was significantly lower than 2006 (TK Post Hoc: $q=3.528$, $P<0.05$), and estimated mean maternal age, calculated from the MI (Inns, 1982), (test statistic=4.33, p value=0.017, df of 2) was significantly lower in 2005 than in 2006 ($q=-4.136$, $P<0.05$) (Figure 2.3.2.2).

Table 2.3.2.1 Compilation of maternal body condition parameters measured during the sample years.

	Maternal Weight(g)	Maternal Body Condition(g/cm ³)	Maternal Age(days)	Kidney Fat Index(%g)
2005 <i>n</i> =30	4766.23	1.698	710.5	115.18
2006 <i>n</i> =21	4637.38	1.778	861.70	136.63
2007 <i>n</i> =28	4663.43	1.746	763.4	75.25

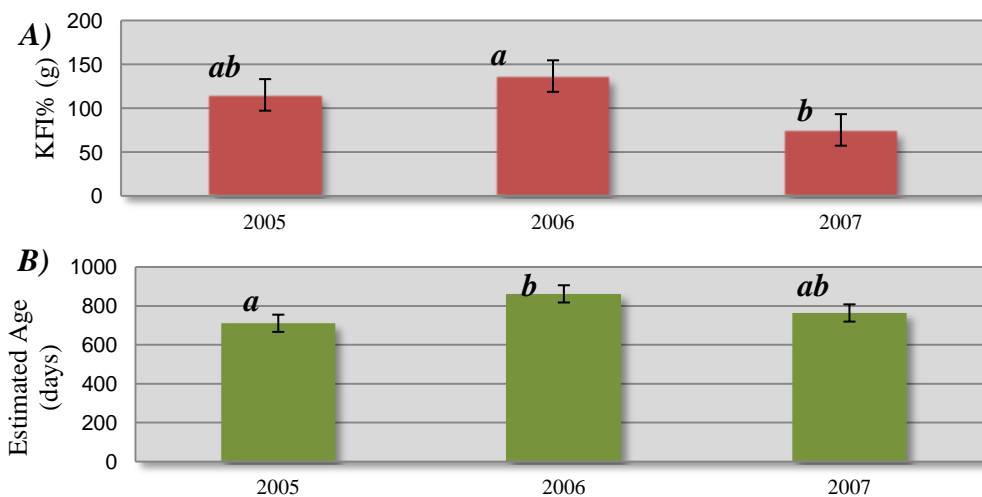


Figure 2.3.2.2 Maternal parameters with significant differences between rainfall years. **A.** Kidney Fat Index % (g) (Monson et al., 1974), **B.** Estimated Age (Inns, 1982), which was deduced from the Molar Index (Kirkpatrick, 1964). Data points with differing postscripts are significantly different. Error bars represent standard error.

Full maternal body weight (test statistic=0.15, p value=0.862, df of 2), and MBC (test statistic=0.52, p value=0.597, df of 2), both showed equal variance and ANOVA indicated there was no significant relationship with the year of sampling when controlled for age (Figure 2.3.2.3).

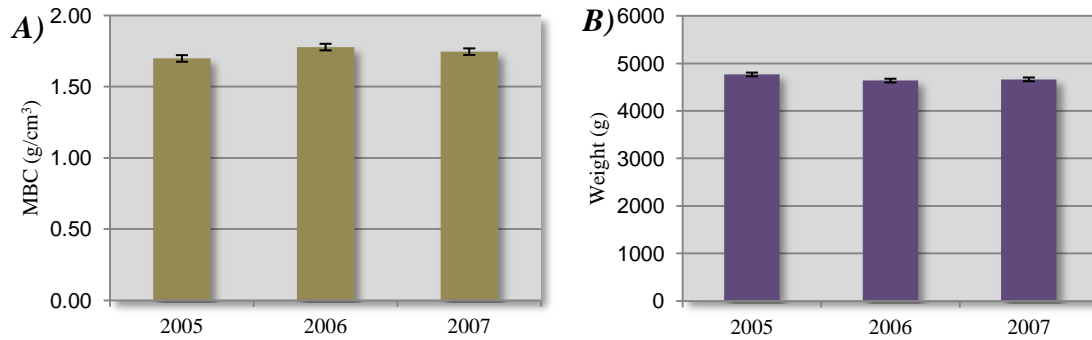


Figure 2.3.2.3 Maternal parameters not significantly different between rainfall years, **A**. Maternal Body Condition (Krebs & Singleton, 1993; Green, 2001; McGowan et al., 2008), **B**. Maternal Full Weight.

However, when this parameter was dissected further to account for the gut weight, as this changes based on the availability and quality of feed in each month and can be used as a short-term measure of fitness, a significant difference appeared between the years. The proportion of gut weight to full weight was normally distributed and significantly higher (test statistic= 3.95, p value=0.023, df of 2) in 2007 than 2006 (TK Post Hoc $q=-3.873$, $P<0.05$) (Figure 2.3.2.4). When a Bonferroni Correction was performed ($0.05/n=4$ comparisons) establishing an independent significant level of 0.0125, none of the parameters were independently significant.

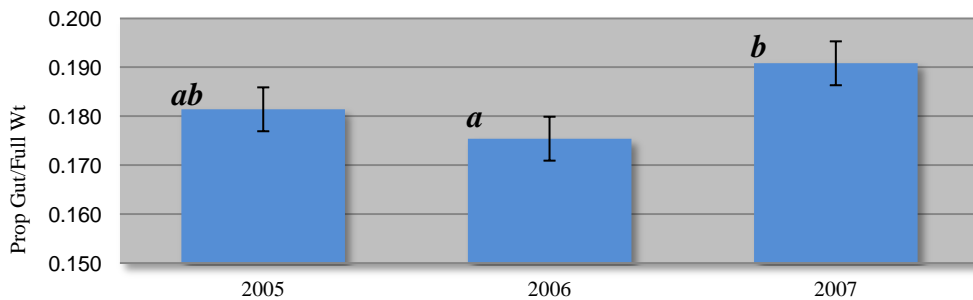


Figure 2.3.2.4 Proportions of weight distribution in the Kangaroo Island *M. eugenii* over the sample years, gut weight (g)/full weight (g). Data points with differing postscripts are significantly different.

Age (or $\sqrt{\text{Age}}$) appeared to be a heavily influencing factor for body condition in the sampled females. Backward elimination, general multiple regression showed significant relationships with full body weight (test statistic=21.95, p value= <0.001 , df of 1) (Figure 2.3.2.5), and MBC (test statistic=21.57, p value= <0.001 , df of 1) (Figure 2.3.2.6). The association of maternal age and KFI only occurred in 2005 (test statistic=3.47, p value=0.073, df of 1), while there was also a significant impact of $\sqrt{\text{Age}}$ on the proportion of gut weight/full weight in 2007 only (test statistic=7.21, p value=0.013, df of 1).

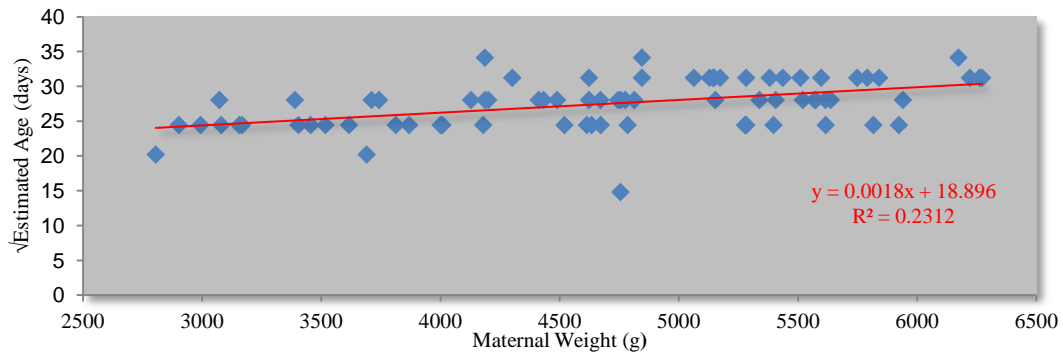


Figure 2.3.2.5 Relationship of estimated age (Inns, 1982) and maternal full weight (g) in the Kangaroo Island *M. eugenii*.

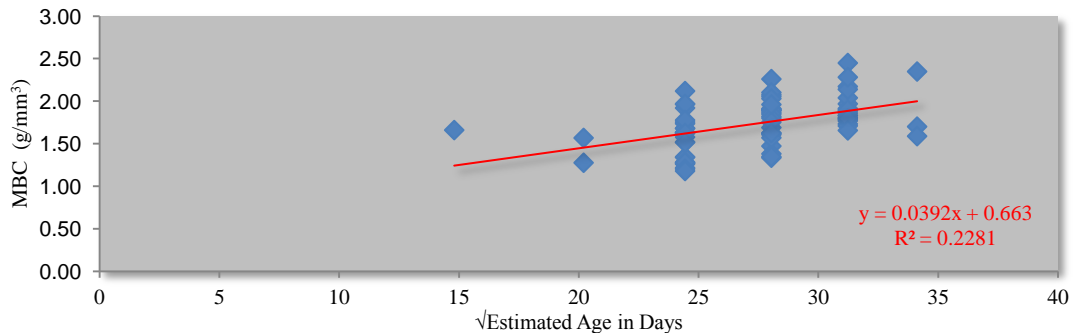


Figure 2.3.2.6 Relationship of estimated age (Inns, 1982) and Maternal Body Condition, full weight (g)/foot length (mm)³ (Krebs & Singleton, 1993; Green, 2001; McGowan et al., 2008) in the Kangaroo Island *M. eugenii*, this relationship is significant.

2.3.3 Pouch Young Condition

Overall pouch young body condition average statistics are given in Table 2.3.3.1. Shapiro-Wilks tests of normality showed that PY weight had a normal distribution, while $\log(\text{PYBC})$ and $(\text{PY Age})^3$ were used as best fit. General Regression shows PY weight was positively related to maternal weight (test statistic=7.33, p value=0.008, df of 1) (Figure 2.3.3.2), $\sqrt{\text{KFI}}$ (test statistic=12.50, p value=<0.001, df of 1) and MBC (test statistic=9.07, p value=0.004, df of 1). There was no effect of sampling year on PY weight (ANOVA: test statistic=0.62, p value=0.543, df of 2).

Table 2.3.3.1 Compilation of pouch young body condition parameters measured during the sample years.

	Pouch Young Weight(g)	Pouch Young Body Condition(g/mm ³)	Estimated Pouch Young Age(days)
2005 n=30	121.5	0.00104	131.6
2006 n=21	137.5	0.00083	138.6
2007 n=25	127.4	0.00107	127.7

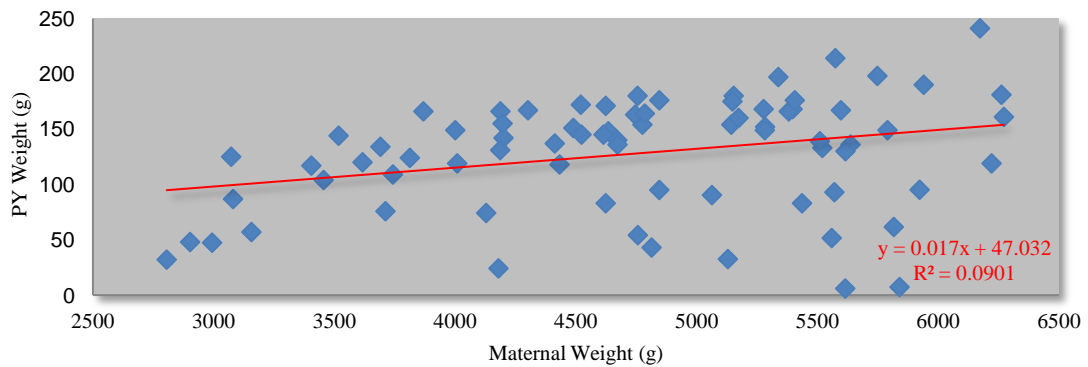


Figure 2.3.3.2 Relationship of Maternal full weight (g) and Pouch Young weight (g) in the Kangaroo Island *M.eugenii*, this relationship is significant.

The estimated age of the PY, (PY Age)³, (Murphy & Smith, 1970; Inns, 1982) was significantly associated with year of collection (ANOVA: test statistic= 3.11, p value=0.050, df of 2), as pouch young were significantly younger in age in 2007 than 2006 (TK Post Hoc: $q=3.322$, $P<0.05$) (Figure 2.3.3.3), although a Bonferroni Correction of the ANOVA analysis ($0.05/n=3$) suggested a significance level of 0.0167 was needed for independence. The $\sqrt{\text{KFI}}$ (controlled for maternal age and collection year) (test statistic=5.59, p value=0.002, df of 3) and maternal weight (logarithmic regression: test statistic=5.10, p value=0.027, df of 1) were significant predictors of estimated age in the pouch young, although the association with maternal weight was not significant when the analysis was controlled for the influence of $\sqrt{\text{KFI}}$. In addition, the proportion of maternal gut weight to maternal full body weight in mothers had a significantly negative relationship with PY estimated age (test statistic=5.35, p value=0.024, df of 1).

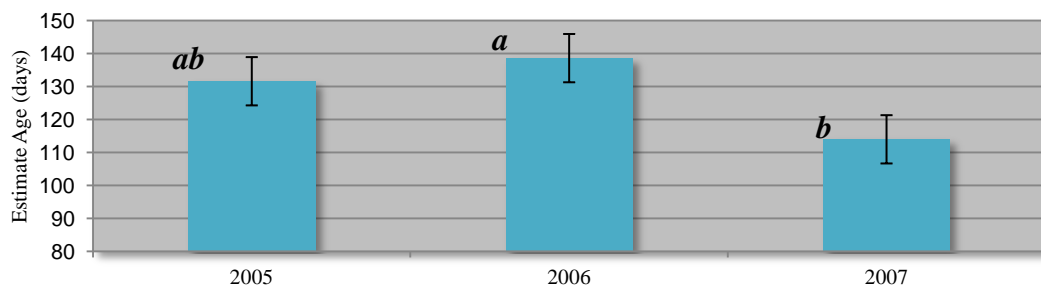
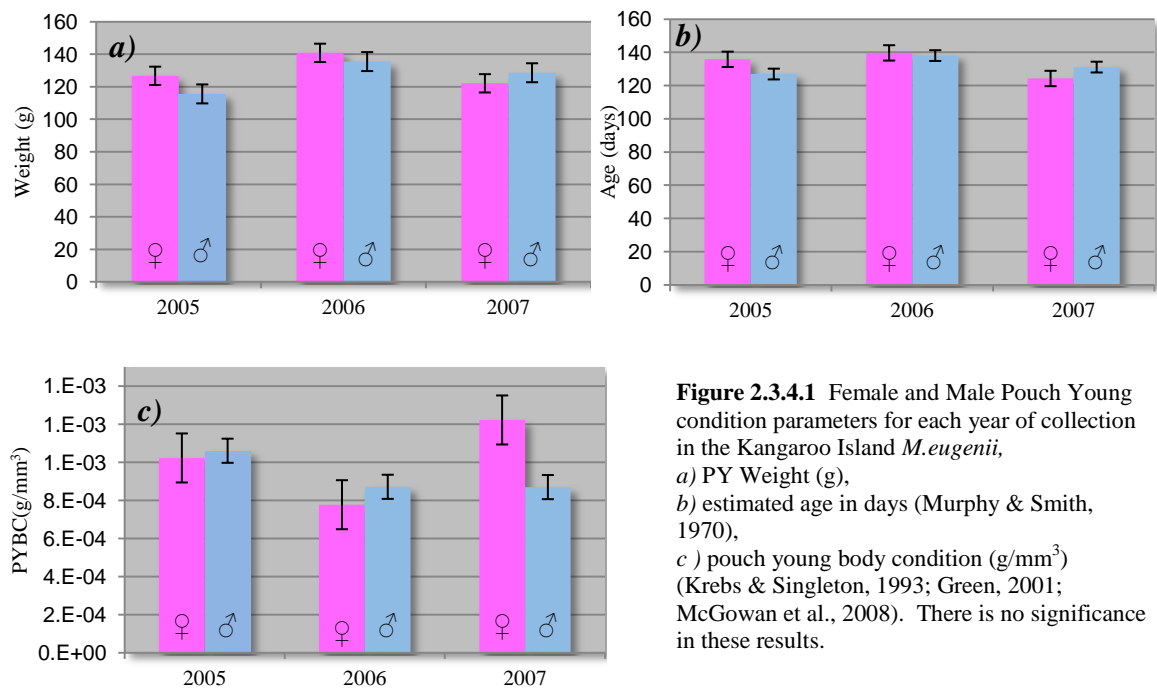


Figure 2.3.3.3 Pouch Young estimated age as per formulas from Murphy & Smith (1970) for each year of collection in the Kangaroo Island *M.eugenii*. Data points with differing postscripts are significantly different.

There was no treatment effect of sample year on $\log(\text{PYBC})$ (test statistic= 0.48, p value=0.619, df of 2), nor was $\log(\text{PYBC})$ associated with maternal weight (test statistic=0.53, p value=0.463, df of 1) or $\sqrt{\text{Age}}$ (test statistic=0.57, p value=0.724, df of 1). However, in the sample there was a negative relationship between maternal $\sqrt{\text{KFI}}$ and pouch young PYBC (test statistic=10.15, p value=0.002, df of 1).

2.3.4 Maternal Resource Allocation and Progeny Sex

Using two-way ANOVA, there was no significant impact of sex (PY Weight: test statistic=0.11, p value=0.738, df of 1; PY estimated age: test statistic=0.00, p value=0.980, df of 1; log(PYBC): test statistic=0.14, p value=0.711, df of 1) or sample year (PY Weight: test statistic=0.68, p value=0.508, df of 2; PY estimated age: test statistic=1.03, p value=0.363, df of 2; log(PYBC): test statistic=0.57, p value=0.568, df of 2), or interaction between them (PY Weight: test statistic=0.19, p value=0.826, df of 2; PY estimated age: test statistic=0.50, p value=0.611, df of 2; log(PYBC): test statistic=0.41, p value=0.665, df of 2), on PY weight, estimated age or log(PYBC) (Figure 2.3.4.1).



However, general multiple regression analysis shows maternal full weight was positively associated with both male PY weight (interaction of maternal weight and $\sqrt{\text{Age}}$ on male PY weight: test statistic=3.63, p value=0.065, df of 1) and female PY weight (interaction of maternal weight and $\sqrt{\text{Age}}$ on female PY weight: test statistic=3.12, p value=0.086, df of 1), though the relationship was different for male and female offspring (Figure 2.3.4.2).

Regression analysis also showed a difference between the two sexes when looking at the relationship between (PY age)³ and maternal $\sqrt{\text{KFI}}$ (controlled for maternal age) (Female PY: test statistic=14.05, p value=<0.001, df of 1; Male PY: test statistic=3.49, p value=0.070, df of 1). There was also a difference between the two groups in the relationship between maternal $\sqrt{\text{KFI}}$ and PY weight (controlled for maternal age) (Female PY: test statistic=8.40, p value=0.006, df of 1; Male PY: test statistic=5.36, p value=0.027, df of 1).

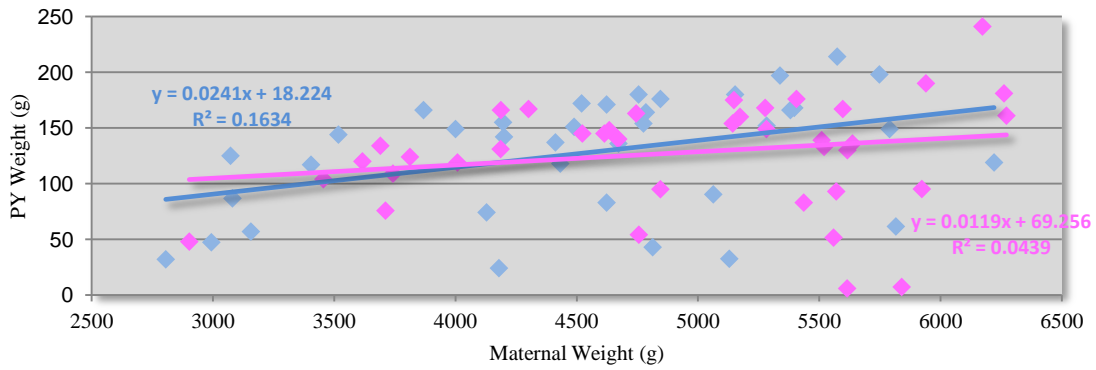


Figure 2.3.4.2 Relationship between maternal full weight and weight of male and female offspring in the Kangaroo Island *M.eugenii*. The blue dataset is the male PY relationship to maternal age, which is significant.

2.3.5 Pouch Young Sex Ratio

The PY sex ratio did not appear to be significantly associated with year of collection (Figure 2.3.5.1). There was also no significant deviation from the overall expected sex ratio of 1:1 in any year of collection ($\chi^2=2.324$, $n=76$, $P<0.10$). Therefore, the Null Hypothesis was retained, as the observed values were not significantly different from expected (Figure 2.3.5.2).

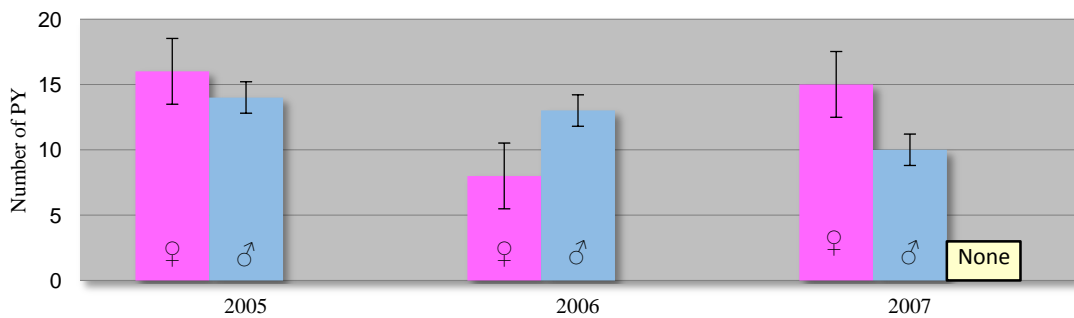


Figure 2.3.5.1 Total numbers of Pouch Young females and males over the sample years for the Kangaroo Island *M.eugenii*. The overall sex ratio is 1:1. Any trends towards producing one sex over the other are non-significant.

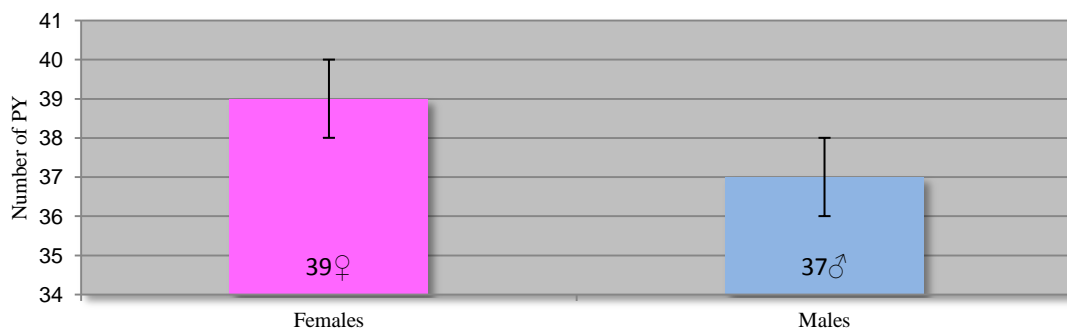


Figure 2.3.5.2 Total numbers of Pouch Young females and males collected for the entirety of the study of the Kangaroo Island *M.eugenii*. Overall sex ratio does not differ significantly from 1:1.

Logistic Regression analysis shows that the sex ratio of PY was significantly associated with maternal weight (test statistic=3.65, p value=0.056, df of 1) and non-significantly related to maternal age (test statistic=2.82, p value0.093, df of 1), but not with MBC or KFI. When the

whole data set was examined, the data from the older and heavier females was significantly different from the 1:1 ratio (χ^2 statistic 4.263, $n=26$, $P<0.10$).

If each year was studied independently, the relationship between gender on the one hand and maternal weight and age on the other became more significant. The sex of a PY in 2005 was significantly related to maternal age (Log Regression: test statistic=4.29, p value=0.038, df of 1) and maternal weight (test statistic=4.07, p value=0.044, df of 1), and the relationship between the two was highly predictive of PY sex (test statistic=5.27, p value=0.022, df of 1). There were significant differences in the average maternal weight of mothers who had female and male PY in 2005 (test statistic=4.20, p value=0.050, df of 1). Yet, in 2006 (test statistic=0.42, p value=0.524, df of 1) and 2007 (test statistic=0.04, p value=0.850, df of 1), there was no relationship between these maternal factors and PY sex, as the only significant relationship between maternal weight and PY sex was in 2007, with mothers without PY being significantly smaller than other mothers (test statistic=3.58, p value=0.043, df of 2; $q_{\text{Male vs. None}}=3.40$, $q_{\text{Female vs. None}}=3.71$, $P<0.05$) (Figure 2.3.5.3).

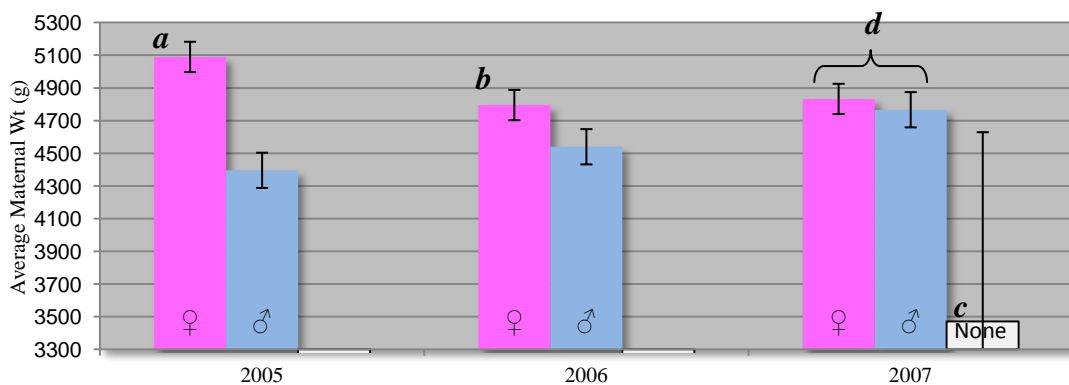


Figure 2.3.5.3 Average weight (g) of Mothers with Pouch Young, females and males collected each year for the Kangaroo Island *M.eugenii*. There are significant differences between *a* & *b* and between *c* & *d*.

As Sunnucks & Taylor (1997) found that females on Kawau Island, NZ showed sex biases in mothers weighting below 3.2kg (female) and above 4.2 kg (male), suggesting 3 separate groups: Group 1 (<3.2kg) with a higher proportion of female young, Group 2 (3.2-4.2kg) equal probability of having either sex, and Group 3 (>4.2kg) with a higher probability of producing male offspring. When this grouping system was tested on this sample, it proved a poor fit for the data set (test statistic=2.04, p value=0.153, df of 1). In this data set, the probability of having sons increased under the weight of 3.5kg and the probability of having daughters increased over the weight of 5.5kg. The current data lent itself to partitioning into 3 slightly modified weight groups (Figure 2.3.5.4): Group 1 included mothers below 3.5kgs, Group 2 comprising mothers between 3.5 - 5.5kgs and Group 3 consisting of mothers above 5.5kgs (Log regression: test statistic=6.64, p value=0.010, df of 1), the maternal weight

group appeared more predictive than just maternal full weight and maternal age, as the effects of weight group and maternal age were slightly less influencing (test statistic=6.07, p value=0.014, df of 1). Year of sampling had no significant impact here, although individually, 2005 was the only year to show significant relationships between weight group, age and PY sex (Weight Group: test statistic=4.73, p value=0.030, df of 1; Weight Group & Maternal Age: test statistic=5.99, p value=0.014, df of 1), while years 2006 and 2007, analysed individually, did not show significant relationships for PY Sex and maternal weight group. The sex ratio of Group 1 was 3:1, which was significantly different to the sex ratio of Group 3, 1:2.8 (TK Post Hoc: $q=3.347$, $P<0.05$). When taken separately, Groups 1 and 3 both deviated significantly from the expected ratio of 1:1 (Group 1: $\chi^2=25.0$, $n=5$, $P<0.001$; Group 3: $\chi^2=23.040$, $n=10$, $P<0.001$).

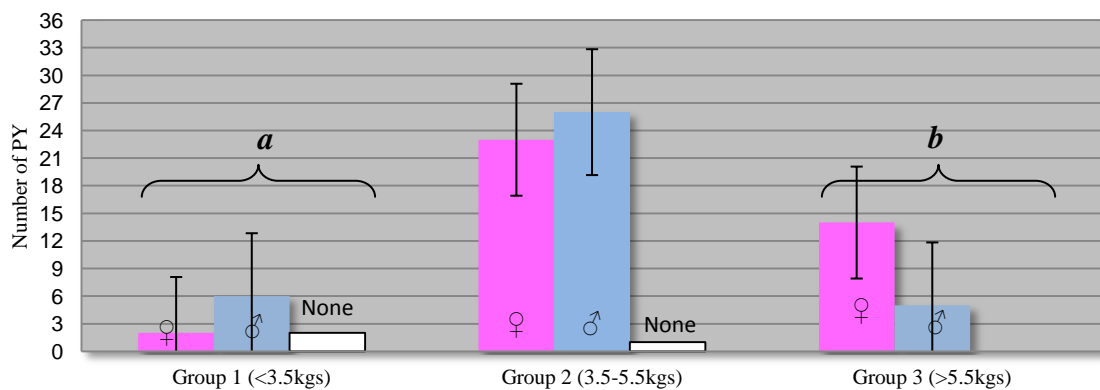
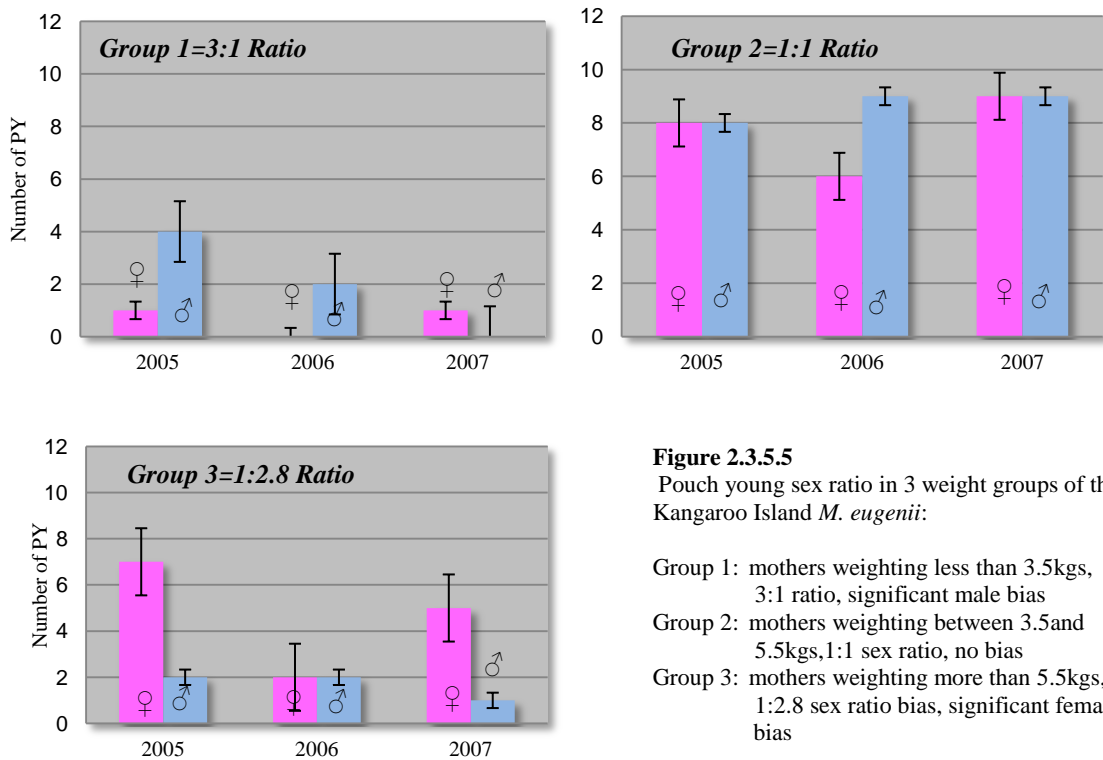


Figure 2.3.5.4 PY Sex Ratio per weight group in *M. eugenii* collected over the entirety of the study on the Kangaroo Island. Data points with differing postscripts differ significantly from each other and from the expected 1:1 ratio.

When the groups were treated separately, with year as the explanatory variable, there was no significant relationship between year (representing rainfall) and the sex ratio variation in Groups 2 (test statistic=0.33, p value=0.565, df of 1, $n=35$) and 3 (test statistic=1.27, p value=0.261, df of 1, $n=10$), although Group 1 (test statistic=3.82, p value=0.051, df of 1, $n=5$) did show a predictive relationship, although this result may be influenced by the small sample size. . The KFI in Group 2 was significantly correlated with year of collection (ANOVA: test statistic=8.44, p value=0.007, df of 1, $n=49$), though not in Groups 1 (test statistic=3.08, p value=0.178, df of 1, $n=8$) & 3 (test statistic=0.69, p value=0.429, df of 1, $n=19$), with 2007 Group 2 females ($n=18$) being significantly lower than both 2005 ($q=3.24$; $P<0.05$, $n=16$) and 2006 ($q=3.65$; $P<0.05$, $n=15$) Group 2 females (Figure 2.3.5.5).

**Figure 2.3.5.5**

Pouch young sex ratio in 3 weight groups of the Kangaroo Island *M. eugenii*:

Group 1: mothers weighting less than 3.5kgs, 3:1 ratio, significant male bias

Group 2: mothers weighting between 3.5 and 5.5kgs, 1:1 sex ratio, no bias

Group 3: mothers weighting more than 5.5kgs, 1:2.8 sex ratio bias, significant female bias

Maternal age showed a non-significant predictive trend with PY sex (Log regression: test statistic= 2.82, p value=0.093, df of 1), yet if the influence of sample year and weight group classification were included in the analysis, the relationship was close to significance at the 0.05 level (Log regression: test statistic=10.49, p value=0.062, df of 1). The effect of the relationship between maternal age, sample year and weight group was more significantly associated with PY sex than maternal age, sample year and maternal weight (Log regression: test statistic=8.42, p value=0.077, df of 1).

2.4 Discussion

2.4.1 Does rainfall impact on maternal condition, and is maternal condition an indicator of pouch young condition?

The present data on maternal condition show that rainfall (represented in the data by sample year) during the breeding season, January to June (Andrewartha & Barker, 1969; Berger, 1970; Inns, 1980), was significantly associated with maternal condition. The rainfall from January to March 2006 (predominant mating and parturition months) was significantly higher than the norm, and the other sample years. This corresponds with maternal condition parameters that year, as the KFI and maternal estimated age were both higher in 2006 than in the other sample years. Conversely, the pattern of rainfall over the entire summer period in 2005 and 2007 differed in their effects on maternal body condition.

Mating and pregnancy in *M. eugenii* occurred predominantly in the months of January and February. In 2005, January and February received close to average rainfall before declining from March to May, while in 2006, the rainfall was well above average and in 2007, the accumulated rainfall was poor. However, the average growing season on Kangaroo Island is mid-April to late October (Burrows, 1979), and most body fat in the tammar accumulates in late winter and spring (Inns, 1980). In accordance with the rainfall during this period, females in 2005 showed a median KFI, with females in 2007 producing the lowest KFI, as the preceding winter and spring showed the least accumulated precipitation for the period. Only the 2007 cohort of adult female wallabies contained individuals without pouch young, which is rare in a species of high fecundity like *M. eugenii* (Andrewartha & Barker, 1969). Such drought induced loss of pouch young has been observed in other macropodoid species with high fecundity (Frith & Sharman, 1964; Fleming et al., 1983). Other studies on Kangaroo Island *M. eugenii* found that out of 282 mothers observed from 1964 to 1969, only 15 reproductively capable females did not have pouch young (Andrewartha & Barker, 1969), while Inns (1980) observed fecundity rates as high as 100% and as low as 88%, Wright & Stott (1999) observed rates of 83% on culled farms and 92% on uncultured farms. In the present study, the three empty females noted in 2007 resulted in a fecundity rate of 89.3%, as opposed to 100% in 2005 & 2006.

Maternal age was significantly impacted by rainfall patterns, as females in 2005 were younger than in other sample years, indicating a “die-off” of older mothers. An increased mortality rate usually corresponds to a year when the growing season (April to October) is

shortened. Yet, mothers in 2007 were not quite as young, showing a median age between 2005 and 2006 females, despite the reduced total rainfall for the 2007 growing season, as compared to that in the 2005 growing season. Inns (1980) observed similar die-offs of older adults affected by the drought of 1977, as in other marsupial species exposed to prolonged periods of drought, although die-offs are usually coupled with changes in body weight, which was not observed in the current data (Caughley et al., 1984; Fisher et al., 2001a; Parrott et al., 2007). According to the current data, 2007 should have shown a greater mortality rate in older females than 2005 that was not evidenced in the data.

Rainfall, although influencing KFI and maternal age, did not appear to impact full body weight or MBC, despite the relationship of these two parameters to increased maternal age, *i.e.* older mothers tending to be larger mothers. However, this data might have been confounded by the timing of sampling, as sampling occurred in mid-June each year, and rainfall in June 2005 was especially high. The proportion of maternal gut weight to maternal full body weight gave a slightly clearer picture, as the gut will include any weight of ingesta food matter. The females in 2006 had a significantly lower gut/full weight ratio than those in 2007, despite increased resource availability. Mothers in 2006 were not nutritionally stressed or required to fill their digestive system to capacity and were exposed to higher quality feed, while females in 2007, in an attempt to “catch up” after a period of low resource availability, had increased their feed intake, causing a higher percentage of their overall weight to be filled gut weight. Further confounding effects may be attributed to land-use practices, as water supplied for livestock or crops is often freely available to native species, as observed in other macropod species habituating in farming communities (Jonzen et al., 2005).

It is not surprising that maternal body condition had a direct and significant correlation with PY body condition, due to maternal resource transfer (Green et al., 1988), yet fluctuations in maternal condition caused by variations in rainfall appeared to be slightly mitigated for *M. eugenii* pouch young. Progeny weight was directly linked to maternal full weight, but not with rainfall, while KFI was a significant predictor, whether negatively or positively, of all the PY body condition parameters. This indicates that fat stored during the growing season was instrumental in the production and development of offspring. The estimated age of PY was closely linked to KFI, evidenced by the reduced age of young in 2007, in accordance with low KFI that year, as marsupial milk composition changes with differing planes of nutrition, which causes differences in growth rates of PY (Rose & Flowers, 2005). In

considering the estimated age of young, the comments of Inns (1982) must be considered, although the growth curves used by Murphy & Smith (1970) are useful for predicting age, the accuracy is questionable. Inns (1982) found that in years of drought, these growth curves actually show a retarded development process, rather than a younger age. Despite this conjecture, the result of this study remains the same; drought conditions significantly affected maternal body condition enough to delay birth or to retard the growth of the pouch young. Similar evidence for drought-induced growth restriction during lactational dependency has been observed in other marsupial species, such as the *P. tapoatafa* (Rhind & Bradley, 2002) and *M. rufogriseus banksianus* (Higginbottom, 2000).

The impeded skeletal growth rate observed in the 2007 pouch young of mothers with poor fat stores was not corroborated by reduced PY weight or PYBC in the sampled population. Neither of these parameters showed any rainfall dependent difference, and despite sharing significant relationships with KFI, the annual variation in adipose tissue deposits evident in these data did not produce annual variance in PY weight or PYBC. Given the difference in skeletal development as represented by the estimated age of the PY, PY weight and PYBC must be indicators of body condition irrespective of body size. As maternal weight is a function of age in this population and maternal weight is an indicator of PY weight, it is likely that younger females are using a different resource transfer mechanism than older females to supplement body condition enough to remove differences in weight irrespective of skeletal size.

Suppositions based on this data suggest that younger mothers were transferring their resources directly to their young, initially bypassing their own body condition improvement, similar to some females in captive studies (Cork, 1991), while older females were able to retain their body fat stores and the condition of their PY. This corresponded with the increased mortality of young mothers apparent in 2007, as late winter, spring and summer of 2006-2007 was extremely dry. In addition, there seemed to be an adaptive pattern of resource allocation during recovery from nutritional stress, although this may be employed by all mothers, not just younger mothers. In 2007, as evidenced by the increased maternal gut/full weight ratio and the reduced amount of KFI (there is a significantly negative relationship between the two), all mothers appeared to be allocating resources to their PY's condition first, despite skeletal growth lagging behind other years. The relationship shared between PYBC and maternal gut/full weight and KFI is negative, as the more condition was applied to the young, the less fat stores and feed were available for maternal utilization.

2.4.2 Does maternal investment vary between sons and daughters?

As there was no significant impact of rainfall on sex ratio bias in the sample presented here, it is likely that species specific adaptations, irrespective of rainfall, were responsible for the observed skew. Unlike many ungulate species (Kojola, 1998), the ability of tammar mothers to buffer their young from external resource variation, with only a slight delay in development, did not translate into adaptive variation in investment between the sexes. There was no significant difference in condition or size between the male and female young at the point of sampling, which is consistent with other macropodoid species (Stuart-Dick & Higginbottom, 1989; Fisher, 1999). This may be due to the divergent life history traits of eutherian and metatherian species, as resource transfer *in utero* is partially regulated by the embryo in eutherians (Clutton-Brock, 1991), and is maternally controlled through lactation in metatherians (Trott et al., 2003; Menzies et al., 2007). The only minor deviation in condition between the sexes was a small increase in female PYBC in 2007. However, this was not mirrored by any other parameter and was not significant. Given the lactational pattern of this species, as with other marsupial species there are three different stages in lactation for the *M. eugenii* (Green et al., 1988; Cork & Dove, 1989; Young et al., 1997), this does not preclude sexually biased resource transfer later in pouch life, *i.e.* before or after emergence from the pouch or at weaning. This data only confirms that there was no discrepancy in resource allocation between the sexes at this early stage of pouch development.

The findings of Murphy & Smith (1970) correspond with the present results, as they reported no discrepancy between the sexes at this stage of development, or that of *Petrogale canthopus* at emergence (Poole et al., 1985). Sexual dimorphism has not been observed until the young emerge permanently from the pouch, at which point males prioritise resource allocation to growth while females prioritise reproduction (Nurse & Renfree, 1994; Williams et al., 1998; Loison et al., 1999). A cross-fostering experiment in *M. eugenii*, also failed to find any significant difference in size between sons and daughters, although mothers that birthed sons had a higher probability of weaning an offspring, regardless of the cross-fostered sex that they had weaned (Robert et al., 2010).

In other weakly polygynous species, such as the tammar, there is evidence that heavier mothers produce heavier daughters and lighter mothers produce lighter daughters, while the weight of sons remains unaffected by maternal weight (Hewison et al., 2005). A similar comparison in the current data set showed that the body mass of sons increased faster with

maternal weight than that of daughters, even when maternal age was included. However, the age and weight of daughters showed greater increase with maternal condition parameters, *i.e.* KFI, than that of sons, especially when maternal age was accounted for in the analysis. Therefore, heavier mothers are producing heavier sons, but not heavier daughters, although this mother is not necessarily in good condition, merely possessing a more robust frame that achieves greater weight. A son with a larger, more robust frame is more likely to win a contest than a better-conditioned male with a smaller frame, but better body reserves. However, the older, better-conditioned mothers produced older, better-conditioned daughters, but not older, better-conditioned sons, despite the lack of a discernible difference in size or condition at the time of sampling in the present data. Thus, there might be some form of terminal reproductive effort in older females, by increasing their resource transfer to their daughters (Isaac & Johnson, 2005).

2.4.3 Does maternal condition alone bias sex ratio?

The overall sex ratio of pouch young in the sampled population was 1:1, which supports Fisher's (1930) theory of sex ratio equality (Kolman, 1960). However, there was an increased possibility of producing female PY in older, larger mothers, which was offset by younger mothers producing sons, while intermediate mothers were producing both sexes equally, irrespective of body condition parameters such as KFI. Similar results were noted for other macropod species, in which the overall ratio was 1:1, although specific sections of the population showed sex ratio bias (Caughley & Kean, 1964; Robinson et al., 1994). The production of a male or female offspring was not dependent on maternal body condition or rainfall stimuli, but solely on maternal weight and age. This result is in stark contradiction to much of the literature available on highly dimorphic ungulate species (Cote & Festa-Bianchet, 2001). However, the pattern does correspond with examples observed in weakly dimorphic mammalian species (Hewison & Gaillard, 1999; Rosenfeld et al., 2003; Rosenfeld & Roberts, 2004; Hewison et al., 2005; Cunningham et al., 2009; Shibata & Kawamichi, 2009), where high quality mothers produced females, based on age, diet or dominance. Thus, the overall sex ratio skew pattern did not correspond with the TWM.

The relationship between maternal age and weight with the production of daughters, although not significantly correlated with sample year at the 0.05 level, appeared to be magnified in 2005 mothers. Collectively, mothers in 2005 produced a sex ratio closer to parity than mothers in 2006 or 2007, but the probability of older, larger mothers producing daughters was higher than in other sample years. Mothers in 2005 were slightly younger than in other sample years, and their KFI was at median levels, as was their feed intake. The

intensity of the 2005 sex ratio bias amongst older, heavier mothers may have been caused by another form of stress during pregnancy or mating, as stress is shown to change hormone levels (Lane & Hyde, 1973; Kinsley & Svare, 1988; Hattingh & Petty, 1992; Yamaguchi et al., 2005) and may be caused by any number of external factors. Stress on the sampled populations during the breeding season, coupled with possible nutritional stress, is linked to female biased sex ratios in other species (Marealle et al., 2010). Yet, this stress might be mitigated by alleviating high population density at a time of intensified resource competition (Swihart et al., 1998). For example, Wright & Stott (1999) showed a difference in weight and condition between tammars sampled on culled and uncultured properties, with the culled sample showing higher weights and lower worm rates, but there was no effect on the sex ratio between the two populations.

As the direct cause of the magnified trend in 2005, and the overall relationship in the population between maternal age, weight and likelihood of producing daughters, was not easily discernable, comparisons with other observed sex ratio skews in the *M. eugenii* literature were necessary. However, such comparisons produced some interesting contradictions. For instance, Sunnucks & Taylor (1997) found that *M. eugenii* mothers on Kawau Island in New Zealand exhibited a sex ratio bias for which *a posteriori* application of the TWM appears suitable. Similar to the present study, an overall sex ratio parity was observed, along with a relationship between maternal weight and sex ratio bias, their data also showed a scale of maternal weight into three weight classes, each with a separate probability of producing sons or daughters. Such maternal weight groupings have been noted in other mammalian species, though most often as a function of dominance class (Berman, 1988; Nevison, 1997; Cote & Festa-Bianchet, 2001; Brown & Silk, 2002). However, the lightest group in the 1997 data, comprised of females below 3.2kg, had a female biased ratio; the intermediate group in 1997 (including females between 3.2 and 4.2kg) had no sex ratio bias; and the heaviest group, including females above 4.2kg, had a male biased sex ratio (Sunnucks & Taylor, 1997). These results are opposite to the results of the present study.

The disparity between the two studies represents two distinct adaptive patterns, with opposing results. Both rely on maternal weight and produce an overall equal sex ratio, although with contradictory mechanisms. Such intraspecific divergence might be caused by changes in the local climate, as described in the Bennett's wallaby (*M. rufogriseus*), which has evolved different breeding cycles in distinct habitats (Fleming et al., 1983), or by

geographic isolation causing fixed differences in genetic makeup, as described between Kangaroo Island and Garden Island tammar populations (Hinds et al., 1990a). However, although there are climatic differences between Kawau Island and Kangaroo Island (BOM, 2008; WWO, 2008), the analysis of the current sample from Kangaroo Island suggests this should have no bearing on sex ratio skewing in the species. Thus, there must be another explanation. Review of the literature available on intra-specific discrepancies in sex ratio bias suggests that in some cases it is caused by the omission of maternal age as a causal factor in analysis (Saltz, 2001; Saltz & Kotler, 2003), or due to stochastic variation caused by small sampling sizes (Brown & Silk, 2002). As many of the factors correlated with sex ratio bias are age-dependent in populations, the omission of maternal age in the analysis by Sunnucks & Taylor may be significant for the present comparison.

Due to the variation between the results in the Sunnucks & Taylor (1997) study and the present data, it is more useful to assess the similarity in results to other projects on Kangaroo Island *M. eugenii*, as this removes the possible confounding influence of geographic variance. Both Inns (1980) and Andrewartha & Barker (1969) found no deviation from the 1:1 sex ratio of pouch young in tammar populations, but neither study looked at the probability of sex ratio bias based on maternal age or weight. Unlike these early studies, Wright & Stott's 1999 survey of several tammar populations on Kangaroo Island found a male biased ratio in pouch young, as high as 2.47:1 in uncultured populations.

The male biased sex ratio in pouch young observed in Wright & Stott's (1999) survey was not easily explained, when compared with the data presented here. The average weight of wallabies in the 1999 study was 5.36kg, with a KFI between that of mothers in 2005 and 2007, and an age slightly older than the females of the present study. However, Wright & Stott did not differentiate in their statistics between male and female adults, possibly confounding the statistics, as females tend to live longer than males (Andrewartha & Barker, 1969; Inns, 1982), and fluctuate less in adipose tissue deposits and weight than males, though they remain heavier than females throughout the year (Inns, 1980).

2.4.4 Is the TWM present in this population? If not, then what?

The present data set showed little significant support for the TWM. The fact that smaller, possibly primiparous (Inns, 1980; Lloyd & Rasa, 1989), mothers "chose" to produce sons and larger, more experienced, older mothers "chose" to produce daughters, despite the polygynous mating structure, contradicts the philosophy of the TWM. This leaves the

question, if the TWM is not the overall governing model of sex ratio allocation in this species, then there must be another model.

There are several different theories as to why the production of daughters could be more desirable than sons in polygynous species. One such theory, which is almost as widely tested as the TWM, is the Local Resource Competition (LRC) model (Clark, 1978), in this instance competition for mates is included as possible resource competition. Although first described for primate species, the LRC model is now widely used as an alternative when support for the TWM is lacking. The LRC was originally discussed as a model for species where high dispersal rates of one sex (normally the male) causes increased resource competition amongst the non-dispersed sex (mothers and daughters), providing impetus for a male biased sex ratio skew amongst progeny. Although this paradigm is inconsistent with the present data, a modified age dependent version of the LRC might correspond. The literature on *M. eugenii* suggests that mothers and daughters live in the same or overlapping home ranges, competing for resources and mates (Inns, 1980). There is no natal dispersal by either sex in the tammar wallaby, and adult breeding dispersal appears to be only under conditions of environmental necessity, such as after fire (Christensen, 1980).

As there is no evidence of dispersal amongst either sex in the Kangaroo Island *M. eugenii*, resource competition between mothers and offspring of both sexes will occur. However, as females live longer than males in the wild (Andrewartha & Barker, 1969; Inns, 1980; Speakman, 2005), and seasonal shifts in grazing behaviour, as adult males are more predominant in open clearings during summer months while females are more predominant during winter months (Inns, 1980; Andrewartha & Barker, 1969), will alleviate some competition between a mother and her sons. This means that mothers having daughters earlier in their breeding career will compete more intensely for resources for a longer period with their daughters (Kokko & Jennions, 2008), including competition for mates. Such competition has been observed in red-necked wallabies (*M. rufogriseus*), where females living in family groups have lower reproductive success than more solitary females (Johnson, 1986). Therefore, it is beneficial for a mother to have sons earlier in her breeding years and daughters later (Guillon & Bottein, 2011), favouring a negative correlation between the production of daughters and maternal age (Clutton-Brock et al., 1981; Clutton-Brock & Albon, 1982; Cockburn et al., 1985a; Cockburn et al., 1985b; Clutton-Brock & Iason, 1986; Cockburn, 1990). Moreover, although in most macropod species the male disperses more widely than the female, when such dispersal is absent, as in the tammar and

the Quokka (*S. brachyurus*) where dispersal is absent due to habitat restrictions, there is evidence that a female biased ratio will occur (Johnson, 1989).

The current population did not represent a female biased sex ratio *per se*, but rather an equal population sex ratio where older, larger females preferred to have daughters and smaller, younger females preferred to have sons. Similar effects are observed in other mammalian species, where sex ratio is dependent on maternal body condition, but only amongst high-ranking females (Dittus, 1998). Some of the literature on eutherian species, such as the bighorn sheep (*Ovis canadensis*), supports this concept, as only older mothers showed an interaction between environmental conditions and reproductive strategy (Martin & Festa-Bianchet, 2011). Thus, there must be a perceived benefit realised by older, larger mothers investing in daughters for such a mechanism to be adaptive.

Weakly dimorphic species, such as the tammar, where sexual dimorphism is based solely on body size, and the differential between the sexes is less than 20% (Poole et al., 1992; Hewison et al., 2005; Shibata & Kawamichi, 2009), exhibit a dynamic of older, larger females producing more daughters. These species produce less variation in reproductive success between the sexes, reducing the need to bias increased investment in one sex over the other. This is in contrast to highly dimorphic macropod species, which show consistency with the TWM (Fisher, 1999) and in accordance with other weakly dimorphic species, which show less support for the TWM and studies often report other models as causal factors (Delean et al., 2009). The male dominance in *M. eugenii* is a weak predictor of paternal success, as although the dominant male in the group will secure roughly 60% of first matings, females mate with several males during their oestrus cycle, and the dominant male only sires roughly 50% of the young in the group, with secondary males contributing significantly to the population (Hynes et al., 2005). In the absence of highly polygynous social structures, as sexual dimorphism is directly linked to the level of polygyny in a species (Loison et al., 1999), it becomes more likely that female fitness may vary just as highly as that of males. For example, high quality mothers will produce high quality daughters, which will produce high quality granddaughters, while high quality sons will produce more offspring, but not always of superior quality (Leimar, 1996).

Once high discrepancies in reproductive success are removed from the population, other population parameters become important influences on sex ratio bias. For example, female dominance or hierarchy may occur in large groups, where relatives compete for resources

and mates, and larger daughters may inherit the home ranges of larger mothers. When females also present with some form of dominance ranking or territoriality, it is likely that a female biased sex ratio will occur amongst older, larger mothers (Hewison & Gaillard, 1999; Koskela et al., 2009). Several mammalian species show a sex ratio bias based on dominance hierarchies, rather than maternal condition (Berman, 1988; Lloyd & Rasa, 1989; Krackow, 1995; Flint et al., 1997; Dittus, 1998; Kinahan & Pillay, 2008; Grant & Chamley, 2010). However, some primate species have defined dominance groupings, similar to the present weight groups, with discrete sex ratio bias probabilities which mirror the data presented here (Berman, 1988; Nevison, 1997). Although such female dominance rankings have not been observed in *M. eugenii*, it is possible that dominant males, given a choice, will prefer to breed with older, larger females and that these females are better able to compete for resources against smaller ones.

Such dominance hierarchies in females have not been observed in Macropodidae (Stuart-Dick & Higginbottom, 1989), suggesting there are other reasons why older, larger females prefer to invest in daughters. In *M. eugenii* family groups, there is likely to be intergenerational competition for resources amongst mothers and daughters as discussed above, though there is also an increased competition between siblings and other relatives. Evidence from eutherian species suggests that in societies where siblings or close relatives compete for mates and resources, where male and female offspring carry equal costs of production, mothers bias their young according to the sex that competes less intensely for mates (Hamilton, 1967; Charnov, 1982; Clutton-Brock & Iason, 1986). Reviews of the literature suggest that, across species, response to competition or cooperation between relatives is more important than environmental stimuli in determining sex ratio bias (West et al., 2005). Thus, in tammars, producing more daughters will reduce the intensity of competition for mates amongst males, consequently increasing the reproductive success of sons (Grafen, 1984).

In the sampled population, it is unlikely that these forms of competition are mutually exclusive, but that both are operating on maternal investment simultaneously. Therefore, younger, smaller mothers are producing sons to alleviate competition for resources and mates, as females are capable of mating at emergence from the pouch (Williams et al., 1998), and males reach fecundity much later, at 18-20 months of age (Inns, 1980), producing daughters early in a female's reproductive career will only increase competition for mates and resources at a point in their life history where they are the most vulnerable to

fluctuations in resource availability (Inns, 1980; Caughley et al., 1985; Parrott et al., 2007; Pople et al., 2007). This vulnerability is alleviated as females mature, at which point the investment in daughters is necessary to increase the fitness of sons produced in previous breeding cycles, by reducing male competition for available females in the family group. Changes in population dynamics created by these two forms of competition could favour changes in sex ratio, and explain the discrepancy evident in some of the data on sex ratio in *M. eugenii* (namely between the present data and that presented by Sunnucks & Taylor (1997)).

Such a reproductive life history cycle may also serve to maximize the total fitness of each female wallaby, in conjunction with minimizing competition with and between relatives. As there is no marked difference between the size of male and female offspring at emergence from the pouch, and male dominance is only weakly related to the number of progeny sired, younger mothers producing sons are unlikely to impact on their son's fitness or survival post-weaning. Inns (1980) found that during stressful years, juvenile male mortality rates were higher than juvenile female rates during their first year after emergence from the pouch, and this discrepancy continued into their second year, with 58% of females surviving compared with 50% of males in a good year and 56% female and 36% male survival rate in a poor year. This increased life expectancy may be due to higher water turnover rates in YAF in extreme environments, mass-specific metabolism twice that of adults and energy requirements 60-70% that of adults, although adults are three times their size (Munn & Dawson, 2001). If life expectancy is diminished for sons, then female biased sex ratios in older, more experienced mothers might be an attempt to maximize the number of successful progeny produced, rather than the average reproductive potential of each progeny (Myers, 1978; Frank, 1990). The differential energy expenditure shown by sons and daughters emerging from the pouch suggests that, as primiparous females are increasing fat stores for their inaugural breeding cycles, males are concentrating on growth (James, 1996), which might lead to increased mortality in male offspring. This period in the life cycle of many macropods is the most sensitive to fluctuations in environmental stimuli (Frith & Sharman, 1964; Munn & Dawson, 2001). Inns (1980) found the highest mortality rates were for juvenile and old adult males, with pronounced annual cycles in body weight and fat stores in adult males. Similar results were found in the present data set, as males showed significantly lower KFI than females with more extreme annual fluctuations (see Chapter 6).

The possibility of increased mortality in male progeny, irrespective of maternal investment, suggests that older, better-conditioned, larger mothers are investing in daughters to guarantee that these daughters reach maturity. There is evidence in other mammalian species that progeny of older females have higher survival rates (Clutton-Brock, 1984; Cameron et al., 2000) and that mothers in unpredictable environments will have daughters, as they mature faster (Kempe et al., 2009). These mothers are better able to withstand resource variation, recovering more quickly by eating greater amounts of feed and accruing KFI faster, thus they are less likely to abort PY under nutritional stress. As there was no discrepancy in resource allocation between tammar daughters and sons, assuring the maturity of daughters later in life might have the same impact on gamete success as having larger males in other species (Leimar, 1996). Fecundity in the tammar increases with age, as the fecundity of younger mothers tends to fluctuate annually, while adult females remain consistently above 80% (Inns, 1980), there is also evidence that females become more selective in mate choice as they grow older (Kokko & Mappes, 2005). Kangaroo Island suffered a prolonged drought during the years 1977 & 1978, yet, despite the reduction in resource availability, mature females remained at fecundity rates of 93% and 84%, while younger females had a rate of 40% and 0%, respectively. Thus, smaller, stressed mothers were having sons or no offspring at all, while older mothers were having an increasing proportion of daughters as they became better able to cope with fluctuating environmental stimulus. Since daughters have a longer reproductive lifecycle than sons, and will reliably remain at fecundity levels of 80-100%, they provide a more rapid, secure and prolonged investment (Kruger et al., 2005). Increased reproductive effort as females mature has been noted in other marsupial species, exhibited as terminal reproductive effort (Isaac & Johnson, 2005), and is especially important for populations at or near carrying capacity (Clutton-Brock & Iason, 1986). Other populations at carrying capacity, where intergenerational competition occurs in one sex more than the other, *i.e.* the shorter-lived sex (tammar males) (Werren & Taylor, 1984), show population dynamics that favour the production of the longer-lived sex (tammar females according to Inns (1980)).

Recent literature predicts that adjustments in sex ratio allocation may not be as straightforward as Trivers & Willard originally hypothesized, as there is evidence that high quality mothers, in systems where both the LRC and TWM are working simultaneously within the population, will invest in either sexed offspring to increase fitness returns (Wild & West, 2007). Roberts & Schwanz (2011) and Isaac *et al.* (2005) reached similar conclusions for marsupial species. The data presented here did not correspond with a single model, but

with several (Linklater, 2007). It is therefore quite reasonable to theorise that previously conceived independent models were working simultaneously on the same population, and that mothers employed a lifelong reproductive strategy to maximize total reproductive output, rather than each breeding episode individually. This may account for intraspecific discrepancies between studies, as previous examinations have focused on individual breeding episodes and extrapolated assumptions *a posteriori* for entire populations. Yet, there are still questions to be answered regarding the role of the TWM and its involvement in sex ratio bias in the tammar. There is some evidence apparent in the current data, but only a complete examination of the underlying assumptions of the TWM will eliminate the model as an effect in this population. Thus, an understanding of the ability of tammar offspring to compensate for poor starts in life, and exactly what role maternal resource transfer plays in any compensation, is necessary. In addition, the adaptive nature of the sex allocation process is still in doubt. Further investigation in controlled environments will shed light on whether females are actively selecting the sex of their PY based on population pressures and environmental cues, or whether their choices are ecologically predisposed.

CHAPTER 3 ***Experimental Manipulation of Maternal Nutritional Intake and Body Condition, To Affect Change in Offspring Sex Ratio***

3.1 Introduction

The ability for species to adapt to their environment is an important part of development, as it maximizes fitness by allowing individuals and populations to develop phenotypes that best match the surrounding habitat and environment (Scheiner, 1993; Nylin & Gotthard, 1998; Pigliucci, 2001; West-Eberhard, 2003; Kasumovic et al., 2009). The adaptive nature of a species in its local environment is usually broken into specific ‘traits’ (Gould & Lewontin, 1979) that are studied independently, *e.g.* foraging, breeding, hibernating, etc. To understand the physiological mechanisms and environmental controls, most scientists will manipulate certain known aspects of the species’ habitat and observe the effects. For example, the manipulation of photoperiod to mimic the end of the breeding season in some species produces a direct effect on juvenile development, causing maturity sooner and at a smaller than normal body size (Abrams et al., 1996; Johansson et al., 2001; Davidowitz et al., 2003; Shama & Robinson, 2006). To understand the true adaptive nature of sex ratio skewing in a species, the species must be tested under varying environmental conditions.

Adaptive behaviour relies on information, and a correct interpretation of the relevant environmental cues by an individual (Dall et al., 2005), to allow predictions of future conditions. Predicting environmental circumstances includes processing cues obtained from variable weather patterns, resource availability and interspecific and intraspecific animal behaviour and competition, which means most individuals must continuously gather information, sometimes at the expense of other biological processes (Dall & Johnstone, 2002).

The adaptive nature of sex ratio bias in mammals can be quite hard to confirm, as there is no consensus in the literature on the subject of mechanism or association of the phenomenon, and the distribution of findings does not conform to the predictions of any single adaptive model (Clutton-Brock & Iason, 1986). In order to show a true adaptive nature for sex allocation, parents must change their pattern to increase their fitness when external stimuli vary. The literature suggests that several sex allocation models can act on the same population simultaneously, making it difficult to assess actual adaptive patterns of sex ratio bias (Isaac, 2005; Linklater, 2007; Robert & Schwanz, 2011).

The data obtained in the wild populations of the tammar wallaby (*Macropus eugenii*) on Kangaroo Island in 2005, 2006 & 2007, and presented in Chapter 2, did not support the Trivers-Willard Model (TWM) (Trivers & Willard, 1973), as younger, smaller mothers produced sons and older, larger mothers produced daughters. This result deviates from the common theory of LRC and TWM, which predict that parents, usually mothers, should adjust the sex of their progeny in response to environmental conditions to maximize fitness, usually sons in polygynous species. As the results presented in Chapter 2 deviated from the premise of these common theories, as with other species with sexual allocation patterns that deviate from the models (Charnov et al., 1981), it is also possible that the pattern observed in *M. eugenii* varies from the adaptive nature attributed to sex allocation patterns in these predicative paradigms.

Sex ratio skew the sampled tammar pouch young did not correlate with annual rainfall patterns discussed in Chapter 2, rather centring on maternal size and age, which could suggest that a non-adaptive sex ratio biasing mechanism, such as a life-history strategy that was genetically based or learned through social cues. Differences in learned and genetically inherited traits are seen throughout the animal kingdom (Hogan & Bolhuis, 1994), and there is evidence that learned behaviours may enhance reproductive fitness of mothers (Rutkowska & Adkins-Regan, 2009), including influencing progeny sex ratio. Recent studies on human sex ratios suggest that the neural pathways may gradually evolve genetically to constrain adaptive behaviour, such as sex allocation processes (Lewis et al., 2010).

In order to answer the question of adaptability for the population presented in Chapter 2, it is necessary to follow the path set by previous literature, and manipulate aspects of the environment in which *M. eugenii* are kept and observe the impact on specific traits, in this case sex ratio skew. The relevant parameter, due to the ambiguous nature of support in the observed data for a relationship between rainfall and sex ratio skew, is nutritional stress. The manipulation of all or specific individual stressors in experimental parameters helps to unravel their separate influence on population dynamics (Sibly, 1999), which will allow the understanding of how such stressors impact on an individual in a population (Maltby, 1999). As food quality is linked to reproductive success in other marsupial species (Higginbottom, 2000), and there is evidence that the quality and composition of the maternal diet may influence breeding and sex ratio (Rosenfeld et al., 2003; Cameron, 2004; Rosenfeld &

Roberts, 2004; Green et al., 2008), the manipulation of this variable will allow greater understanding of other factors causing sex ratio skew, namely maternal size and age. In addition, by isolating or eliminating nutritional stress, as variations in stress response between populations of the same species are often attributed to local adaptations of distinct populations based on historic exposure to specific stress events (Lam, 1999), insight into the different sex allocation strategies observed in *Macropus eugenii* on Kawau Island, New Zealand (Sunnucks & Taylor, 1997) and on Kangaroo Island (Chapter 2) may be obtained. As both studies were field studies, and manipulation of nutrition in the field is not feasible, it is necessary to use controlled breeding experiments, as it will allow for more in depth understanding of mechanisms through manipulation of nutritional stress.

Captive and experimental breeding is widely used in species with declining population numbers, and the majority of the literature suggests that fertility and fitness in captive populations equates to that of free ranging populations (Trewick, 1997; Shibata & Kawamichi, 2009; Ganan et al., 2010; Malo et al., 2010), including marsupial species (Murphy & Smith, 1970; Merchant, 1976; Inns, 1982; Fleming et al., 1983; Poole et al., 1985; Poole & Merchant, 1987; Rodger, 1990; Taggart et al., 1999), as long as inbreeding and human influence are avoided (Zschokke, 2002; Martinez-Pastor et al., 2008; Nees et al., 2009; Moreno et al., 2011). Captive breeding experiments have been used to investigate a host of reproductive parameters, including suckling behaviour (Pluhacek et al., 2010), calving success (White et al., 1997), artificial manipulation (Rodger, 1990; Rodger et al., 1993; Taggart et al., 1998), lactation (Rose & Flowers, 2005) and sex ratio bias (Merton et al., 1984; Trewick, 1997; Zschokke, 2002; Pluhacek et al., 2010; Moreno et al., 2011). The aim of the present experiment was to clarify the relationship between maternal age and weight and sex ratio bias observed in the population discussed in Chapter 2. Evidence suggests that age-related variations in breeding tactics are only exhibited when food supplies are scarce (Bunce et al., 2005; Lee, 2011), as otherwise young and old parents are equally capable of breeding success. Thus, in an attempt to unravel the influence of the predictive models, nutritional stress was removed, to allow maternal age and weight to operate independently. If there was no change in the sex ratio bias, then the reproductive life history cycle of tammar mothers may not be a reaction to change in resources, but genetically based. It is predicted that under reduced nutritional stress, larger mothers will have female offspring and smaller mothers will have male offspring, as observed in Chapter 2.

3.2 Methods & Materials

Fifty tammar wallabies (*Macropus eugenii decres*) were captured on Kangaroo Island under Department of Environment and Heritage scientific permits. They were captured using a stunning technique (Robertson & Gepp, 1982) under Department of Environment and Heritage (SA) Scientific Permit Q25076 and University of Adelaide Animal Ethics Approval S-079-2005B. Once captured the wallabies were transported to the Roseworthy campus of the University of Adelaide, where they were held in 2 large pens, males and females segregated from each other to prevent mating. Before being released into the large holding pens, the pouches of all females were checked for the presence of Pouch Young (PY), which were removed if present. The females were checked for PY again several months later, as blastocysts in diapause would have been activated following the removal of the sucking stimulus of a PY from the teat (Renfree, 1993; Renfree & Shaw, 2000). These PY were also removed if found, to ensure that females returned to normal oestrus patterns (Merchant, 1979). Animals in large holding pens were fed *ad libitum* on specialized commercially produced kangaroo and wallaby feed formulation, and water was freely available. The feed contained 18% protein and .39% NA (nitrogen) yielding 10.17mJ/kg. *M. eugenii* requirements are c. 1.73mJ day⁻¹ for an average female weighting 5.6kg, and 2.19mJ day⁻¹ for an average male weighting 7kg, and 16.9% protein (Hume, 1999).

Sixteen adult female tammars were randomly selected from the captive population, placed in individual 5x3m² mesh pens, and fed *ad libitum* of the same feed, water was freely available in each pen. Another sixteen females were randomly selected, placed in the same pen structure and fed a maintenance diet, based on their weight, requirements as stipulated by Hume (1999) and the parameters of the available feed, while water remained freely available. The animals were able to see and smell each other through the mesh, to minimize stress caused by social isolation during the experiment (Hynes et al., 2005) (Figure 3.2.1). Males were then chosen at random to mate with the females, and kept in the same pen for 5 days. After mating, the males were removed, and the females were kept isolated in their pens for roughly 40 days, while their pouches were checked for PY starting at 30 days (Figure 3.2.2). Sex of the PY was noted once parturition had been detected.



Figure 3.2.1
Captive female *M. eugenii*, being released into the experimental 5x3m² pens after the pouch was checked for parturition and the presents of pouch young.



Figure 3.2.2
The pouch of a captive *M. eugenii* mother after parturition, pouches were checked for PY and then the PY were sexed without removal from the teat. The scrotum and pouch are visibly distinct, although further sexual differentiation occurs later in pouch life, for sexing of young after parturition (Tyndale-Biscoe & Renfree, 1987; Butler et al., 1999; Isaac et al., 2005; Linklater, 2007).

ANOVA and the Tukey-Kramer Post Hoc Test were used to establish any significant differences between the captive and wild populations. Transformation of the data was undertaken if Shapiro-Wilk normality tests exposed skewed distributions, and a Bonferroni Correction was used to establish independence of multiple ANOVAs performed on the same data set. Captive females were assigned to the same weight groups presented in Chapter 2, Group 1: <3.5kg, Group 2: 3.5-5.5kg, Group 3: >5.5kg, and sex ratio was compared between the two groups. Logistic Regression for Binomial Distributions was done on the captive population, to find any relationship between maternal weight or maternal weight group, and Chi² statistical analysis was used to assess any deviation from the expected 1:1 ratio. Then, logistic regression was used to compare the wild data to the captive data.

3.3 Results

None of the females on the maintenance diet produced offspring, with an average weight 1kg less than those females that did have young (average of 4934g) and an average MBC of 1.66, which is significantly different from those captive females on the *ad libitum* diet that did have PY (1.987) (ANOVA: test statistic=3.91, p value=0.005, df 4, $q=4.546$, $q_{0.01,120,4}=4.50$), but not significantly different from the wild samples. Thus, analysis of sex ratio can only be performed on the *ad libitum* females, and comparisons between mothers and pouch young in the captive population and the wild population of Chapter 2 was performed on the basis that the reproductive biology of this species has shown a consistency between captive and wild breeding, as exhibited in other species (Fleming et al., 1983; Zschokke, 2002; Ganan et al., 2010; Renfree & Lewis, 1996). Shapiro-Wilk tests for normality showed that maternal weight and MBC produced normal distributions in the captive population, with standard error of variance of 78300 and 0.011 respectively. There was a significant difference between the MBC (test statistic=10.20, p value=0.002, df of 1, $n=92$) and the maternal weight (test statistic=10.38, p value=0.017, df of 1, $n=92$) of females when using status (captive or wild) as the treatment factor. Females in the captive population were significantly heavier than all wild sample years (2005: $q=3.89$; 2006: $q=4.278$; 2007: $q=3.55$; $q_{0.05,60,4}=3.74$), while the MBC of 2005 females was significantly different to that in the captive population (2005: $q=4.698$; 2006: $q=3.17$; 2007: $q=3.09$) (Figure 3.3.1). A Bonferroni Correction of this data ($0.05/n=2$) indicates that an significance level of 0.025 is needed for independence, and both MBC and maternal weight were significantly correlated with captive/wild status.

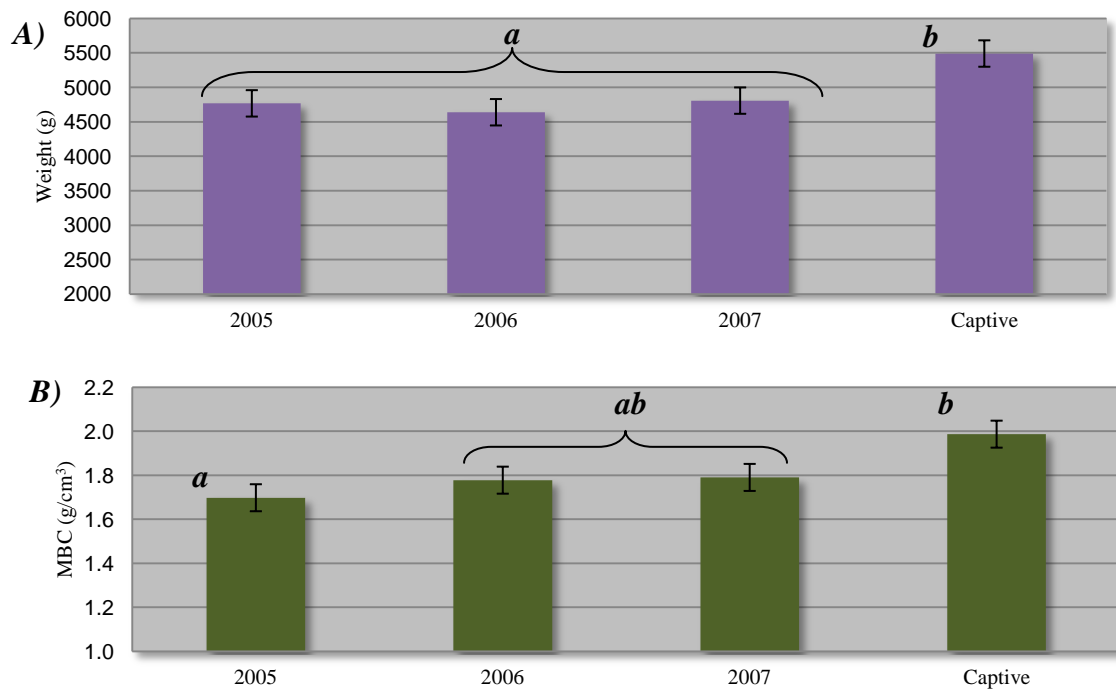


Figure 3.3.1 Comparison of A) Maternal Full Weight and B) Maternal Body Condition (weight(g)/foot length (cm)³) of female Kangaroo Island *M. eugenii* caught in the wild and held in captivity, data points with differing postscripts are significant. Error bars mark standard deviation, captive_n=16, 2005_n=30, 2006_n=21, 2007_n=25.

The sex ratio of the captive pouch young was significantly different from the expected (and observed in the wild) 1:1 ratio, with a ratio of 4.3:1 ($\chi^2=6.250$; $P<0.05$, $n=16$) (Figure 3.3.2).

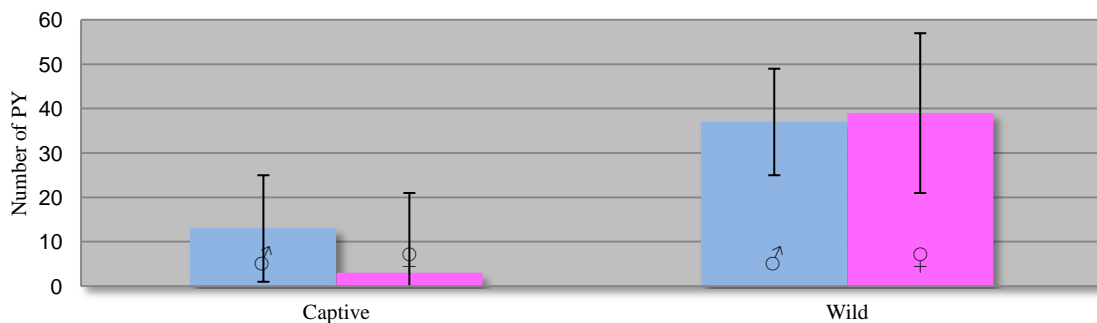


Figure 3.3.2 Comparison of sex ratio in captive and wild Kangaroo Island *M. eugenii*, the 4.3:1 sex ratio in captivity is significantly different from the 1:1 ratio seen in the wild, captive_n=16, wild_n=76.

The sex of pouch young in the captive population was not significantly associated with maternal weight (test statistic=0.57, p value=0.462, df of 1), MBC (test statistic=0.00, p value=0.981, df of 1) or weight group (test statistic=0.73, p value=0.409, df of 1), as found in the wild population, see Chapter 2 results. As expected from these results, logistic regression did find a significant relationship between PY sex and status (captive or wild) (test statistic=6.09, p value=0.014, df of 1), while weight group was the only maternal condition parameter that was significant across both populations (test statistic=4.52, p value=0.033, df of 1), though not as predictive to PY sex as status. In addition, when the relationship between status and maternal weight group was assessed, the relationship was a

highly significant predictor of PY sex (test statistic=6.59, p value=0.001, df of 2) (Figure 3.3.3).

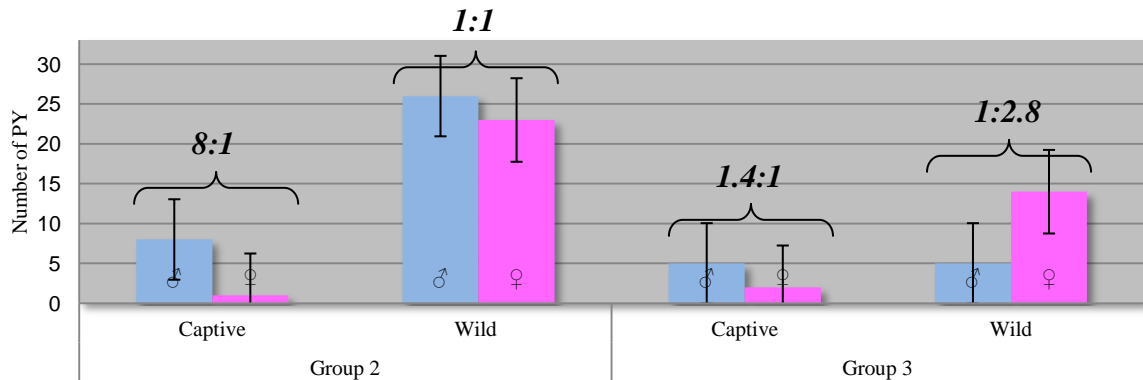


Figure 3.3.3 Comparison of sex ratio in captive and wild Kangaroo Island *M. eugenii*, for Weight Groups 2 & 3. Captive Weight Group 2 has a 8:1 sex ratio, while the Captive Weight Group 3 sex ratio is closer to the 1:1 than the wild group, and significantly more male biased, Group 2: captive_n=9, wild_n=49; Group 3: captive_n=7, wild_n=19.

The weight groups classified in Chapter 2 (Group 1:<3.5kg, Group 2: 3.5-5.5kg, Group 3: >5.5kg) had significantly different overall PY sex ratios when comparing wild and captive mothers. In the captive population there were no animals in the Group 1 weight range, so only Groups 2 & 3 were compared. The sex ratio of Group 2 in the captive sample was significantly different from the expected 1:1 ratio as seen in the wild ($\chi^2=5.44$; $P<0.05$, $n=8$) and the captive Group 3 was not significantly different from the expected 1:1 ($\chi^2=1.286$; $P<0.05$, $n=5$), unlike in the wild. The change in ratio for Group 3, depending on status, was highly significant, as these females had gone from a sex ratio bias dominated by daughters, to one dominated by sons ($\chi^2=78.231$; $P<0.001$, $n=7$).

There were also significant differences between the captive population sex ratio and that of each year, in relation to the weight groups. The Group 2 captive sex ratio bias was significantly different from that in 2005 (log regression: test statistic=4.21, p value=0.040, df of 1, $n=25$) and 2007 (test statistic=4.36, p value=0.037, df of 1, $n=27$), but not from that observed in 2006 (test statistic=2.51, p value=0.113, df of 1, $n=24$). Group 3 also showed significant differences, with the captive sex ratio different to 2005 (log regression: test statistic=4.02, p value=0.045, df of 1, $n=16$) and 2007 (test statistic=4.16, p value=0.041, df of 1, $n=13$), but not to 2006 PY (test statistic=0.50, p value=0.480, df of 1, $n=11$) (Figure 3.3.4).

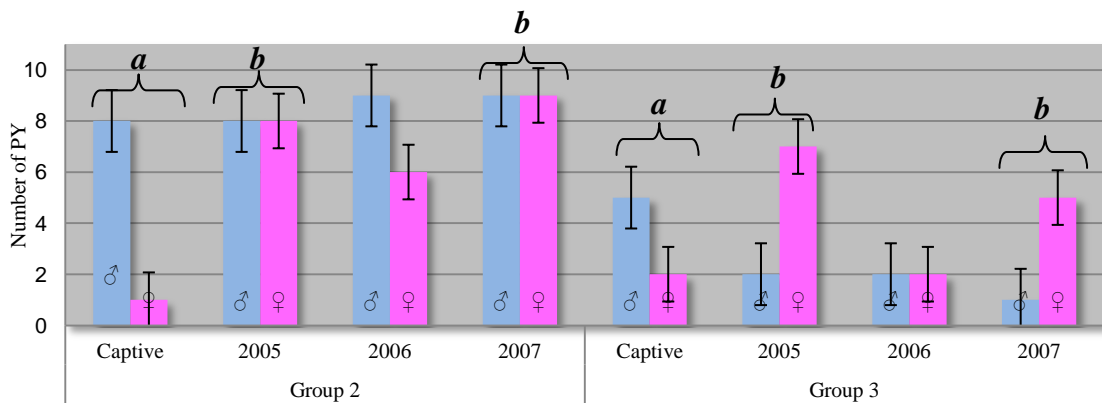


Figure 3.3.4 Annual comparison of sex ratio in captive and wild Kangaroo Island *M. eugenii*, for Weight Groups 2 & 3. Data points with differing postscripts are significantly different in each group, Group 2: captive, $n=9$, 2005, $n=16$, 2006, $n=15$, 2007, $n=18$; Group 3: captive, $n=7$, 2005, $n=9$, 2006, $n=4$, 2007, $n=6$.

3.4 Discussion

None of the mothers fed the maintenance diet produced offspring for comparison, despite having MBC and weight equivalent to that of the wild populations. This severely limited the ability of the experiment to assess the adaptive nature of sex ratio skew based on manipulation of maternal resource intake and body condition. Thus, only comparisons between the wild and *ad libitum* captive females were possible. Comparisons between the captive wallabies in the current data and the wild samples discussed in Chapter 2 indicated that the experimental population of mothers and PY were in good condition. The tammar mothers in this captive experiment were significantly heavier, with a higher MBC, than tammar mothers in all Kangaroo Island sampled populations. As the captive females were not dissected after the experiment, KFI and estimated maternal age were not available. However, this had negligible effects on the comparison of the two populations (captive and wild), as only maternal weight and maternal age were significant predictors of PY sex in the wild population. Given the positive correlation between maternal weight and age observed in the wild samples, the assumption that heavier females were most likely older females may be applied to the captive population as well.

The heavier body weight of captive females, however, did not translate to more female young, as would have been predicted if the pattern observed in the wild samples had presented in the captive mothers. In fact, the pouch young sex ratio of the captive sample was not associated with maternal body weight, MBC or weight group (as seen in Chapter 2), and was significantly different from the 1:1 overall ratio observed in the wild, with an overall captive sex ratio that was significantly male biased at 4.3:1. The most significant maternal predictor of PY sex was status, either captive or wild, with a highly significant correlation between maternal weight group and captive status across the sampled population as a whole (captive and wild). Overall, intermediate mothers, despite showing a 1:1 ratio in the wild, had a heavily male biased sex ratio in captivity, while larger (assumed to be older) mothers, despite having a female biased ratio in the wild, had a captive sex ratio much closer to parity. When the captive weight groups were compared with the same weight classes from each year of wild sampling, captive intermediate mothers showed a sex ratio bias significantly opposed to that seen in the 2005 and 2007 samples, but not with the 2006 mothers, while captive larger (assumed to be older) mothers showed the same juxtaposition in sex ratio bias.

The extreme result observed in the captive population suggests that sex ratio bias in Kangaroo Island *M. eugenii* is an adaptive process, which is influenced by maternal size and captive status alone. As the maintenance fed mothers did not produce PY for comparison, any conclusions on what external influence of captivity caused such an extreme male skew in offspring is purely supposition. However, it is clear that removal of nutritional stress appears to have removed the age related distinction in reproduction (Bunce et al., 2005; Lee, 2011). Initial explanations also appear to support the TWM as the overarching predictive model for sex ratio bias in this species, as these mothers appeared to be in better condition than similar females in the wild. In addition, the captive females equated to the wild 2006 sample, suggesting that a larger sample size in that year might have produced a significant effect to provide greater support for the TWM in the data presented in Chapter 2. However, the picture might be more complicated, as there was no association between maternal weight, MBC and PY sex in captivity, and the association with maternal weight was lost when the entire sample population was considered as a whole. In fact, the most significant predictor of PY sex was captive status, not maternal condition, suggesting an arbitrary *a posteriori* application of the TWM to this data may overlook any other bias that was associated with captivity. For instance, all experimental mothers were fed the same high energy, high protein diet for the duration of the experiment, possibly influencing the sex ratio of their offspring, as maternal diet, rather than maternal body condition, has been linked to sex ratio variation (Rosenfeld et al., 2003; Green et al., 2008), with excess glucose (Cameron, 2004), fatty acids (Green et al., 2008) and high levels of fat and carbohydrate (Rosenfeld et al., 2003) favouring increased production of sons (Robertson et al., 2006). This has also been observed in ungulate species, where increased dietary energy during specific seasons increased the proportion of sons to as high as 75% (Enright et al., 2001).

The stress of captivity may directly influence sex ratio in females for a number of reasons other than diet and nutrition (Moriya & Hiroshige, 1978; Rosenfeld & Roberts, 2004; Marealle et al., 2010). Sex ratio skewing has been linked to episodes of high social stress in humans (Zorn et al., 2002; Helle et al., 2009; Bruckner et al., 2010; Ohlsson & Shah, 2010), possibly by changing hormone levels in mothers (Kinsley & Svare, 1988; Kemper, 1990; James, 1996; Yamaguchi et al., 2005; Grant, 2007). Linklater (2007) also found the effects of captivity induced a strongly male-biased sex ratio (67% male) for conceptions occurring after arrival in captivity, the data for the present study exhibited an 81.2% male bias. The size of the male bias in Linklater's study increased with increased time in captivity before conception.

Despite the tammar being highly studied in captivity, and for the most part garnering results that have been correlated with wild populations (see Tyndale-Biscoe & Renfree, 1987 and Renfree & Shaw, 1996 for a review), captivity has been known to produce varying effects on the reproduction of other species. For example, females born in captivity have been known to reach maturity and breed at younger ages than those in the wild (Baskaran et al., 2009), though not in *M. eugenii*, as captivity tends to delay maturity in tammar females (Williams et al., 1998). The increased handling in captivity is also associated with maternal stress in other macropodoid species, causing loss of pouch young and suppression of regular oestrus cycles (Poole et al., 1985). Increased handling stress was observed in the current experiment, typified by increased saliva production from the mouth and nose and thermoregulatory licking of the forearms (characteristic of macropods) during capture episodes. The nature of the tammar wallaby is quite skittish, and the impact of chasing was unlikely to produce levels of stress unseen in the natural habitat, but the inability to escape from the chaser did increase stress. Similar conditions have produced reduced sex ratios in captivity before (Lane & Hyde, 1973) and, in rodents, the stress caused by overcrowding (Krackow, 1997) has been shown to reduce the proportion of sons in the next generation, while artificial manipulation of female dominance hierarchies is also correlated with reduced sex ratio bias (Pratt & Lisk, 1989, 1990). However, these stressors associated with captivity are suggested to produce more daughters than sons, while the tammar mothers in the current experiment produced a high proportion of sons. Psychological stress in captivity may also cause mothers to produce more sons (Schuster & Schuster, 1972).

If the main driving force behind sex ratio bias in the sampled population of wild *M. eugenii* mothers was competition for resources and mates within a family group, the parameters of this study may have contributed to the overproduction of sons by changing the group dynamics. For individual female wallabies, the experimental parameters designed to control mating produced an unstable environment and erratic social groupings, both of which have been shown to affect reproductive effort and sex ratio (Wise et al., 1985; Kemme et al., 2009; Shibata & Kawamichi, 2009). Prior to the experiment, captive females were kept in large single sex pens for several months, with a transparent wire fence separating them from the males. They were then transferred into the individual pens, to ensure feed levels, mating success and for ease of capture. The female experimental block was separate from the large holding pens, and although individuals could see and smell each other in an attempt to mitigate any stress, this solitude could have increased stress levels. This stress was

evidenced by the number and manner of escapes, as females were more likely to escape into another female's pen than out of the enclosure altogether, despite the ease of escape being equal for both outcomes, suggesting that social interaction was highly important (Sibbald & Hooper, 2004; Sibbald et al., 2005; Jorgensen et al., 2009).

These groupings may also have inadvertently simulated a reduced male presence in the captive population, indicating increased mate competition for experimental females. As adaptive behaviours are indicative of perceived social cues, the lack of physical contact with male tammars, except during periods of designated breeding, may have cued (Danchin et al., 2004; Moore et al., 2005) these mothers to produce more sons. Theory does predict that when the operational sex ratio skews in favour of one sex or the other, parents should increase their fitness by producing progeny of the uncommon sex (Werren & Charnov, 1978; Burley, 1982; Charnov, 1982), *i.e.* when disease has caused a population crash in males, females will produce an excess of sons until sexual parity is returned (Clutton-Brock & Iason, 1986). In addition, a study on female mouse lemurs (*Microcebus murinus*) found that similar domiciliary arrangements had a profound effect on sex ratio, as females grouped together prior to conception produced a highly significant proportion of male offspring (Perret, 1990), which has been confirmed in studies on polygynous human relationships (James, 1996).

The literature also suggests that mothers are capable of anticipating the future environmental conditions of their young as a vital part of adaptive behaviour, influencing sex ratio patterns and their evolution (West & Sheldon, 2002). The anticipation of reduced mate availability for themselves and their young could have caused overproduction of sons in this experiment (Wedell et al., 2002; Kokko & Mappes, 2005; Smith & Reichard, 2005), which corresponds with the population dynamics observed in Chapter 2, and could be the explanation for the discrepancy in sex ratio bias between the Chapter 2 data and that from Kawau Island (Sunnucks & Taylor, 1997). As the population on Kawau Island is heavily managed due to the introduced species status of *M. eugenii*, and that access is easier during the summer months when more males are grazing on open pastures (Andrewartha & Barker, 1969; Inns, 1980), a male biased operational sex ratio (which was not tested by Sunnucks & Taylor) may have caused the observed bias. Interestingly, similar population cues have been observed in female fish who responded to female-biased adult sex ratios by increasing reproductive effort and producing more sons, in anticipation of future difficulties in acquiring mates (Heubel et al., 2008).

The experimental females, normally part of a polygynous mating system where they mate with several males in their social group, were unable to choose their mating partners, only exposed to a single male partner during oestrus. It is also highly likely that designated breeding partners were novel to these females, as capture in the wild occurred over several large properties during several separate trips and males and females were kept apart during the acclimatisation process. Mate conditioning, especially in social animals, has been linked to the production of sons, i.e. if the female has observed the male in the family group and is familiar with his traits prior to mating, she is likely to increase reproductive output, while females mated unexpectedly, with novel males are shown to reduce output (Rutkowska & Adkins-Regan, 2009). In addition, females obtain chemical cues from attentive males, indicating nutritional status, disease levels and reproductive fitness (Giaquinto et al., 2010), which have a direct influence on female hormones during mating (Pryke et al., 2011), cues from these males may have caused the production of sons due to high nutrition prior to mating. On the other hand, given the limited choices available to the experimental females, if males were viewed as poor quality mating partners, the constraint on her ability to choose another partner may have increased stress, leading to an adaptive male biased sex ratio (Pryke et al., 2011). While, females in the wild are free to choose breeding partners they deem of good quality, producing less stress and an equal sex ratio.

Thus, the adaptive nature of sex ratio skewing of *M. eugenii* cannot be isolated from population dynamics; one trait must impact on another (Fawcett et al., 2011), as the individual operates as a whole in its environment, rather than a collection of mutually exclusive traits (Gould & Lewontin, 1979). In a sense, the pattern of sex ratio bias in this population of *M. eugenii* did fit with theoretical models, because mothers are adaptively changing sex ratio in response to environmental cues, but perhaps the cues suggested by conventional adaptive sex allocation hypothesis are incorrect, explaining why there is so much deviation in the literature on cue accuracy (Moore et al., 2005). A simple explanation for the overwhelming male bias observed in the experimental sample is the TWM, and the data from the wild population supports the conclusion that this model might be acting on distinct weight classes, yet without the ability to compare younger, smaller mothers in this experimental situation, it is difficult to make sweeping *a posteriori* generalisations. In fact, the lack of relationship between maternal condition and offspring sex ratio in the experimental sample just as strongly suggests that there are other responsible factors, and in support of the discussion of Chapter 2 results, several forces are interacting at the individual

and population level, to produce the observed sex ratio pattern. Stress, perhaps mediated through elevated plasma cortisone levels, may well be one of those forces. Further testing of the TWM assumptions, such as the ability of juvenile *M. eugenii* to rebound from poor starts with compensatory growth, might clarify the role of the TWM in sex ratio patterns of this species. Attempts to pinpoint physiological control mechanisms of this adaptive behaviour might also shed further light on the environmental sources of control, as both parents may have conflicting agendas in contributing to sex ratio bias, either through semen quality (Ganan et al., 2010) or maternal hormone levels at conception (James, 1996; Grant, 2007), as there was no evidence of foetal abortion or resorption as the control mechanism in either the wild or captive populations.

CHAPTER 4 *Testing Assumption 2 of the Trivers-Willard Model, by Assessing the Applicability of Compensatory Growth Theory to the Tammar Wallaby*

4.1. Introduction

Mammalian mothers bear the full cost of gestation and lactation, which means they bear a disproportionate share of the overall burden of reproduction (Sikes, 1996). Due to the extreme unbalanced nature of reproduction in many mammalian species, several theories regarding the ability of a mother to maximize reproductive fitness have been proposed. One such theory is the Trivers-Willard Model (TWM) (Trivers & Willard, 1973), which assumes that in polygynous mammals, where the male competes for access to receptive females, the reproductive ability of sons is more dependent on maternal resource transfer. This suggests that mothers in good condition should produce sons, who will benefit from their mother's increased ability to transfer resources to become bigger, better males who secure more copulations, while females in poor condition or with access to limited resources should produce daughters, as size will have little impact on a daughter's fitness as an adult. Sex ratio hypotheses are based on specific assumptions: the TWM criteria are that (1) maternal condition is correlated with progeny condition, (2) offspring condition will be maintained into adulthood, and (3) differences in maternal resource allocation will affect sons more than daughters (Trivers & Willard, 1973).

In order to test the predictive capacity of models like the TWM, it is important to test the underlying assumptions. Assumptions 1 and 3 of the TWM were tested in Chapters 2 and 3 of this thesis; assumption 2, that offspring condition is an indicator of adult condition, meaning that young with a poor start in life will become inferior adults, thus influencing their ability to reproduce effectively, remains untested in the tammar wallaby (*Macropus eugenii*). Assumption 2 has not been widely tested in the literature in other species either, but, as phenotypic quality, especially body mass, has been associated with increased reproductive output and survival rates in adults (Roff, 1992; Stearns, 1992), it is essential to explore the foundations of between-individual variation in adult body mass to expose the reliability of assumptions linking juvenile quality with adult quality. The limited data available is ambiguous in nature (Mangel & Munch, 2005), some showing support for the assumption (Kennedy, 1969; Falconer, 1973; Trivers & Willard, 1973; McClure, 1981; Pond et al., 1990; Green & Rothstein, 1991) and some contradicting it (Meyer et al., 1962; Andreassen & Ims, 1990). The failure to satisfy empirically the underlying assumptions of

sex ratio theories like the TWM raises serious questions about the use of the hypothesis as a tool for understanding maternal investment between the sexes (Sikes, 1996). In the case of *M. eugenii*, where evidence to date shows support for several different theories, failure of evidence to meet the specific parameters of the TWM would eliminate it from the suite of possible explanations for the observed patterns.

When a species fails to meet the criteria of assumption 2 of the TWM, the species usually shows some form of compensatory growth. Compensatory growth occurs when juveniles, who were previously nutritionally restricted partially or completely, exhibit faster growth rates during recovery than juveniles exposed to continuous food availability (Wilson & Osbourn, 1960; Jobling et al., 1994), resulting in the growth-depressed animals reaching the same size-at-age as conspecifics subjected to more favourable conditions (Ali et al., 2003). The phenomenon of compensatory growth was first discussed by Waters in 1908 and then by Osborne & Mendel in 1915 (Wilson & Osbourn, 1960) and has since been noted in a wide variety of species, including goats (Wuliji et al., 2003; Joemat et al., 2004), domesticated ruminants (Hoch et al., 2003; Tomkins et al., 2006; Gonzaga Neto et al., 2011), many species of fish (Broekhuizen et al., 1994; Jobling & Koskela, 1996; Hayward et al., 1997; Ali et al., 2003), pigs (Edmonds & Baker, 2003; Whang et al., 2003), invertebrates (Bradley et al., 1991), mice (Yarmolinsky et al., 2009; Jones et al., 2011), lizards (Radder et al., 2007), turtles (Roark et al., 2009; Wang et al., 2011) and other vertebrates (Wilson & Osbourn, 1960; Sibly & Calow, 1986). Interestingly, compensatory growth has also been observed in some ungulate species, the prime example discussed by the authors of the TWM theory (Solberg et al., 2004; Dale et al., 2008; Rughetti & Festa-Bianchet, 2010), but not in others (Solberg et al., 2008), possibly contributing to the uncertainty associated with experimental support for the TWM.

In an optimal environment, with no growth limitations, individuals are expected to follow an 'ideal' growth trajectory (Jobling et al., 1994; Ali et al., 2003), which could be considered the 'normal' growth pattern (Jobling, 2010). Implicitly, the size achieved by individuals displaying normal growth is viewed as 'optimum' in all cases, so the ratio between the amount of compensation and the size of the control animals, when the increased growth rate has abated, provides a measure of the compensatory response (Ali et al., 2003). This increase in growth rate suggests that organisms are programmed to achieve a target trajectory (Tanner, 1963; Monteiro & Falconer, 1966; Hubbell, 1971). The convergence of growth trajectories amongst organisms exposed to different nutritional circumstances reflects

the ‘target-seeking’ manner of compensatory growth, and provides evidence that growth rates are regulated by individuals. These animals ‘evaluate’ their growth trajectories and adjust them to remove variations from the ‘ideal’ trajectory (Hubbell, 1971; Broekhuizen et al., 1994; Ali et al., 2003). These trajectories are written in an individual’s genetic material

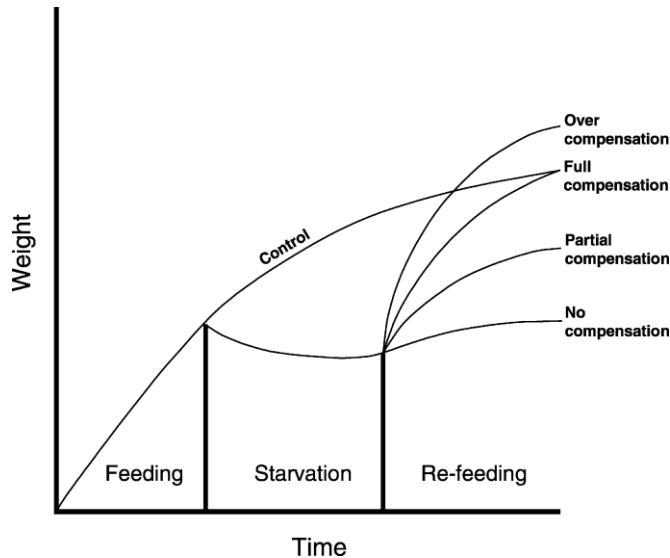


Figure 4.1.1. Idealised patterns of growth compensation based on Jobling (1994), figure from Ali *et al.* (2003).

and powered by environmental resources (Tanner, 1963).

Compensatory growth is most likely an adaptive coping mechanism for species living in environments of fluctuating resource availability (Dale et al., 2008), as many animals inhabit localities where periods of high feed quantity/quality are interspersed with periods of low quantity and quality, including periods of famine

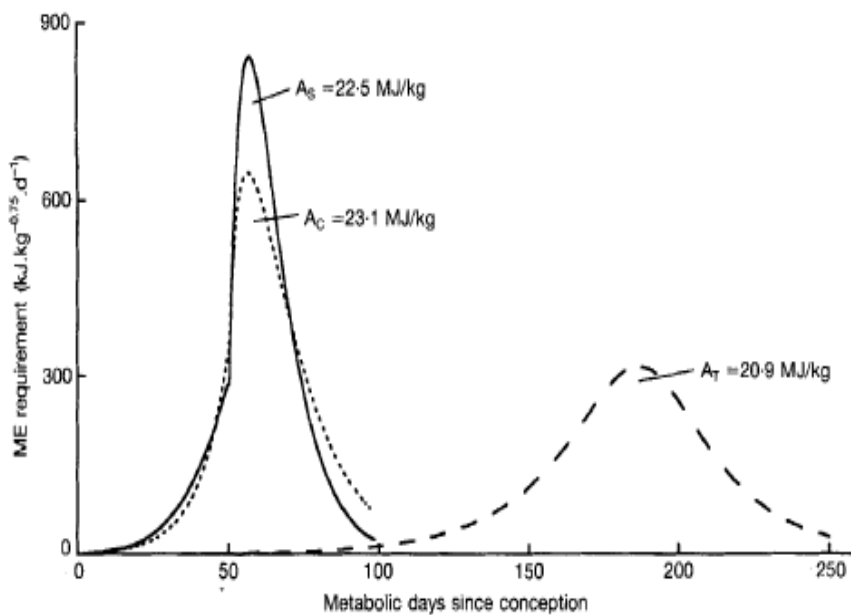
(Broekhuizen et al., 1994; Gurney et

al., 2003). Plasticity in the control of growth rates allows individuals to reduce rates during periods of nutritional depression to conserve energy for vital functions (Lindstrom et al., 2005) and increase during recovery periods to recompense the mass lost during slower growth, effectively restoring an individual’s reproductive fitness and ability to compete for resources. As such, the amount of change during realimentation, and the power of the compensation, is directly related to the intensity and timing of the nutritional depression, *i.e.* animals who have suffered a more intense nutritional insult will experience higher growth rates during recovery than individuals exposed to a less intense injury (Jobling et al., 1993; Jobling et al., 1994; Taborsky, 2006; Gonzaga Neto et al., 2011; Jones et al., 2011), as there are several types of compensatory response. In full compensation, deprived individuals will achieve size parity at the same age as non-deprived individuals (Figure 4.1.1). Partial compensation occurs when the deprived animals fall short of attaining size parity at the same age, but still exhibit rapid growth rates. Overcompensation is also possible, occurring when deprived individuals show not only a more rapid growth rate than their control counterparts, but also demonstrate more effective food conversion ratios (Shadnouch et al., 2011), reaching a larger size than the control, although this is a rare outcome (Hayward et al., 1997). The amount of compensation is also linked to the stage of development of the individual at commencement, the rate of maturity of the species or the pattern of re-alimentation (Wilson & Osbourn, 1960). It has been suggested that compensatory growth

occurs about 25% of the time in viable parameter sets, with long term compensation occurring most often, and over compensation occurring the least often (Mangel & Munch, 2005).

The literature indicates that the increase in growth rate during recovery relies on an increase of food intake. Hyperphagia, coupled with the manipulation of internal allocation patterns to route a fixed fraction of net production to body reserves, produces the compensation (Jobling & Koskela, 1996; Gurney et al., 2003; Rommers et al., 2004; Gonzaga Neto et al., 2011), reducing deposition of adipose tissue during recovery in favour of allocation to structure (Broekhuizen et al., 1994; Jones et al., 2002; Gurney et al., 2003; Gurney & Nisbet, 2004). This connection between reduced adiposity and hyperphagia is known as the “lipostatic model” (Kennedy, 1953) and is controlled by brain cues produced by adipose tissue deposits to “indicate” an individual’s fitness (Nishizawa & Bray, 1980; Blum, 1997; Schwartz & Seeley, 1997; Jobling & Johansen, 1999). Broekhuizen *et al.* (1994) hypothesize that body mass is a twofold model, in which tissues are broadly partitioned into reserves (adipose tissue deposits and the mobilizable parts of musculature) and structural components (skeleton, circulatory and nervous tissues), and that there is a “normal” ratio of these, and when abnormality is detected, compensation mechanisms occur when possible. When starvation occurs, initial weight loss is rapid, followed by a period of stability at this lower level, until death by starvation occurs. Once food is reintroduced, the rate of growth will accelerate until all the body mass lost during starvation is regained, and normality (as compared with conspecifics) is achieved. Thus, the accepted recovery strategy is hyperphagia, followed by rapid growth and then repletion of energy reserves (Broekhuizen et al., 1994; Jobling et al., 1994; Jobling & Johansen, 1999). Studies on ungulate species suggest that balancing mechanisms, such as the lipostatic model, control several aspects of phenotypic growth, including allocation to ornamental or sexual growth (Mysterud et al., 2005). Well fed, fast growing ungulate males face weak costs of ornament growth compared with nutritionally stressed males (Bergeron et al., 2010), and tend to reach maturity first, lengthening their reproductive life.

The reproductive cycle, as one of the more energetically expensive stages of life history (Oftedal, 1985), may also be subjected to balancing trade-offs. For mammals, lactation is the most energy draining stage of reproduction, and has been linked to compensatory growth patterns of offspring. For example, nutritionally stressed lactating mothers have shown an increased level of immunoglobulin transfer to their young over their non-stressed cohorts, boosting the infection fighting capabilities of their milk despite the nutritionally depressed effect on offspring growth (Landete-Castillejos et al., 2002). Feed restriction in eutherians during early gestation and alleviated prior to the first parturition has been shown to affect mammary gland development, increasing mammary cell proliferation and milk production by 14% in the recovery phase (Kim & Park, 2004), which in turn causes corresponding changes in the growth rate of offspring. Although the tammar wallaby expends total lactational energy levels similar to eutherian herbivorous mammals, the daily output and peak output are much lower. However, lactation is longer in metatherians than their



eutherian equivalents (Cork & Dove, 1989) (Figure 4.1.2), and this trend is exaggerated for macropods who show even longer development stages than other marsupials (Russell, 1982b).

Figure 4.1.2 Estimated requirement for metabolizable energy (ME) ($\text{kJ} \cdot \text{kg}^{-0.75} \cdot \text{d}^{-1}$) to support pregnancy/lactation in 500kg dairy cows (—), 50kg sheep (···), and 5kg tammar wallabies (- - -). Areas under the curves (A_C , A_S and A_T , for cattle, sheep and tammars, respectively) represent total energetic requirements. Figure from Cork & Dove (1989).

There are two distinct stages of reproductive development in the tammar, pouch life and post-pouch life. To assess the species' adherence to assumption 2 of the TWM, both stages must be tested for compensatory growth. The data presented in Chapter 2 of this thesis suggested that mothers were able to buffer their young from environmental fluctuations, yet the young sampled in the wild during the breeding season of 2007 showed a marked increase in body condition relative to their age and skeletal growth, which may be evidence of a compensatory growth effect. Maternal effects at this stage of lactation are also known to

impact on post-weaning development of offspring (Gendreau et al., 2005), including on post-weaning compensatory growth patterns. During weaning, young-at-foot macropods are not markedly dependent on milk when high-quality feed is available, but do show more dependence on maternal milk supplementation when external resources are poor (Munn & Dawson, 2003). At this stage of development, as most females reach maturity in late October-November when they reach a 2kg weight threshold (Williams et al., 1998) immediately post-weaning, poor quality feed could have deterring effects on maturity and reproduction. In red-necked wallabies (*Macropus rufogriseus banksianus*) poor food quality at this stage of development is associated with reduced ability to rear offspring to emergence from the pouch and weaning, and reduced time spent in the pouch by pouch young (PY) and delay in the age of first parturition of daughters (Higginbottom, 2000). In primiparous sows, nutritional restriction caused increased mortality of embryos, especially female embryos, during gestation and a reduction in weight and condition of young (Vinsky et al., 2006).

Male juvenile tammar post-weaning concentrate their resources on growth, as maturity occurs at 18 to 20 months of age (Inns, 1980), while females are focused on reproduction. If assumption 2 of the TWM applies to the tammar, and there is a link between juvenile condition and adult condition, there may be a difference in the way cohort effects are articulated between the sexes, indicating variations in sensitivity between males and females to stress events at certain stages of development (Kruuk et al., 1999; Toigo et al., 1999; Festa-Bianchet et al., 2000; Kruuk et al., 2000; Solberg et al., 2004). Some eutherian mammals have displayed sex specific compensatory growth patterns at the same stage of development (Festa-Bianchet et al., 2000; Jones et al., 2011), with females better able to recover from a poor start in life than their male counterparts.

The experiment presented in this chapter was designed to test for violations of the second assumption of the TWM in the two stages of development of *M. Eugeniei*, PY and juveniles. If compensatory growth occurs in *M. eugeniei*, in violation of the TWM assumptions, juveniles initially nutritionally restricted should experience depressed growth, with subsequent amelioration of the depression when the restriction is removed, reaching the same size-at-age as non-restricted juveniles. As Chapters 2 and 3 have provided little evidence in support of the TWM in this species, it is hypothesized that juvenile tammar wallabies, of both sexes, will exhibit compensation. The assessment of pouch young *M. eugeniei* growth during lactation under different planes of nutrition is designed to confirm the observations of the Chapter 2 wild pouch young, and it is predicted that there will be no

difference in growth trajectories of male or female pouch young whose mothers are fed *ad libitum* and maintenance diets.

4.2. Methods & Materials

Tammar wallabies (*Macropus eugenii decres*), both juvenile and adult female, were captured on Kangaroo Island, under Department of Environment and Heritage scientific permits and with University of Adelaide Animal Ethics approval, using a stunning technique (Robertson & Gepp, 1982), as per approvals and methods listed in Chapter 3. The animals were captured in December 2007, when young-at-foot wallabies were becoming juvenile adults, and February 2008, when adult females have very small pouch young. The captive *M. eugenii* were then kept as a group in large pens on Roseworthy Campus, University of Adelaide for several weeks to acclimatize to captivity.

4.2.1 Juveniles

Twelve juvenile tammar wallabies, 4 females and 8 males, were randomly chosen to participate in the experiment, the numbers were unequal for each sex due to the



Figure 4.2.1.1
Juvenile wallaby in experimental pen.

misclassification of some juvenile males as females during capture, as juvenile male testes are much smaller than adult male testes until roughly 18 months of age (Renfree et al., 1996) which resulted in an overabundance of juvenile males in the captive population. The juveniles were placed in 3x3m² pens, and randomly assigned to 2 different diets, High (H) nutritional input and Low (L) nutritional input. Water was available *ad libitum* (Figure 4.2.1.1). The animals on the H diet were fed *ad libitum* on the same specialized wallaby feed used in Chapter 3. Adult wallaby

requirements are roughly 1.73mJ for females weighting 5.6kg, and 2.19mJ for an average male weighting 7kg, and 16.9% protein (Hume, 1999). The wallabies on the L diet were fed maintenance levels of the same diet, though a vitamin/mineral supplement was added, assuring only levels of protein and energy were affected. The animals were fed for 96 days on their assigned diets and then the individuals on the L diet were swapped to an H diet and fed for a further 84 days, animals on the H diet remained on the H diet. Thus, two diet groups were created, an HH group and an LH group, 2 of each were females and 4 of each were males. Measurements of foot length and weight were taken on a fortnightly basis as per Chapter 2 Methods & Materials.

Growth in the juvenile wallabies was analysed using 2 parameters, body weight (g) and Juvenile Body Condition (JBC), which was calculated as per Chapter 3, weight by the foot length³ (Krebs & Singleton, 1993; Green, 2001; McGowan et al., 2008). The Growth Rate (GR) was then calculated by dividing it by time (days), for example $GR_{\text{weight}} = \frac{\text{weight}_{54 \text{ days}} - \text{weight}_{40 \text{ days}}}{14 \text{ days}}$. Normality of the parameter distributions was checked and Restricted Maximum Likelihood (REML) analysis for repeated measurements over time was used to assess the impact of time, diet and sex on the measured parameters. Mean differences were assessed using Student's *t* test and ANOVA measurements. Comparison of the Growth Rate curves slopes was also done using correlation tests.

4.2.2 Mothers with Pouch Young

Twelve adult female tammar wallabies with small pouch young (PY), no more than 5.5cm in crown/rump, were randomly assigned to 2 different diets, HH and LH as above and kept in the same pens, once the juvenile experiment was completed. As there is no difference in size or condition in the pouch young of *M. eugenii*, sex of the PY was not recorded. The females were fed for 112 days on their assigned diets and then the LH group was swapped, all were then fed a further 84 days, for a total of 196 days, which is close to the end of the exclusive pouch life of PY (Murphy & Smith, 1970; Hinds & Tyndale-Biscoe, 1985). Fortnightly body condition measurements of maternal full body weight (kg) and foot length (cm) were taken and Maternal Body Condition (MBC) calculated (weight/foot length³) as per Chapter 2 Methods & Materials (Krebs & Singleton, 1993; Green, 2001; McGowan et al., 2008). Measurements were taken while the mother was anesthetized using Isoflurane/O₂ gas to reduce stress during PY body measurements. Body condition measurements on PY were obtained as per Chapter 2 Methods & Materials, and weight (g) and foot length (mm) were measured and PYBC calculated (g/mm³). As the PY were very small at the start of the experiment and permanently attached to the teat (Merchant & Sharman, 1966), the crown/rump (CR) measurement was also obtained using callipers (Ullmann, 1993; Shackell et al., 1996; Frigo & Woolley, 1997) (Figure 4.2.2.1), weight being too difficult to obtain during the early stages of development as removal and reattachment is difficult and increases the likelihood of PY loss (Russell, 1982b). Weight was obtained from day 84, as PY could be reliably removed from the pouch and reattached without loss.

Analysis was performed as per the juvenile data set above, using REML analysis, ANOVA and Student's *t* test, with correlation tests to compare the growth rate slopes between the two diet groups.



Figure 4.2.2.1 *A)* a pouch young permanently attached to the maternal teat, with a crown/rump measurement being taken, *B)* a pouch young, no longer permanently attached to the teat, being weighed.

4.3. Results

4.3.1 Juveniles

When the whole data set was analysed regardless of sex, the distributions of weight (Shapiro=Wilk: SW=0.9856, P=0.105) and JBC (SW=0.9887, P=0.293) are both normally distributed with equal variance. The juvenile weight at the start of the experiment (day 0) showed no significant difference between the individuals randomly assigned to each starting diet ($t=0.00$, p value=0.998, df of 10). An REML repeated measures antedependence test of the two groups over the entire experiment indicated that diet (HH or LH) ($F=32.90$, p value= <0.001 , df of 1) and time (test statistic=24.46, p value= <0.001 , df of 12) had significant impacts on the weight of the juveniles, although there was not significant interaction between the two (test statistic=0.61, p value=0.830, df of 12) (Figure 4.3.1.1).

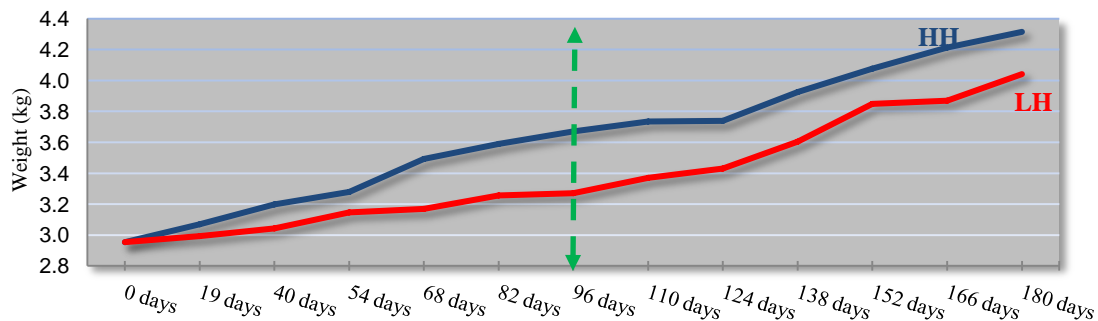


Figure 4.3.1.1 Comparison of average weight of juvenile Kangaroo Island *M. eugenii* held in captivity and fed High and Low nutritional input diets, the green arrow marks the point at which the diets were swapped.

Analysis of juvenile weight on day 96, the point at which the juveniles in the LH group were swapped from the L diet to the H diet, showed a significant difference between the two groups ($t=2.62$, p value=0.026, df of 10) (Figure 4.3.1.2). However, after 180 days, the difference between the two groups was no longer significant ($t=1.80$, p value=0.103, df of 10).

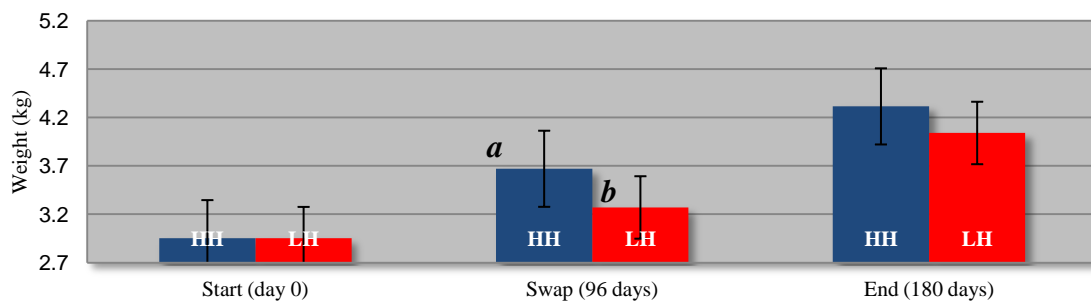


Figure 4.3.1.2 Comparison of average weight of juvenile Kangaroo Island *M. eugenii* held in captivity and fed HH and LH diets for 180 days, diets were swapped at day 96, data points with differing postscripts are significantly different, error bars represent standard error.

For this discrepancy at day 96 to be slightly compensated for by day 180, Growth Rate_{weight} must be different between the groups. The GR_{weight} at the start of the experiment is not significantly different between the 2 groups ($t=0.43$, p value= 0.674 , df of 10), while the GR_{weight} of the LH group was significantly depressed compared with that of the HH group at the diet swap ($t=2.62$, p value= 0.026 , df of 10) and no significant difference in GR_{weight} after day 166 ($t=0.00$, p value= 0.996 , df of 22). A REML analysis of the growth rate also showed significant impacts of diet ($F=34.68$, p value= <0.001 , df of 1) and time ($F=508694.59$, p value= <0.001 , df of 11), but no interaction between the two ($F=0.89$, p value= 0.555 , df of 11).

There was no significant difference in JBC between the two groups at day 0 ($t=-0.08$, p value= 0.936 , df of 10), while at the diet swap JBC was significantly different between the two groups ($t=2.95$, p value= 0.015 , df of 10) (Figure 4.3.1.3). However, by the end of the experiment there was no longer any significant difference in JBC between the groups ($t=0.58$, p value= 0.574 , df of 10) (Figure 4.3.1.4). Repeated measures analysis shows that diet ($F=11.04$, p value= 0.001 , df of 1) and time ($F=3.17$, p value= <0.001 , df of 11) are both significantly associated with JBC, but there is no interaction between the two ($F=0.56$, p value= 0.857 , df of 11). Comparison of the slopes of the growth curves using correlation analysis showed a significant difference between the two groups (correlation score -0.0267), suggesting the GR_{JBC} of the two groups was significantly different.

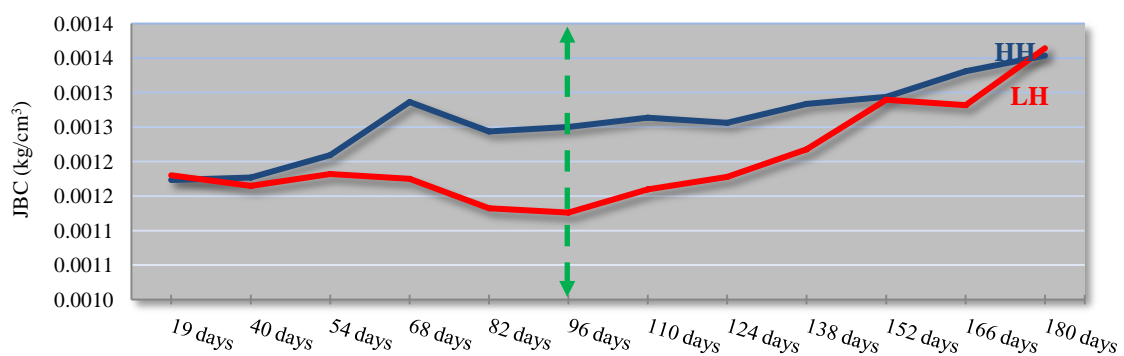


Figure 4.3.1.3 Juvenile Body Condition (weight (kg)/foot length³(cm)) of juvenile Kangaroo Island *M. eugenii* held in captivity and fed two different combinations of the H and L diet for 180 days. The green line marks where the LH group was swapped from the L diet to the H diet.

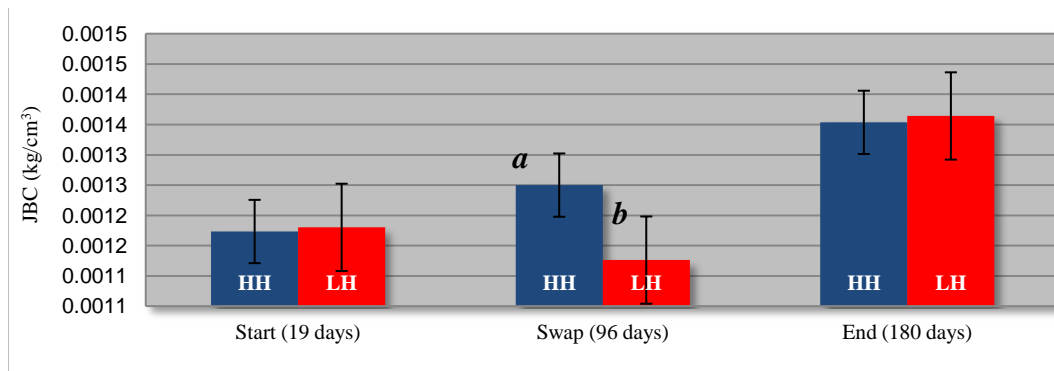


Figure 4.3.1.4 Average Juvenile Body Condition (weight (g)/foot length³(cm)) of juvenile Kangaroo Island *M. eugenii* at day 0, day 96 and day 180 of the experiment, data points with differing postscripts are significantly different.

Given that the LH group recovered JBC to be equal to the HH group in 84 days, GR_{JBC} was significantly affected by the diet swap. The GR_{JBC} of the LH group was significantly depressed when compared to the HH group at day 96, before the diet swap ($t=2.95$, p value=0.015, df of 10), but the LH group had recovered GR_{JBC} by the end of the experiment ($t=0.58$, p value=0.574, df of 10). Repeated measurements analysis indicated that time alone ($F=807.89$, p value= <0.001 , df of 11) influenced growth rate, with no impact of diet ($F=1.50$, p value=0.223, df of 1) or interaction between the two ($F=0.12$, p value=1.00, df of 11).

4.3.2 Juvenile Growth between the Sexes

Due to the small sample size of female juveniles in this data set, the ability of this data to pick up correlations lower than 40% (power test for 40% with 4 replicates is 0.059) is reduced, yet the data suggested some differences between the sexes despite this issue. Repeated measures analysis (REML) suggested that sex, with no relationship to time or diet, had a significant impact on juvenile weight during this experiment ($F=17.70$, p value= <0.001 , df of 1). In fact, over the entirety of the experiment, females showed a significantly reduced weight ($t=-2.76$, p value=0.007, df of 146.62) and JBC ($t=8.59$, p value= <0.001 , df of 142) when compared with their male cohorts.

Prior to the diet swap, females showed a significantly higher JBC than their male counterparts ($t=8.43$, p value= <0.001 , df of 70) (Figure 4.3.2.2), but were equal in weight ($t=1.28$, p value=0.47, df of 47) (Figure 4.3.2.1). The impact of sex on weight was only apparent over time when comparing the two groups ($F=3.63$, p value=0.060, df of 1), with no association to diet ($F=0.08$, p value=0.784, df of 1), while JBC was highly influenced by sex ($F=96.98$, p value= <0.001 , df of 1) and showed significant interactions with diet ($F=3.89$, p value=0.053, df of 1) and time ($F=5.28$, p value=0.025, df of 1). After the swap, there were significant differences between the sexes in both weight ($t=-4.05$, p value= <0.001 , df of 70)

and JBC ($t=5.06$, p value= <0.001 , df of 70), with a highly significant influence of the interaction of sex and diet on JBC ($F=19.11$, p value= <0.001 , df of 1), but not on weight ($F=0.53$, p value= 0.468 , df of 1), although diet ($F=45.66$, p value= <0.001 , df of 1) and sex ($F=50.37$, p value= <0.001 , df of 1) were independently associated with weight after the swap.

Correlation analysis of the slopes of the growth curves showed that male juveniles on the HH diet significantly increased their GR_{weight} (correlation score 0.0066) and GR_{JBC} (correlation score 0.0588) after day 96, when compared with females on the HH diet. Female juveniles showed no significant difference in weight between the two diet groups at the start of the experiment ($t=1.41$, p value= 0.293 , df of 2), or in JBC ($t=-2.58$, p value= 0.123 , df of 2), at the swap point (Weight: $t=2.68$, p value= 0.116 , df of 2; JBC: $t=1.28$, p value= 0.329 , df of 2) or at termination (Weight: $t=1.39$, p value= 0.299 , df of 2; JBC: $t=-1.49$, p value= 0.275 , df of 2). However, analysis of the slopes of the JBC growth curves (GR_{JBC}) suggests that the LH group was influenced by diet when compared the HH group (correlation score 0.0660), before and after the swap. Repeated measures analysis suggests that diet did have a significant impact on weight ($F=30.11$, p value= <0.001 , df of 1), but not on JBC ($F=0.09$, p value= 0.766 , df of 1), but this is most likely due to the overcompensation of females in the LH group, which showed significantly higher JBC after day 152 than their HH cohorts ($t=-2.72$, p value= 0.022 , df of 10).

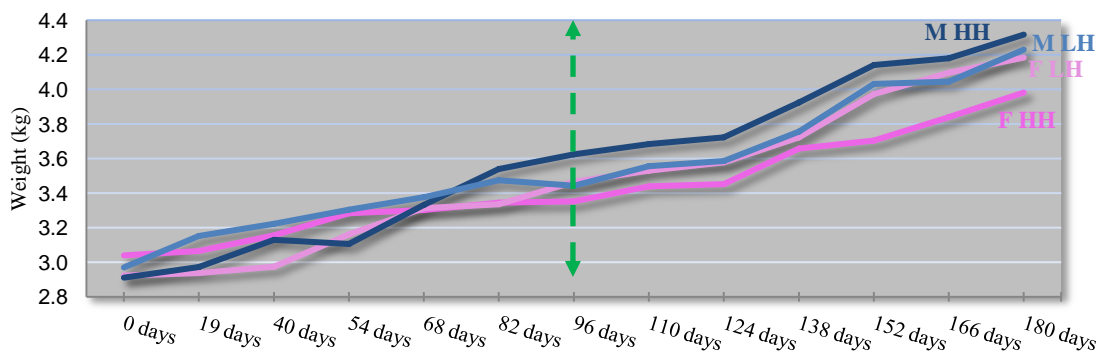


Figure 4.3.2.1 Comparison of average weight in both sexes of juvenile *Macropus eugenii* on the LH die and the HH diet, the green arrows marks the point of diet swap in the LH group.

Male juveniles also demonstrated no significant difference in weight at day 0 ($t=-0.24$, p value= 0.816 , df of 6) or JBC ($t=0.84$, p value= 0.435 , df of 6). However, by day 96 there was a significant impact of diet on weight ($t=2.72$, p value= 0.017 , df of 14) and JBC ($t=7.70$, p value= <0.001 , df of 14) between the groups. By termination of the experiment at day 180, the difference in JBC between the groups had been reduced to a non-significant level ($t=1.64$, p value= 0.151 , df of 6), but the difference in weight between the two groups remained ($t=2.26$, p value= 0.065 , df of 6), indicating that no overcompensation occurs in male juveniles, though compensation does occur. The growth rate curves indicated that GR_{weight} (correlation score -0.0388) and GR_{JBC} (correlation score -0.0175) in LH males were significantly depressed when compared with the HH males.

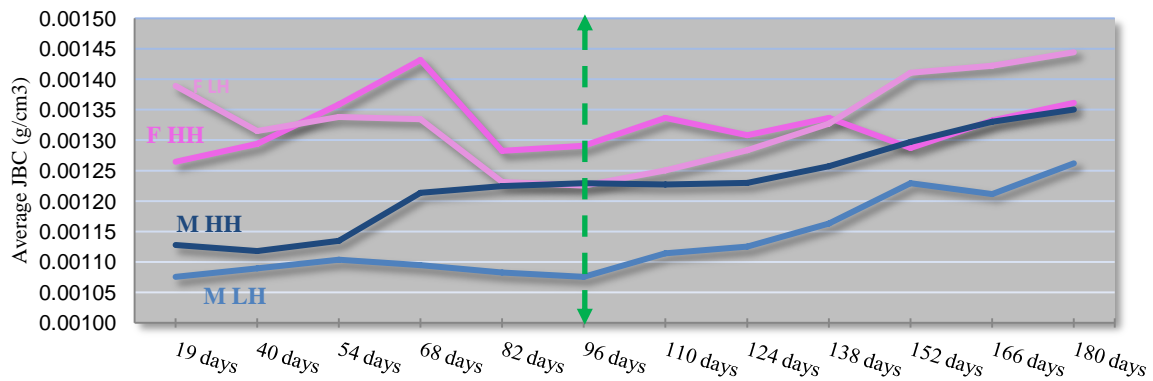


Figure 4.3.3.2 Comparison of average JBC (weight(kg)/foot length (cm)³) in male and female juvenile *Macropus eugenii* on the HH and LH diet, the green arrow marks the point of diet swap in the LH group.

4.3.3 Mothers with Pouch Young

Maternal weight was significantly associated with diet throughout the experiment ($F=72.74$, p value= <0.001 , df of 1) (Figure 4.3.3.1). Maternal weight at the start of the experiment was significantly different between the two groups, despite the random allocation of the treatment ($t=2.33$, p value= 0.053 , df of 7), while at day 112, the point at which the diet for the LH group was swapped, the difference in weight between the groups was highly significant ($t=6.32$, p value= <0.001 , df of 5.55). By the termination of the experiment at day 196, after the diet swap, there was no significant difference between the groups ($t=0.46$, p value= 0.661 , df of 6), despite the LH mothers surpassing their initial body weight by day 126, both groups had lost a large amount of weight by day 196, thus LH mothers returned to their original weight ($t=0.05$, p value= 0.963 , df of 3), while HH mothers lost a large proportion of their weight after day 182, reducing to a level not significantly different from the LH group ($t=0.46$, p value= 0.661 , df of 6). The growth curve slope of the LH mothers is significantly different before and after the diet swap (correlation score 0.0490), though not significantly different from the HH group at any point during the experiment.

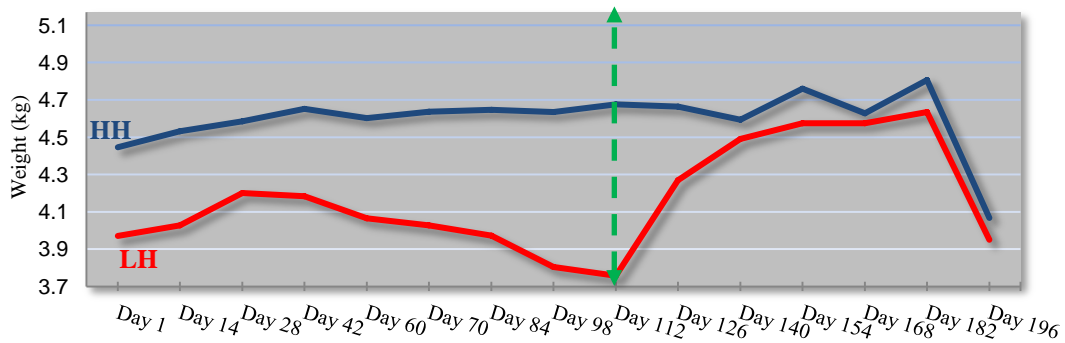


Figure 4.3.3.1 The average weight in Kangaroo Island *M. eugenii* mothers over 196 days of the experiment, the green line marks the point at which the diet was swapped for the LH group, day 112.

The MBC of the LH group was influenced by the assigned diet, when compared with the HH group ($F=3.62$, p value=0.060, df of 1) (Figure 4.3.3.2). The influence of diet on the weight groups was most influential prior to the swap, as there was no significant difference between the two groups at the start of the experiment ($t=0.23$, p value=0.821, df of 7), but day 112, there was a significant difference ($t=3.24$, p value=0.005, df of 16). After the diet swap, the LH group had reclaimed enough condition by day 126 to ameliorate any disparity in weight between the groups ($t=0.38$, p value=0.716, df of 6).

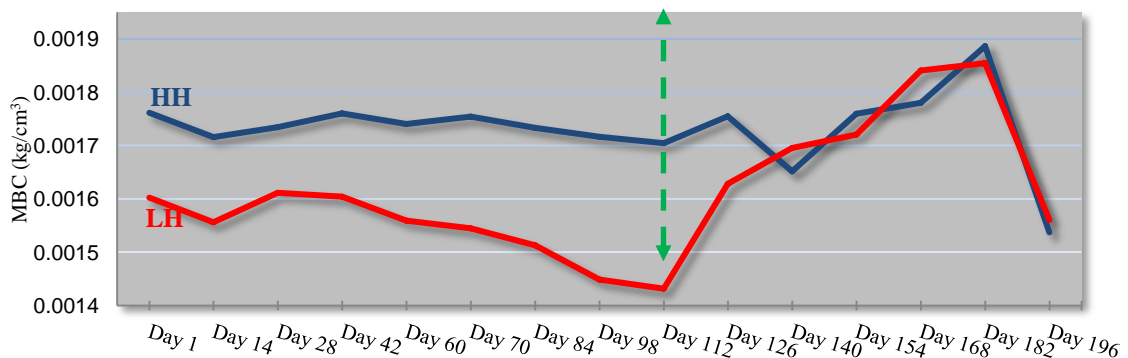


Figure 4.3.3.2 The average Maternal Body Condition (weight (kg)/foot length (cm³)) (Krebs & Singleton, 1993; Green, 2001; McGowan et al., 2008) in Kangaroo Island *M. eugenii* mothers over 196 days of the experiment, the green line marks the point at which the diet was swapped for the LH group, day 112.

Although the female condition parameters were significantly impacted by the assigned diets, the PY body condition parameters did not mirror this pattern. There was no significant

difference in PY CR between the two groups ($F=0.32$, p value= 0.576 , df of 1) (Figure 4.3.3.3).

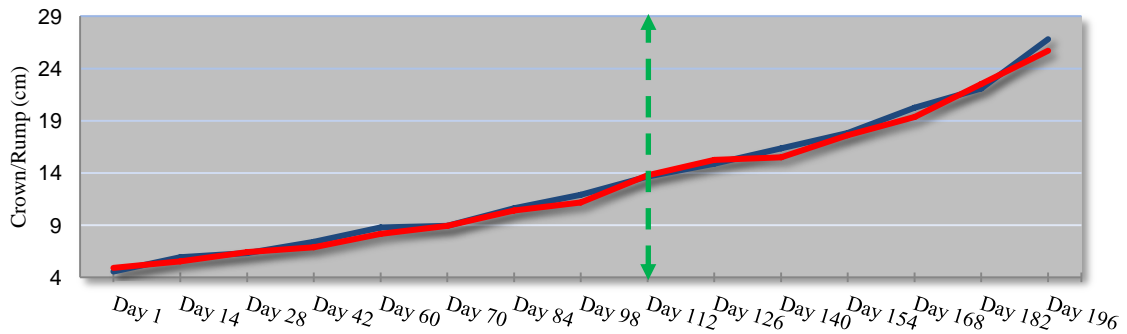


Figure 4.3.3.3 The average crown/rump measurement in pouch young Kangaroo Island *M. eugenii* of mothers fed differing diets over 196 days of the experiment, the green line marks the point at which the diet was swapped for the LH group, day 112.

In addition, analysis of PY weight, which was only taken from day 84 onwards, shows no significant difference in weight or PYBC between the two diet groups ($F=0.34$, p value= 0.560 , df of 1) (Figure 4.3.3.4 & 4.3.3.5).

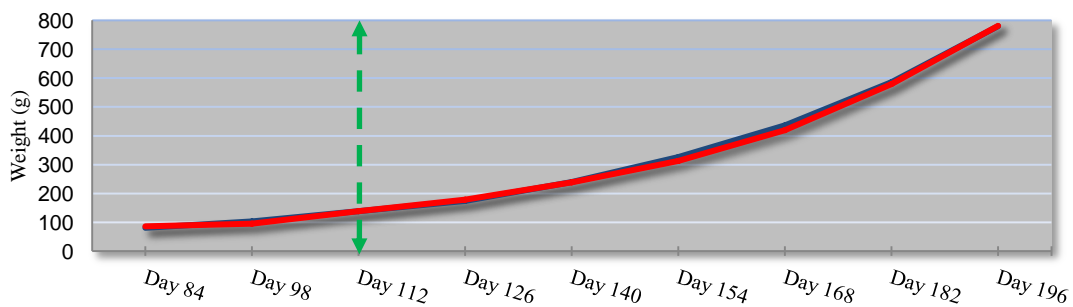


Figure 4.3.3.4 The average weight (g) in pouch young Kangaroo Island *M. eugenii* of mothers fed differing diets over 196 days of the experiment, the green line marks the point at which the diet was swapped for the LH group, day 112.

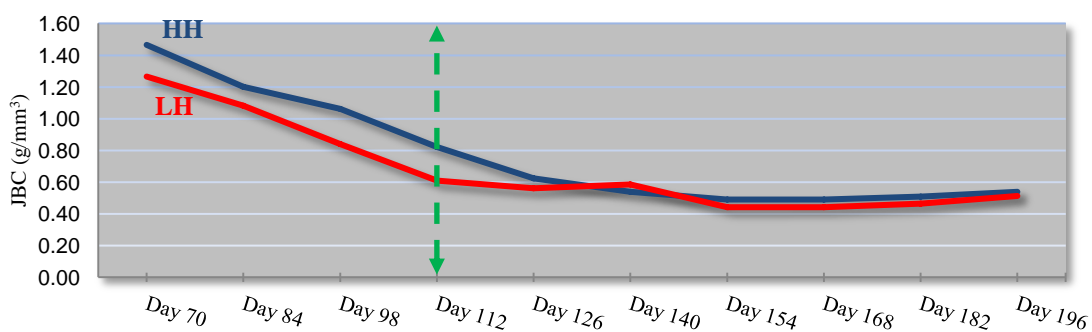


Figure 4.3.3.5 The average Juvenile Body Condition (weight(g)/foot length(mm)³) in pouch young Kangaroo Island *M. eugenii* of mothers fed differing diets over 196 days of the experiment, the green line marks the point at which the diet was swapped for the LH group, day 112.

4.4. Discussion

4.4.1 Juveniles

Nutritional depression caused reduced growth rate in this population of juvenile *M. eugenii*, which was then alleviated by the removal of the restraint. Overall, when the sample was considered as a whole without introducing sex as a variable, the restricted group showed compensatory growth in the amelioration of differences with the unrestricted group in both weight and condition (JBC) after the diet swap at day 96. Although, the average weight of the restricted group did not quite reach parity by the end of the experiment, the difference between the groups was no longer significant.

When the sexes were analysed separately, it became clear that males exhibited a different compensatory pattern to their female cohorts, which may have had a confounding impact on the dataset when analysed as a whole. The sex of the juvenile had a significant effect on compensatory patterns, although males tended to be heavier than females throughout the experiment and females tended to have a higher body condition parameter throughout. In fact, despite the impact of the diet on growth trajectories, the sex of the juvenile was more important during the recovery phase of the experiment than the increase in dietary input. The 'optimum' trajectory in this population, namely the *ad libitum* group, displayed sexually distinct 'normal' growth patterns for this sample, in the early stages of juvenile development, post weaning, males allocated their resources to structural development, reaching a significantly higher weight than the females, while females focused their resource allocations on body condition, or condition development that was maintained at a constant level throughout the experiment, similar to other eutherian species, where females concentrate their early developmental resources on condition and reproduction rather than growth (Solberg et al., 1999). Male condition eventually reached parity with the females, but females never reached the same weight as their male counterparts. This corresponds with data available on dimorphic ungulate species, where a large percentage of variability in body mass is explained by sex and age (Solberg & Saether, 1994; Loison et al., 1999). In these species, males are more influenced by nutritional variation in development than females (Solberg et al., 2004) and do not compensate as well, though they grow for a longer period of time and reach larger sizes.

The compensation strategy differed markedly between the sexes in the restricted group (LH group). These males had a weight growth rate slower than their sated conspecifics, but after the swap, the growth illustrated a parallel pattern between the groups, rather than a

compensatory pattern, although the difference in weight between the groups was not quite significant at the 0.05 level. Male condition suffered greatly during the restricted phase, as a significant amount of condition was lost, which was not recovered after the diet constraints were removed. It appears that constrained males, similar to the satiated males, focused their energy on structural development even during nutritional restriction. This targeted development, at the expense of condition, may be a fundamental cause of increased juvenile mortality rates for young males in the wild (Inns, 1980). As the difference in male weight at the end of the experiment was almost significantly different at the 0.05 level, more so than at the point the nutritional depression was removed, compensation did not appear to have occurred in this sample. Instead, it appears that the 'normal' growth rate was resumed during realimentation, just from a lower starting point (Broekhuizen et al., 1994). However, since the juvenile tammar male does not reach maturity until 18-20 months (Inns, 1980), it is possible that the growth trajectory of the recovering group could increase later in development, as in some ungulate species where compensation responses can take up to 16 months (Dale et al., 2008).

The lack of concrete evidence for compensatory growth in the sampled juvenile male *M. eugenii* may indicate that assumption 2 of the TWM is true in this species. If life history traits in males of a species have evolved through sexual selection to maximise growth rate to grow as quickly as possible over a given period, it is suggested that compensation mechanisms may not have developed and a bad start in life may never be recovered (Clutton-Brock et al., 1988). There is no evidence of compensatory growth in the male tammars of this experiment. Thus, assumption 2 cannot be rejected for this sample, and the TWM cannot be ruled out as a possible acting force in the sex ratio skew of this species based on this data. However, given the small increase in reproductive success of dominant males of this species (Hynes et al., 2005), the tammar wallaby may not present the best model for testing of the TWM assumptions.

The permanent impact of a poor start in life may also impact on physiological reproduction, not just on phenotypic size and ability to secure access to females, as in some domesticated ruminant males feed restriction during specific periods of sexual development in calthood impacted on Luteinizing hormone activity, delaying puberty and testicular growth (Brito et al., 2007), with no compensation observed during recovery feeding. In the tammar, growth is age dependent and continues after maturation (Inns, 1980), with dominant males showing only a slightly increase in reproductive success than less dominant males (Hynes et al.,

2005), and the lack of compensation may indicate that nutritionally deprived males allocate more resources to their testes in early life than males with a good start in life (Sarasa et al., 2010). Therefore, although there was no significant evidence in the present study that juvenile tammar males demonstrate complete compensatory growth, compensation may occur later in development and increased resources of the restricted group during the observed period of development may have been allocated to increased growth of reproductive organs instead of condition, as condition did not appear to be a resource priority in the sampled males.

Females in the restricted group revealed an overcompensation pattern in both weight and body condition (JBC). Nutritionally deprived females displayed a slowing of weight growth trajectory during the constrained phase and a rapid drop in body condition, followed by a steadier decline prior to removal of restriction. This corresponds with the hypothesis of Broekhuizen *et al.* (1994), who suggested that weight loss during starvation periods is initially rapid, and slows and remains at a lower value until death or food is reintroduced, and that the decline in weight loss rate during a nutritionally constrained episode is due to a reduction in costs by the individual. This reduction of costs, in combination with hyperphagia, is the driving force behind the compensation growth rate during realimentation. However, the reduced trajectory of restricted female weight was alleviated by day 68, at which point the weight growth rate of both groups reached parity prior to the removal of the nutritional constriction, an intriguing result as overcompensation in the deprived group started prior to *ad libitum* feeding. In addition, this overcompensation trajectory increased during the recovery phase, although there was never a significant difference in weight during the experiment. This suggests, in accordance with more recent conjecture (Jones et al., 2002; Gurney et al., 2003; Gurney & Nisbet, 2004), that hyperphagia alone cannot explain the overcompensation seen in the sampled population, as it began during restricted feeding.

The issue with Broekhuizen's model is that it proposes that the primary purpose of recovery growth is to restore nutritional condition, which routes additional nutritional intake primarily to reserves. Gurney *et al.* (2003) insisted that hyperphagia and the reduced costs associated with modified behaviour will not produce enough of a compensatory response for an individual to overcompensate for a starvation period. Instead, they advocated that hyperphagia, coupled with a mechanism for rerouting resource allocation from reserves to structure during high feeding rates, will produce not only compensation effects, but also overcompensation (Gurney & Nisbet, 2004). The results from the data presented here

agreed with this hypothesis. The body condition of the females in the constrained group demonstrated more plasticity than female weight in response to the changing dietary parameters, dropping below that of the *ad libitum* group after 54 days. When the restraint was removed, females on the nutritionally stressed diet increased their growth rate to overcompensate after 42 days of unrestricted feed availability. The drop in body condition during the restricted phase corresponded with the increase in weight growth rate, indicating that body reserves were mobilised to boost the flagging structural growth. Body reserves continued to deplete prior to realimentation, while mass growth rate increased. After the removal of the restriction, overcompensation in the growth of structure increased, while condition took much longer to recover. It appears that females were able to activate and utilise their body reserves to preserve their growth rate, most likely regulated by the changes in adipose tissue and related hormone profiles (Jobling & Johansen, 1999), in accordance with Gurney's hypothesis.

The overcompensation observed in the restricted juvenile females may have occurred in an effort to increase size for reproduction. The effects of compensation episodes on maturity and early reproduction in the tammar wallaby are currently unknown. Female maturity in *M. eugenii* occurs at roughly 9 months of age (Inns, 1980), at a weight of 2kg (Williams et al., 1998), immediately post-weaning in late October-November. In other mammalian species, females from good maternal environments reach maturity earlier than females from poor maternal environments who have undergone some sort of compensatory growth (Monteiro & Falconer, 1966) and restricted primiparous females show reduced offspring development (Vinsky et al., 2006). In addition, fecundity in primiparous tammar females is more variable annually than in adult females, which may indicate that nutritionally stressed juvenile females will abandon reproduction in favour of growth (Inns, 1980). However, as female juvenile tammars are capable of extreme overcompensation, as shown in the current study, it is possible that, as long as nutritional depression is not prolonged, disadvantaged females could reach maturity at the same time or before their privileged counterparts.

The impact of juvenile sex on growth rate in the recovery phase of the nutritionally depressed group eliminated the possibility of viewing the entire data set as a whole. When analysed without incorporating juvenile sex, the resource allocation pattern of realimentation in the data presented here did not conform to the accepted eutherian pattern of rapid structural growth followed by the salvage of body condition (Broekhuizen et al., 1994; Jobling et al., 1994; Jobling & Johansen, 1999), in fact confirming a contradictory pattern

altogether. However, when sex was accounted for, females followed the traditional pattern, while male juvenile *M. eugenii* showed much less compensation on the restricted diet. During the nutritional depression period, males lost less weight than the females, while maintaining their body condition at a relatively constant level, and females proved much better at recovering their lost mass than males. Similar results have been observed in other ruminant and ungulates species, where males fail to show compensation and females prove more adaptable to changes in resource availability (Solberg et al., 2004; Gendreau et al., 2005; Dale et al., 2008; Solberg et al., 2008). There is also evidence of sexually distinct compensation targeting different aspects of anatomy (Jones et al., 2011; Shadnouch et al., 2011), indicating that recovery growth can take complex and targeted growth trajectories, and individuals with flexible and responsive development patterns may have an advantage over those programmed into a single size.

4.4.2 Mothers with Pouch Young

The compensatory response observed in the juvenile *M. eugenii* was not mirrored in the pouch young in this experiment. Although restricted mothers dropped significant amounts of weight and MBC under nutritional constraints, which were recouped during the satiation phase of the experiment, PY displayed no significant differences in size, *e.g.* crown/rump length or weight, at any point of the experiment. This result indicated that external nutrition in *M. eugenii* mothers had little to no impact on development of pouch young, unlike data observed in other macropod species, where rates of emergence from the pouch and weaning reduced with maternal nutritional depression (Higginbottom, 2000) and age of first parturition of daughters was correlated with maternal feed quality. This places more emphasis on tammar development after emergence from the pouch, as young-at-foot wallabies are affected by poor environmental resources during weaning, becoming more reliant on lactational supplements (Munn & Dawson, 2003). The data presented here suggests that maternal care completely buffers the pouch young against nutritional adversity, and hence food shortages during pouch life have little, if any, long-term consequences compared with reduced nutrition post-weaning (Clutton-Brock, 1991).

The only slight difference between the groups appeared in pouch young JBC prior to the diet swap at day 112. Such a result could mean that, though structural development was maintained as a constant regardless of maternal nutrition and resource allocation, PY body condition was more flexible. These findings somewhat contradict the result found in the wild population sampled in 2007 (Chapter 2), where nutritionally stressed mothers had heavy PY that were less skeletally developed (or younger in age) than PY of non-stressed

mothers (as presented in Chapters 2 and 3), but did correspond with the data collected in 2005 and 2006 (Chapter 2). However, as resource allocation during lactation is regulated by the mother in macropods (Merchant & Sharman, 1966; Trott et al., 2003) and maternal milk production is timed precisely to coincide with specific developmental phases of the PY (Menzies et al., 2007), maternal apportioning of resources to PY in the data presented here prioritized skeletal and structural development, even if it meant redirecting allocations earmarked for body condition during periods of nutritional stress to maintain development.

Similar trade-offs in maternal resource allocation during lactation in response to maternal dietary restrictions have been seen in other species (Wright et al., 1998; Landete-Castillejos et al., 2002), *i.e.* when mothers are forced to reduce their allocation to offspring growth, they will increase immunity provisions in their milk to boost the diminished growth rate. There is also evidence from investigations on ungulate species that mothers under nutritional stress shift the flow of nutrients from soft tissues, like muscle, to tissues of more importance, such as structure or the immune system (Davis, 1998), though such trade-offs may have consequences linked to increased mortality rates later in life (Quigley & Drewry, 1998). Evidence from research on rodent species indicates that mothers subject to nutritional restriction during early stages of lactation, as in the present study, increased their milk production during recovery (Kim & Park, 2004). As larger tammar mothers produce more milk (Green et al., 1988), it is possible that a similar compensatory process helped the restricted mothers recover body weight and condition, while keeping pouch young development at parity with non-restricted mothers. In light of the juvenile male tammar data presented here, with an apparent lack of compensatory growth after weaning, the ability of a tammar mother to buffer PY may do little to improve the reproductive success of her sons. Previous studies on rat pups have shown that pups exposed to pre-weaning lactational restriction, but with free access to resources post-weaning, recovered from any deleterious effects of the restriction by adulthood, while pups exposed to post-weaning nutritional restriction, and not restricted during lactational development, caused effects that were not alleviated during adult reproduction cycles (McGuire et al., 1995).

The allocation of resources throughout pouched life in this population changed as development progressed. During early pouched life, the body condition in the current population of pouch young was high, even in the nutritionally constrained group, while weight and crown/rump were low. As PY development progressed, body condition decreased as PY size increased. Intriguingly, mothers in both groups experienced a severe

decline in mass and condition at day 182, which coincided with a slight steepening of the PY weight and crown/rump growth trajectories. Green *et al.* (1988) found that *M. eugenii* pouch young have low daily milk consumption rates early in pouch life, which gradually increase until week 18. After week 18, milk consumption increased substantially until week 30, which represented a five-fold increase in consumption from week 18. In addition, tammar mothers increased their energy content of milk from 205kJ/100g at week 1 of their experiment to 500kJ/100g at week 26, which was followed by a rapid increase to 1150kJ/100g during the final stages of lactation. These increased lactational outputs correspond with increased maternal nutritional intake (Cork, 1991). It seems likely that the decrease in maternal body condition in the present population at day 182, or week 26 of the experiment, was due to the increased milk consumption and energy loading seen in the literature (Green *et al.*, 1988; Cork & Dove, 1989), similar to patterns that have also been observed in other marsupial species (Rose & Flowers, 2005).

Previous data collected on *M. eugenii* indicates that tammar mothers mobilize little or no body reserves during such episodes of peak lactation (Cork & Dove, 1989; Cork, 1991), and that daily dietary intake is solely responsible for meeting the energy requirements of lactation in this species. If this is the case, *M. eugenii* mothers should be highly vulnerable to environmental resource fluctuations during lactation (Sadleir, 1987), which is in slight contradiction to the observed high fecundity rates in the species (Andrewartha & Barker, 1969; Inns, 1980). The low energy levels observed in tammar milk may be an adaptive measure to allow mothers to sustain their lactational load on daily dietary intake, at least during early and mid-lactational loads (Cork, 1991), as with other macropod species (Loudon, 1987). However, the data presented here contradicted these findings, as restricted mothers lost progressively more body condition and weight as PY aged and lactation progressed, suggesting that they utilized body reserves to fund lactational load. In addition, both restricted and satiated groups experienced a steep decline in body mass and condition after day 182 (week 26), despite both groups being fed *ad libitum* at the time. This satiated group of the data presented here maintained a constant weight and MBC, similar to the previous studies that were all fed high quality diets. However, at day 168, MBC increased prior to the marked decline at day 182, which opposes previous research indicating that tammar mothers, as well as some other macropodid species, also do not accumulate body reserves prior to peak lactation (Loudon, 1987; Cork & Dove, 1989; Cork, 1991). Interestingly, the tammars used in Green's and Cork's experiments were all captive reared animals, who most likely were never subjected to any form of nutritional restriction prior to

the experiment and were fed high quality diets during sampling. This is in contrast to the current population, which was collected in the wild and would have been subject to natural environmental resource fluctuations prior to capture, as well as the subsequent experimental restriction.

There is no single “fixed” pattern of compensatory growth in the animal kingdom, but there may be general principles that apply to each species (Mangel & Munch, 2005). In a species like the tammar, where life expectancy in wild populations is relatively short, averaging 2 years in the wild population (Chapter 2) and 3.4 years in Wright & Stott (1999), increased mortality rates and future costs of periods of rapid growth are most likely not felt in a wild population, making compensatory growth a fitness optimizing method of combating fluctuating environmental resources (Mangel & Munch, 2005). Given the overcompensation observed in the current population of juvenile females, feed restriction followed by realimentation could cause more efficient performance associated with lower maintenance requirements (Shadnouch et al., 2011). As the growth rate of an individual at any given size and time is determined by the trade-off between current risks of growth and future benefits of a larger size (Mangel & Stamps, 2001), the pattern of nutritional injury displayed in the current experiment may not honestly represent patterns observed in the wild. As the type of injury is directly associated with the magnitude of recovery (Jobling et al., 1993; Jobling et al., 1994; Jobling & Koskela, 1996; Gonzaga Neto et al., 2011), longer periods of nutritional stress in the wild, such as drought periods, could produce stronger compensatory responses in male juveniles than observed in the sampled population. The TWM assumptions, not corroborated by the sample data of juvenile females, cannot be ruled out due to the lack of significant compensatory growth of juvenile males.

CHAPTER 5 *Attempt to Find a Maternal Post-Conception Control Mechanism in the Wild Population of Kangaroo Island Using Chromosomal Comparisons*

5.1 Introduction

As discussed in the previous chapters of this thesis, the manipulation of sex allocation is observed across the animal kingdom, including reptiles (Pieau & Dorizzi, 2004), turtles specifically (Dorizzi et al., 1991), fish (Burness et al., 2008), birds (Ellegren et al., 1996; Smith & Sinclair, 2004), nematodes (Lively, 1987), mice (Danforth, 1926; Shibata & Kawamichi, 2009), seals ((Proffitt et al., 2008), ungulates (Flint et al., 1997; Gomendio et al., 2006; Garroway & Broders, 2007), including domestic species like sheep (Green et al., 2008) and dairy cows (Roche et al., 2006), marsupials such as antechinuses (Cockburn, 1994) and macropod species (Sunnucks & Taylor, 1997; Fisher, 1999), and including humans (Zorn et al., 2002; Cameron & Dalerum, 2009). However, the predictive theories, like the Trivers-Willard Model (TWM) (Trivers & Willard, 1973), that provide assumptions and hypotheses for testing, merely widen our understanding of the actual pattern of sex ratio bias in a species or group, rather than revealing the mechanism employed by an individual (within a population) to adaptively manipulate the sex of a given offspring.

The physiological mechanisms controlling manipulation of sex ratio have been extensively discussed in the literature and numerous investigators have tried to pinpoint control methods. The only acquiesced information currently available is that there are several mechanisms employed across the animal kingdom, depending on the life history factors of a given species, and that these mechanisms are not mutually exclusive, as several methods of control may be utilized concurrently (Linklater, 2007).

There are several mechanisms proposed for female control of sex ratio bias:

1. Pre-conception: female control of the reproductive tract environment, such as vaginal pH, nutrient/energy makeup of the tract mucus, etc. causing differential motility and mortality of the different classes of sperm (Pratt et al., 1987) or the zona pellucida might have differential binding capabilities dependent on the sex of the sperm (Dominko & First, 1997), female control of timing of insemination in the oestrus cycle (Guerrero, 1974; Harlap, 1979; Martin, 1997; Rorie, 1999);
2. At conception: female causes selective loss pre-placentation, depending on the sex of the early developing embryo; would be most successful for a polytocous species (Flint et al., 1997; Cameron, 2004; Rosenfeld & Roberts, 2004);

3. Post-conception: selective resorption or abortion in later stages of development, in favour of the more beneficial sex (Krackow, 1992; Bazer et al., 1997; Bacon & McClintock, 1999; Larson et al., 2001; Vatten & Skjaerven, 2004);
4. Post-parturition: infanticide of a certain sex after parturition, especially relevant for species producing large litters (Cockburn, 1994; Vinsky et al., 2006; Foster & Taggart, 2008).

The literature is at odds regarding many of these mechanisms. For example, the explanation that the mechanism involves the timing of insemination in relation to the duration of oocyte maturation has just as many advocates (Guerrero, 1974; Harlap, 1979; Martin, 1997; Rorie, 1999) as detractors (Rorie, 1999; Rorie et al., 1999; Rizos et al., 2008). The debate regarding the impact of the pH balance of a female reproductive tract at the time of conception is also contentiously debated by researchers, with some rejecting the hypothesis (Roche & Lee, 2007) and others supporting it (Pratt et al., 1987). Many of the proposed mechanisms involve hormone levels mediated by environmental factors either before, during or after conception (Clutton-Brock & Iason, 1986; James, 1996). Some recent studies on female mammalian mechanisms introduce the ‘good condition’ hypothesis, which proposes a post-conceptual mechanism where the mother’s energy level promotes the development of an embryo of a certain sex, and the ‘maternal dominance’ hypothesis, which suggests a pre-conceptual mechanism where the female influences the ovum to receive a certain sex spermatozoan (Grant & Chamley, 2010). Both of these mechanisms could produce effects corroborating theories like the TWM, where mothers in good condition are more likely to have male offspring to improve their fitness and optimize their reproductive return.

In order to isolate the mechanism in control of sex allocation bias, it is important to understand the method of sexual determination used by the species under discussion. These mechanisms take two distinct forms, genetic control and environmental control, although there are a myriad of combinations in between and in some studies the environmental factors override the influence of the sex chromosomes (Baroiller et al., 2009). In these instances, the genetic or primary sex is adapted during ontogeny so the individual will present as the opposite sex as an adult (Bull, 1983; Valenzuela et al., 2003). This process, or environmental sex reversal (ESR), is a permanent change in phenotype, and individuals will remain the opposite sex and fully capable of reproduction (Stelkens & Wedekind, 2010). Known triggers for ESR take two forms, abiotic (*e.g.* temperature, pH, endocrine-disrupting

hormones, photoperiod, hypoxia) and biotic (*e.g.* crowding, pathogens, population size), which act directly on the developing embryo to initiate sex reversal.

There are two phases acknowledged in mammalian sexual development (Goodfellow & Lovellbadge, 1993; Ramkissoon & Goodfellow, 1996), from the chromosomes to the formation of the gonads and from the gonads to phenotypic development (Vaiman & Pailhoux, 2000). Genetic determination of the first phase is controlled by the heteromorphic sex chromosomes X and Y (*i.e.* XX=female and XY=male), and without the presence of the Y chromosome, the testes will not form and the embryo will automatically feminize (Jost, 1953; Goodfellow & Lovellbadge, 1993; Ogata & Matsuo, 1994; Kent et al., 1996). The second phase is controlled by gonadal hormones secreted by the sexual genitalia, which produce hormones that continue the process of feminization or masculinisation (Vaiman & Pailhoux, 2000).

Mammals, unlike reptile, amphibian, fish and insect species, have sex genes that exert a pleiotropic effect on many phenotypic attributes through these sexual hormones, linking an individual's genotypic sex to metabolism, the nervous system, skeletal structure and other physiological processes (Ogata & Matsuo, 1994; Zanaria et al., 1994; Kent et al., 1996; Ramkissoon & Goodfellow, 1996; Marshall Graves, 2001), making functional reversal difficult. Sexual hormones (Marshall Graves, 2001) have both an organizational and activational effect on the nervous system and brain of most eutherian mammals (Booth, 1979; Goy & McEwen, 1980; Yahr, 1988). For example, androgens present during certain critical periods of eutherian male development permanently "masculinise" the organisation of the brain, sensitising these tissues to sexual hormones in maturity, allowing the same hormones to trigger certain male sexual type behaviours during mating later in life (Phoenix et al., 1959; Maclusky & Naftolin, 1981; Arnold, 1996; Lonstein et al., 2002; Arnold et al., 2003).

Abnormalities during sexual differentiation can cause individuals that exhibit incongruity between genetic and phenotypic sex, or abnormal phenotypic sexual appearance.

Mammalian intersexuality has been documented in 9 of the 19 mammalian orders (Vaiman & Pailhoux, 2000) and 11 different marsupial species (Cooper et al., 1994), and can be caused by discrepancies at either stage of sexual differentiation (Griffin & Wilson, 1992). For example, certain pathologies linked to sex determining genes, like the human DSS genes, can cause divergence between chromosomal and gonadal sex before the hormonal

stage (Ramkisson & Goodfellow, 1996; Vaiman & Pailhoux, 2000), or certain diseases, like *freemartinism* (Short et al., 1969; Jost, 1970; Short, 1970), or mutations in hormonal secretion precursor genes, can cause dysfunctional hormone release (Imbeaud et al., 1995; Achermann et al., 1999), which has been shown to reverse sex (Burns, 1961; Fadem & Tesoriero, 1986).

Although mammalian sexual development has been widely investigated, several species do not conform to the general therian sexual development theory as described. Some mammals follow the XX:XY mammalian genetic sex determination, but do not follow the strict Y driven sex differentiation pathway laid out by Jost (1953), *e.g.* female spotted hyenas (*Crocuta crocuta*), elephants (*Loxodont spp.*) and many marsupials species (Glickman et al., 2005). These mammals are significant, as they begin sexual differentiation prior to the formation of the sexual genitalia (Cunha et al., 2005), suggesting that sexual differentiation is not tied solely to the accepted mechanism of gonadal hormone secretion driven by genetics, as seen in other mammals. Marsupials follow the accepted mammalian genetic chromosome structure of XX:XY (Alsop et al., 2005), although the chromosomes are smaller in comparison with their eutherian counterparts (Toder et al., 1997), yet sexual development in metatherians is not as strictly controlled by these chromosomes.

Even though the genetic mechanisms are very similar to those found in eutherian mammals, marsupials retain some of the flexibility of amphibian gonad to hormonal manipulation, allowing for the reversal of sex when opposing hormone profiles are introduced to young individuals (Catling & Sutherland, 1980; Renfree & Shaw, 2001; Schneider et al., 2010). Unlike eutherian species, although hormone profiles between the groups are similar, marsupial neural pathways are not sexually differentiated by sexual hormones in early development and the sex hormones involved in breeding act purely as an activational mechanism (Rudd et al., 1996). In accordance with this lack of specialization of the neural system, there is evidence that marsupial development can be influenced or reversed with doses of contradictory hormones (Burns, 1961; Fadem & Tesoriero, 1986; Coveney et al., 2001), if administered early enough in development (Shaw et al., 1988; Coveney et al., 2001; Renfree et al., 2001a), although it is unknown if complete fertile reversals occur as most experiments terminated prior to puberty. An early study by Tyndale-Biscoe and Hinds (1989), found that castrating male tammar wallabies and implanting the testis under the flank skin of immature females produced some significant results in both sexes. Although full reversal was not produced, most likely because implantation of the testis took place on day

10 after parturition, grafted females were hermaphrodites in appearance, with mammary glands, Müllerian duct derivatives, vaginal complexes, uteri, oviducts, Wolffian duct derivatives, normal prostate, a normal sized penis and Cowper's glands (Tyndale-Biscoe & Hinds, 1989). A later project exposed ovaries to male-type hormonal protocols in vivo and in vitro, and found that loss of germ cells in the ovary is the critical factor in ovarian sex reversal, which is caused by the toxic effects of Müllerian-inhibiting substance on these germ cells (Whitworth et al., 1996).

Given the evidence for a hormonal roll in the control of adaptive sex ratio in many of the proposed sex allocation mechanisms listed above and the plasticity in hormone response in many marsupial species, testing the hypothesis that the tammar mother could use these hormones after conception to influence the sex of her young becomes an important step in finding a mechanism of control for the sex ratio bias observed in Chapters 2 and 3. As Coveney et al. (2001) produced sex reversal by treating young tammar wallabies with oestrogen essentially 1 day before birth, it is feasible that after conception a tammar mother could use hormones to influence the phenotypic, and thus operational, sex of an offspring, either before pouch life (Wilson et al., 1995) or during (Tyndale-Biscoe & Renfree, 1987; O et al., 1988; Renfree & Short, 1988). If such a mechanism was employed by the *M. eugenii* population examined in Chapter 2 and 3 of this thesis, there should be a large proportion of sex-reversed animals in the general Kangaroo Island population. The following experiment investigated whether such discrepancies between phenotypic and genotypic gender occurred in the wild population of Kangaroo Island tammar wallabies examined in previous chapters, and, in the case divergence did appear, whether such sexual discrepancy occurred in a proportion large enough to explain the observed sex ratio bias. It is presumed that no affect will be observed and that the tammar will not present with high proportions of sex reversed individuals.

5.2 Methods & Materials

Wild tammar wallabies (*Macropus eugenii decres*) were shot by local licensed farmers under Department of Environment and Heritage Destruction Permit Number Q25076 and Animal Ethics Approval Number S-079-2005B and were sampled between the years of 2005-2008, blood was collected from each individual into vacu-tubes with heparin anticoagulant and the phenotype (*e.g.* male or female) of each sample was noted. Once sampled, the blood was refrigerated and transported to a laboratory for culturing. A mixture of 5 ml of culture medium (which is a mixture of 90ml of AmnioMax-C100 (GIBCO-Invitrogen) Liquid Medium, 10ml of AmnioMax Supplement C100 (containing foetal bovine serum), 1ml Glutamine solution (0.2 M), an amino acid, 0.5ml Penicillin, Streptococcus and Fungizone, 0.2ml Heparin solution, 25,000 units/ml and 0.5ml Pokeweed mitogen (Sigma L8777), 1.0mg/ml) was mixed with 0.03ml of blood from each sample. The mixture was then incubated in a 37°C oven with a loose lid while mixing with 5% CO₂. Incubation lasted for 72 hours, with a few drops of colchicines solution (0.1mg/ml) added about 2 hours before the samples were removed from the incubator.

Then each sample was transferred to a 10ml centrifuge tube and centrifuged for 10 minutes at 1200rpm. All supernatant was removed using suction, leaving a small pellet of cells, which was then resuspended using a hypotonic solution, a pre-warmed solution of 10% Potassium Chloride (KCL) in Isotonic H₂O, then incubated again at 37°C for 20 minutes. A small amount (roughly 10-20%) of a fixing solution, 30% Acetic Acid and 70% Methanol, was added and the cells were resuspended before centrifuging the samples at 1300rpm. The supernatant was removed using suction, and three further fixing episodes occurred, centrifuging at 1400, 1500 and 1600rpm increasing with each fix. The final cell pellet was suspended in 0.3ml of fixative solution and 1-3 drops were placed on a cleaned slide (pre-cleaned slides were soaked in a 5% Decon 90 detergent for 2 hours, rinsed 1 hour in flowing tap water, rinsed in RO water and stored in ethanol, prior to use they were rinsed a second time with ethanol before drying) and allowed to dry. Once dry, the slide was rinsed in a 20% Leishman's stain in pH 6.8 buffer for 20 minutes, at which time buffer was used to remove excess stain and the slide dried again. Stained slides were covered with DePex mountant and a clean coverslip, and slides were examined under an epifluorescent microscope to assess genetic chromosome correspondence with the observed phenotype noted in the wild.

The data was then analysed using a two sample binomial test to assess correlations between the 34% of mothers exhibiting sex ratio in the wild population in Chapter 2 and the proportion of sexually reversed individuals observed in the wild population examined here.

5.3 Results

A total of 153 tammar wallabies were sampled and produced successful chromosome cultures during the 4 years of this study. The samples were 100% matches between phenotype and genotype (Figure 5.3.1 & 5.3.2).

As roughly 27 mothers, or 34%, were in groups that exhibited sex ratio manipulation in the wild population sampled between 2005 and 2007, as discussed in Chapter 2, a binomial test of the two populations (sex ratio/reversed=1, non-reversed=0) showed normal approximation of -8.098, and demonstrated a 95% confidence interval that the two proportions were different ($p < 0.001$), that is the proportions of discrepant individuals and those in groups showing sex ratio bias.

A statistical power test of the sex reversal data, where $n = 153$ and the correlation value is 34% at a significance value of 0.05, indicates that the number of replicates (153) has a 99.7% power of finding the phenomenon with the given sample size, and that the minimum number of replicates needed is 69. Thus, the sample size is sufficient to assess whether hormonal manipulation of phenotypic sex post conception is a mechanism used to control sex ratio skewing.

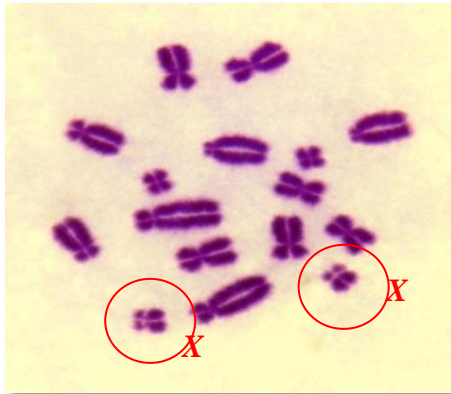
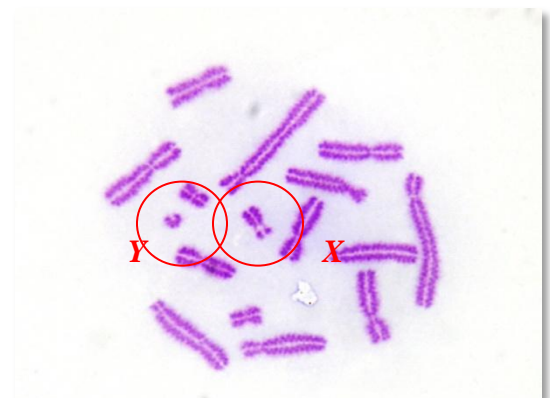


Figure 5.3.1
Female chromosome slide with 2-X
chromosomes indicated

Figure 5.3.2
Male chromosome slide with 1-X
chromosome and 1-Y chromosome indicated



5.4 Discussion

Since marsupial sexual development is more plastic than in eutherians, the flexibility of metatherian gonad to hormonal manipulation facilitates the reversal of sex when opposing hormone profiles are introduced during early development (Burns, 1961; Catling & Sutherland, 1980; Fadem & Tesoriero, 1986; Shaw et al., 1988; Coveney et al., 2001; Renfree et al., 2001a; Renfree & Shaw, 2001; Schneider et al., 2010). This is possible in marsupials because neural pathways are not sexually differentiated by sexual hormones in early development, unlike eutherian pathways (Rudd et al., 1996). However, complete sex reversal has only been achieved when hormones are administered very early in development, which indicates that, if mothers are using hormones to control the sex ratio of their young, treatment prior to or just after parturition would achieve the best results (Fadem & Tesoriero, 1986; Shaw et al., 1988; Coveney et al., 2001; Renfree et al., 2001a). The fertility of such sex-reversed individuals is unknown (Renfree & Short, 1988), as no experiment has continued past puberty. Consequently, as some researchers have found that sex reversal persists only for the duration of hormone treatment, the body returns to homeostasis if injections are discontinued (Coveney et al., 2002), the length of treatment necessary for a permanent reversal, and whether reversal continues in adulthood when maternal hormones are no longer effective, is unknown.

Sex reversal in metatherian species has been observed in both sexes (Tyndale-Biscoe & Hinds, 1989; Whitworth et al., 1996), which contrasts with evidence from eutherian species. The neural pathways of eutherian males are more sensitive to testosterone than pathways in eutherian females, due to their masculinisation during development (Phoenix et al., 1959; Beach et al., 1972; Baum, 1976; Maclusky & Naftolin, 1981; Ulibarri & Yahr, 1996; Lonstein et al., 2002). Earlier investigations in *M. eugenii* demonstrate this lack of differentiation, as both the female and male neural pathways have the same activational sensitivity to testosterone in maturity (Rudd et al., 1996). In addition, male tammar, as well as nulliparous adult female tammar, are capable of producing female parturient behaviour when triggered by prostaglandin F-2 α , evidenced by the adoption of the birth posture and behaviour regardless of genetic sex (Hinds et al., 1990b; Shaw, 1990). Furthermore, female *M. eugenii* implanted with testosterone-secreting cylinders, which produce similar plasma testosterone levels as found in adult males, displayed male type sexual behaviour toward oestrus females that was indistinguishable to control adult males, and significantly different to behaviours shown by control adult females. Rudd *et al.* (1996) also found that castrated

adult males showed no male type sexual behaviour, and were often examined by intact males in a similar fashion to oestrus females.

The plasticity in sex reversal displayed by metatherians indicates that offspring sex ratio could be maternally controlled by an influx of hormones during sexual development, although the duration of hormonal immersion may need to persevere until the end of pouch life. If this were the case, a discrepancy between phenotype and genotype would be evident throughout the population. However, there were no animals with discrepancies between genotype and phenotype in this sample set. As 34%, or 27 of 79 females, in the wild population sampled in Chapter 2 appeared in groups where sex ratio skewing was predominant, and none out of the 153 animals tested here showed sex reversal, sex reversal was clearly not the mechanism used for controlling sex ratio skew in this population. This was not a surprising result, as the presence of such a mechanism would likely cause widely varying and unstable population sex ratios (Stelkens & Wedekind, 2010; Wedekind, 2010), and the wild population sex ratio discussed in Chapter 2 did not deviate from the expected 1:1 ratio (Fisher, 1930). Additionally, an intersexed tammar, which is a possible by-product of such a mechanism if hormonal stimulus is not timed correctly, is likely to exhibit a very mixed phenotype, *i.e.* an XXY male can present with testes, a male urogenital tract and a pouch rather than a scrotum (Watson et al., 1997; Woolley et al., 2003), because of the dual pathway sexual differentiation displayed by the species (Wilson et al., 1995). However, no such individuals were observed in the sample presented here.

As hormone-induced post-conception sex reversal did not appear to be the control pathway used in this population, there is some evidence available on eutherian species that suggests mothers are capable of controlling the sex of the embryo implanting *in utero* prior to placentation (Flint et al., 1997; Cameron, 2004; Rosenfeld & Roberts, 2004) by selective abortion. Studies on eutherian embryos *in vitro* demonstrate that there is a difference in emanation of interferon-tau between conceptuses of the two sexes ((Bazer et al., 1997), which could provide a pregnancy signal to a female as early as the blastocyst stage of development (Larson et al., 2001). This allows the female to ‘decide’ early in pregnancy if the embryo is of the desired sex or if selective abortion of the foetus is necessary in favour of another. These studies found that an increase in glucose levels of the culture medium inhibited early stage development of the female embryo (Gutierrez-Adan et al., 2001; Larson et al., 2001). However, this phenomenon has not been found *in vivo* (Gutierrez-Adan et al., 1996; Catt et al., 1997; Hasler et al., 2002).

Despite these findings in eutherians, it is unlikely that post-conception mechanisms are used in this metatherian. The gestation period for tammars is relatively short, ranging from 26 to 28 days (Merchant, 1979), making selective abortion, or infanticide after parturition, a possible option. However, tammars are highly synchronous breeders and, of the mothers sampled in the wild during the years 2005-2007 (Chapter 2), only 20% possessed young that were significantly smaller and younger than the average, with only 44% of those with younger offspring being part of a group that showed sex ratio skewing. As follows, only 9% of the total sampled wild population possessed young significantly less developed than the average and sex ratio skewing in concurrence, making it highly unlikely that abortion or infanticide after parturition were control mechanisms. If after conception mechanisms such as these existed in the population, then at least 34% of the young would be much smaller and younger by at least 26-28 days, while in the wild sample only 11% showed an age difference of at least 26 ± 3 days (Chapter 2).

Given the genetic control of sexual differentiation apparent in this population (Tyndale-Biscoe et al., 1974; Tyndale-Biscoe & Renfree, 1987; Cooper et al., 1994) and the lack of evidence for post-conception control of sex ratio skew observed in the sampled population, influence over offspring sex ratio bias observed in previous chapters must occur at or prior to conception, by the female, the male or both. There are several at conception mechanisms suggested in the literature available on eutherian species. For example, females of some species may exert control over the intrauterine environment, such as pH balance (Pratt et al., 1987), glucose level (Gutierrez-Adan et al., 2001; Cameron, 2004; Linklater, 2007) or the zona pellucida differential binding capabilities (Dominko & First, 1997), which influence the sex of the spermatozoa that penetrate to successful fertilization of the ovum. Conversely, García-Herreros *et al.* (2010) found that hormone levels at the time of conception did not affect the sex of sperm that fertilized an oocyte. There are also several pre-conception mechanisms proposed in the literature, *e.g.* females controlling the timing of insemination during the oestrus cycle have direct affects on progeny sex ratio (Guerrero, 1974; Harlap, 1979; Martin, 1997; Rorie, 1999). Yet, there is little discussion illuminating the role that males might play in this process, as theories like the TWM assume that males have little motive for controlling sex ratio in polygynous mammals, although there is evidence that males are capable of influencing spermatozoa to increase or decrease fertilization capabilities of one sexed sperm over another (Broer et al., 1977; Moller, 1989; Cui, 1997; Hilsenrath et al., 1997; Preston et al., 2001; Johannisson et al., 2002; Gomendio et al., 2007)

(Chapter 6). As most of the likely contender(s) for control mechanism of sex ratio bias in *M. eugenii* operate at or before conception (Linklater, 2007), any future investigations into this topic should centre on these variables in both the male and female parent.

CHAPTER 6 *Investigation of Sex Ratio of Sperm in Male Tamar Wallabies, to Assess the Existence of a Male Sex Ratio Effect on Sex Ratio*

6.1 Introduction

Most investigations into sex ratio theory have centred on the female parent, as many researchers argue that manipulation of the sex ratio in the F1 generation is logically influenced by the parent that has the most to lose (Trivers & Willard, 1973). This is typically the female parent, as even in cases of shared parental care it is common for the female to invest more resources than the male (Clutton-Brock, 1991); some amphibian, fish and invertebrate species excepted, as parental care among these classes is far more varied than in mammals and birds (Kokko & Jennions, 2008). Thus, males will typically be the more competitive mating partner, subjected to greater intrasexual rivalry (Berglund et al., 1996; Kokko & Jennions, 2008), even at the physiological level (Shimmin et al., 2000; Bonanno & Schulte-Hostedde, 2009; Grant & Chamley, 2010). Thus, such theories as the Trivers-Willard Model (TWM) (Trivers & Willard, 1973) interpret this relationship in a manner that allocates control of sex ratio to the females of polygynous species. This allows females to maximize their fitness by producing larger males when possible, as the reproductive success of sons varies more than daughters with increased maternal resource input. The larger a mother's sons, the more offspring they will produce by securing higher places in the dominance hierarchy, securing more females for mating. However, it is reasonable to assume that the male parent will possess traits that enhance the reproductive success of his sons. In this case, it would be just as much in the male's benefit to control adaptive sex allocation as it is in the female's. As the male is the heterogametic sex in mammals, such an influence is quite feasible (Gomendio et al., 2006). This type of paternal control of the sex of progeny has been noted in birds (Ellegren et al., 1996) and in haplodiploid insects (Henter, 2004).

In mammalian polygynous species, males compete for rank in dominance hierarchies and usually have specific phenotypic attributes related to reproductive success (such as antlers) (Clutton-Brock, 1989; Malo et al., 2005; Grant & Chamley, 2010), the hormones that control fertility often control the other reproductive traits as well (Gomendio et al., 2006). A father could pass on not only the phenotypic attributes necessary to improve a son's fitness, but also physiological traits, like increased sperm swimming velocity, an ability to produce a skewed sex ratio or a higher proportion of normal spermatozoa, assuring reproductive

success of sons, which increases from generation to generation. However, the mechanism for male manipulation of adaptive sex allocation might have more influencing factors than just genetics or hormones (Grant & Chamley, 2010; van Hooft et al., 2010). Many environmental factors can modify a male's ability to reach the full capabilities of his genetic predisposition. For example, a large male, with highly developed sexual phenotypic characteristics, that is extremely attractive to a breeding female, but is under high amounts of stress, may not be able to perform as "advertised" (Reznick et al., 2000; Malo et al., 2005; Bergeron et al., 2010). These external parameters could range from male parasite load to disease, nutrition or resource availability, habitat destruction, drought, predation and migration.

The TWM and other such theories suggest that body condition is a limiting factor of fitness in females which causes sex ratio skew, yet it has a similar impact on the reproductive potential of an otherwise highly successful male (Zedrosser et al., 2007; Burness et al., 2008; Sarasa et al., 2010). The maintenance costs of a large dominant male are quite high, and a nutritionally stressed male is more likely to sustain injury in dominance clashes with other males, or not have the energy to sustain the activities involved in reproduction activities (Parker, 1974; Clutton-Brock et al., 1979; McElligott et al., 1998; Isaac, 2005). As is true for most individuals of a given species, the energy costs of survival will take precedence over reproduction when body condition is poor (Mysterud et al., 2005). Poor body condition does not just influence an individual's ability to undertake the physical aspects of reproduction, it is also shown to impact on testes size (Sarasa et al., 2010), sperm number (Rasotto et al., 2010) and even on ejaculate quality and energetic capacity, important characteristics if sperm competition after copulation is a feature in a species reproductive life-history (Parker, 1970; Burness et al., 2008). In fact, some females appear to be selecting males not solely based on physical body condition and size, but also based on pheromone cues impacted by nutrition (Brown et al., 1996; Olsen et al., 2003; Giaquinto et al., 2010). Giaquinto et al. (2010) found that female Nile tilapia (*Oreochromis niloticus*) were not only discriminating against males in poor condition, but were selecting well-fed males by responding to a food specific chemical cue. Specific dietary supplements have also been shown to impact on semen characteristics post ejaculation (de Graaf et al., 2007). It is therefore reasonable to assume that body condition (impacted by nutrition or resource availability) could have an impact on a paternally controlled sex ratio skew. Such a male mechanism of control could take one of many forms, or even a combination of forms. Firstly, there may be deviations in the rate of production of X or Y sperm (Griffin et al.,

1996; James, 1996; Ishihara et al., 2010). Secondly, there may be deviations in the ability of either X or Y bearing sperm to fertilise the ovum or deviations in the survival rate during storage in the epididymis, although female selection against a specific sex might occur once the sperm have entered the female reproductive tract (Martin, 1997). To assess the working mechanism of a paternal sex allocation, scenario 1 above implies the most control by the male parent, without any confounding maternal effects. It also allows the identification of external factors that might influence individual samples of ejaculate, as opposed to a physiological hereditary trait that would affect all semen samples from an individual.

The study of the impact of environmental factors on sex ratio by manipulating the sex of the sperm in a specific sample can be traced back to the 1920's (Chaudhuri, 1927), when several scientists discovered that the injection of ethyl alcohol into the male mouse, both directly into the testes (Parkes & Bellerby, 1926; Chaudhuri, 1927) and by vapour ingestion (Danforth, 1926), had an effect on the X-bearing spermatozoa, increasing the percentage of male offspring in the secondary sex ratio. Recently, investigations have been focusing on the deliberate human control of such external factors on sex ratio in a semen sample (Quaas & Foote, 1982; Johnson, 1988; Amann, 1989; Welch & Johnson, 1999). These studies focus on either domestic use to increase efficiency in producing desired genetic traits (Seidel & Johnson, 1999; Johnson, 2000), control of X linked genes to avoid expression (Fugger, 1999) or for use in breeding programs for endangered species (Robertson et al., 2006; Behr et al., 2009). Although these studies are not focused on natural male control of the sex ratio, they have provided several technical advancements that allow for the analysis of sperm ratios prior to insemination.

Most of the literature available on this subject considers eutherians mammals, but the development of these techniques has allowed scientists to compare these same physiological processes in other less studied mammals, such as marsupial and monotreme species. As many marsupial species become managed populations in need of conservation, but do not breed rapidly in the wild or reliably in captivity, studies into male reproductive physiology of species like the common ringtail possum (*Pseudocheirus peregrinus*) (Taggart et al., 1996; Phillips et al., 2008), southern hairy nosed wombats (*Lasiorhinus latifrons*) (Taggart et al., 1998), koalas (*Phascolarctos cinereus*) (Wildt et al., 1991; Taggart et al., 1996), agile antechinus (*Antechinus agilis*) (Taggart et al., 1999; Shimmin et al., 2000), the fat-tailed dunnart (*Sminthopsis crassicaudata*) and the long-footed potoroo (*Potorous longipes*) (Taggart et al., 1996) allow for greater understanding of the biological processes involved.

The techniques developed for the manipulation of sex ratio in eutherians can be employed to assess the natural control of such a phenomenon in a marsupial species, to both understand the innate mechanism and manipulate it for conservation purposes.

The small amount of information available on male reproduction and its relationship with body condition, resource availability and nutrition suggests that the situation, at least for polygynous marsupial populations, is very similar to eutherian ungulate species. However, the comparison is of limited value, as there are some distinct differences between the two populations. For example, male common brushtail possums (*Trichosurus vulpecula*) lose a percentage of their body weight during mating season due to breeding activity (Isaac, 2005; Isaac et al., 2005) and are part of a hierarchical polygynous society, where reproductive success is dependent on male-male competition for access to receptive females, similar to many ungulate species (Clutton-Brock et al., 1979; Clutton-Brock, 1989). Although, unlike some ungulate species (Yoccoz et al., 2002; Mysterud et al., 2003) the male possums reduced the corporeal investment in reproduction as they reached optimum reproductive age. The brushtails also lost only 4% of their body weight during this period, where ungulates average a much higher percentage, *i.e.* between 16% (Pelletier, 2005) and 40% (Bobek et al., 1990), probably due to differences in behaviours post-copulation (Isaac, 2005).

The similarities in a comparison between macropodid marsupials, such as the tammar wallaby (*Macropus eugenii*), and eutherian ungulate species are much more pronounced. The tammar wallaby is also a highly promiscuous, polygynous species, where the males compete for access to breeding females, who are monogamous. The male tammar reproductive organs also increase in size during the breeding season, while their body mass decreases (Inns, 1982; Paris et al., 2005a). Pre-copulatory behaviour for the male tammar is predatory in nature, as he chases the female with intermittent attempts to mount, while the post-copulatory behaviour has two strategies (1) mate-guarding and (2) coagulation of the semen to form a fixed plug (Tyndale-Biscoe & Renfree, 1987; Paris et al., 2004). The tammar, as well as other macropodid species such as the bridled nailtail wallaby (*Onychogalea fraenata*) (Fisher, 1999), historically exhibits sex ratio biases similar to that of ungulate species (Sunnucks & Taylor, 1997), *e.g.* in accordance with the Trivers-Willard hypothesis (TWM) (1973).

The tammar wallaby male is part of a multi-male breeding system, and breeding system has been shown to have a bigger impact on testes size and energy expenditure in males than

breeding season (Harcourt et al., 1995). Thus, mammals in these systems tend to have testes that are larger compared with body size (Kenagy & Trombulak, 1986; Reynolds & Harvey, 1994; Harcourt et al., 1995), and expend a larger proportion of energy on growth of testes and reproductive organs than in other single-male breeding species (Mysterud et al., 2005). Males in these societies also have a greater investment in mating behaviours including number of ejaculations and copulations (Ginsberg & Rubenstein, 1990), frequency of mating (Short, 1979) and greater sperm production coupled with more successful competitiveness at the physiological level (Moller, 1989). These characteristics are true of *M.eugenii* and other macropods (Inns, 1982; Paris et al., 2004). If males in good condition should have heavier testes (Piersma & Lindstrom, 1997; Sarasa et al., 2010) in multi-male breeding systems, it is reasonable to assume that these males will have more reproductively robust semen, sperm counts and sperm energetics (Paris et al., 2004) than males in poor condition with smaller testes, as there is some evidence for a relationship between sperm health and %Y sperm (Chandler et al., 2002). Thus, there are similar questions regarding the mechanism of the observed ratio skew, although there may be some subtle differences. Hormone profiles between the two groups are different, as the tammar does not show differential development of the neural sexual pathways, but rather is dependent on the activating effects of testosterone, unlike eutherians that typically have both (Rudd et al., 1996). There is also a discreet difference in semen characteristics, as the coagulation of semen in the tammar occurs within minutes of ejaculation (Taggart et al., 1996; Paris et al., 2004).

As the physiological mechanism of sex ratio skew is relatively unstudied in macropodid marsupials, especially the paternal influence, the method of manipulation may differ markedly from that proposed for similar eutherian species. The aim of this experiment is to test the hypothesis that body condition affects the sex ratio of spermatozoa during spermatogenesis. To do this there are certain assumptions that must be applied. Firstly, the assumption that the probability associated with the production of a male or female young, assuming that each event is independent and both sexes are equally expensive to produce (Fisher, 1930) is 50%. For the purposes of this study, there will be an assumption that the 50:50 chance of having either sex at parturition extends to spermatogenesis (Hamilton, 1967; Johnson, 1988; Amann, 1989; Griffin et al., 1996; Welch & Johnson, 1999), especially given the meiotic cell division involved in the process (Chandler et al., 2002), although deviations from a 50:50 sex ratio have been identified in many species, including humans (Griffin et al., 1996; Chandler et al., 2002). Strategies for the production of progeny of a chosen sex may

change depending on the populations that manifest them, *i.e.* some highly polygynous mammalian species with outbreeding may have male biased sex ratios (Hamilton, 1967) at birth, but the overall population structure will remain 50:50 (Fisher, 1930). Secondly, the assumption that optimum captive breeding will mirror the best conditions available in natural habitats, and that the results of one can infer assumptions about the other when the species in question has a history of breeding with ease in captivity. Several marsupial species, especially the tammar, have been studied in depth, and have reproductive data that correlates well between the field and the laboratory (Taggart et al., 1999). The current study was designed to assess the occurrence of a 50:50 sex ratio among spermatozoa, to infer whether the sex ratio bias observed in Chapters 2 and 3 could possibly be due to a paternal effect, rather than a maternal effect. It is hypothesized that there will be no effect, and that the null hypothesis is supported, and a 50:50 ratio is found.

6.2 Methods & Materials

6.2.1 Wild Males

Data was obtained from a total of 25 adult male tammar wallabies (*Macropus eugenii decres*) shot on Kangaroo Island, South Australia (KI) by farmers licensed under destruction permits issued by the South Australia Department of Environment and Heritage, number Q25076 and the University of Adelaide Animal Ethics Approval Number S-007-2007A. The data was obtained during the month of June over the years 2005, 2006 and 2007 (n=8 or 9 each year). The data represent body measurements that were taken over the several years, which differed markedly in their rainfall totals (Chapter 2), affecting body condition of the wallabies. Once shot, the carcasses were weighed, Kidney Fat Index was measured and calculated and foot measurements taken to calculate Body Condition (BC) (calculated the same way as Maternal Body Condition, weight (g)/ foot length (cm)³) as per Chapter 2 Materials & Methods (Inns, 1980; Krebs & Singleton, 1993; Green, 2001; McGowan et al., 2008).

6.2.2 Captive Males

6.2.2.1 Spermatozoa Collection

Six male tammar wallabies were randomly caught from a wild population and kept in captivity, under the same permissions. The males were kept in pens, separated by transparent netting to allow visual contact with the other wallabies. Females were also placed in pens between the males, so that any pheromone cues necessary for spermatogenesis stimulation would be present, as olfactory stimuli is necessary to signal the reproductive condition of the females to the males (Hynes et al., 2005). Sampling was done during the breeding season of the tammar to maximize the amount of ejaculate available (Paris et al., 2005b; Schneider et al., 2010). Only 6 males were used due to the difficulty in obtaining semen from male wallabies, and successfully treating the semen post-collection to prevent coagulation and obtain usable sperm. Due to the small sample size, two feeding groups, *ad libitum* and maintenance (as in Chapters 3 & 4), could not be supported, or a reduction of the power of the data set would have occurred. Samples were not taken from culled animals in the wild, as ejaculated semen was used for testing, rather than epididimal semen. In addition, the technique used for semen collection and treatment could not be used in the field. At the time of this study, the standardised body measurement protocols performed on the wild male tammars were employed by the same observer to obtain comparable body condition indices, *e.g.* BC and weight.

The males were anaesthetised with an Isoflurane and O₂ mixture, to a surgical level, before ejaculation was stimulated electrically (Paris et al., 2005a) using a Standard Precision Electronics Inc, Broomfield, CO, USA, SPE Electro-Ejaculator and rectal probe. The ejaculate was collected in a 50ml centrifuge tube, allowed to coagulate for 30 mins, and then a 'swim up' technique was employed by layering a 1 to 1 ratio of Phosphate Buffered Saline (PBS) on top of the ejaculate (Molinia & Rodger, 1996). The sperm migrated from the coagulated semen into the PBS for approximately 30 minutes, in an incubator at 37°C in an atmosphere saturated with water containing 5% V/V of CO₂.

The PBS solution containing the spermatozoa was then centrifuged at 1300 rpm, and the supernatant removed. A solution of hypertonic (10% KCL) solution was added to the remaining cell pellet, which was resuspended (as per procedures used in Chapter 5 Materials & Methods), and allowed to incubate at 37°C for 20 minutes, the solution was then centrifuged again. After, the supernatant was again removed and a fixative solution of roughly 70% methanol and 30% acetic acid was added to the cells, which incubate overnight at -20°C. Three further fixes were performed, centrifuged at 1400, 1500 and 1600 rpm, before slides were made, as per Chapter 5. The slides went through an ethanol series, consisting of an 80%, 90% and 100% ethanol rinse for 3 minutes each, and then allowed to air dry.

6.2.2.2 DNA Extraction

Genomic DNA was extracted from *M. eugenii* liver samples. Frozen liver pieces were pestled then collected and resuspended in lysis buffer (50mM TrisPhosphate, 100mM EDTA, 100mM NaCl, 1% SDS) and digested with Proteinase K overnight at 60°C. The homogenous solution was then treated with Rnase A, before being phenol/chloroform extracted, followed by chloroform/isoamyl alcohol extraction. The DNA was then precipitated in absolute ethanol with 0.3 volumes of ammonium acetate and the pellet was washed twice with 75% ethanol. The tammar Y-BAC (M_87K16) was kindly donated by Paul Waters (Canberra). The clone was grown overnight at 37°C, before being pelleted and extracted using the Wizard Plus SV Minipreps DNA Purification System (Promega) following the manufacturer's protocols.

6.2.2.3 Fluorescent in situ Hybridisation (FISH)

Once the BAC DNA was prepared, 300-400mg was directly labelled with 9-mer random primers (Geneworks) using exo-klenow (Monserate Biotech) and either spectrum orange-dUTP or spectrum green-dUTP (Abbott Molecular Inc.). A fluorescently labelled clone was

precipitated, with salmon sperm and sonicated tammar genomic competitor DNA, in 100% ethanol, and dissolved in formamide and hybridization mix (10% dextran sulphate in 2 x SSC pH 8.0), then denatured at 70°C and re-annealed, before being used as a probe on fixed *M. eugenii* sperm FISH slides, as described above.

The FISH slides were pre-treated for the procedure with a 2 x SSC wash, before being treated with RNAase. Slides were then washed in 1% formaldehyde in 1 x PBS, equilibrated in 1 x PBS/50mM MgCl₂ and fixed in 1% formaldehyde in 1 x PBS/50mM MgCl₂ and washed again in 1 x PBS. The slides were then put through a further ethanol series (70%, 90% and 100%) and denatured for 3 minutes in 70% formamide/2 x SSC at 70°C and re-dried in an ethanol series, before being air-dried and hybridized with the precipitated BAC clones overnight at 37°C. Slides were washed in 50% formamide/2 x SSC at 42°C, 2 x SSC at 42°C, 0.1 x SSC at 60°C, then once again in 2 x SSC at 42°C. Slides were then stained with DAPI, and mounted with Vectashield (Vector Laboratories Inc.). The FISH nuclei were visualised under an AxioImager 2.1 epifluorescence microscope (Zeiss) with GFP green, DS red and DAPI filter sets capable of detecting fluorophore labelled DNA.

Once the slides were stained for the Y bearing sperm, the number of Y spermatozoa in a total of 200 were visually counted at 400x magnification (Figure 6.2.1), as that was deemed to be a large enough count to observe a sex ratio if it truly existed (Amann, 1989). Any possibility of skewing the result by counting false positives was negated by the use of a general probe, which allowed for the elimination of any samples where the FISH did not work, by counting just the Y sperm, and not the X as well. The data was then analysed and the body condition parameters compared between the wild and captive groups using Chi² Tests, ANOVA and the Tukey-Kramer Post Hoc test and simple regression.

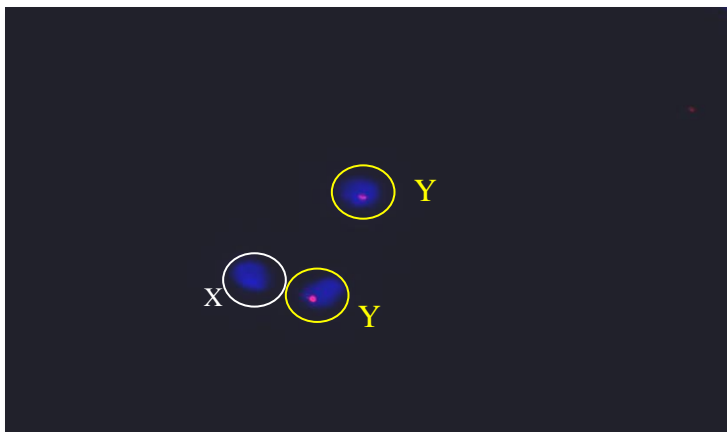


Figure 6.2.1 Slide showing 3 spermatozoa, 2 are male, as indicated by the red staining mark on the Y chromosome, the “X” was not counted.

6.3 Results

6.3.1 Wild vs. Captive Body Condition

A Shapiro-Wilk test of normality showed male weight to be normally distributed for the entire male population (captive and wild samples), with a standard error of variance of 450543. The body condition parameter showed slight skewing, but proved a better fit than any transformation of the data, with a Shapiro-Wilk score of 0.094 and standard error of variance of 0.018. An ANOVA of body condition, with status (captive vs. Wild) and year (captive animals were assigned “2009” as year of collection) as treatment parameters, indicated that year (test statistic=4.02, p value=0.018, df of 3) was more predictive of BC than status (test statistic=3.99, p value=0.056, df of 1). The same treatment of male weight indicated that year (test statistic=2.00, p value=0.139, df of 3) was not predictive of male weight, but status was significantly predictive (test statistic=17.64, p value=<0.001, df of 1). A Tukey-Kramer Post Hoc test shows that the captive BC is not significantly different from BC in the 2006 sample ($q=0.20490$, $P=0.05$, $n=15$), while both are significantly higher than in 2007 (Captive: $q=4.26042$, $P=0.05$, $n=15$; 2006: $q=4.84881$, $P=0.05$, $n=17$) (Figure 6.3.1.1). A Bonferroni Correction ($0.05/n=2$) indicates a significant independent threshold of 0.025, indicating that MBC is independently influenced by year of collection.

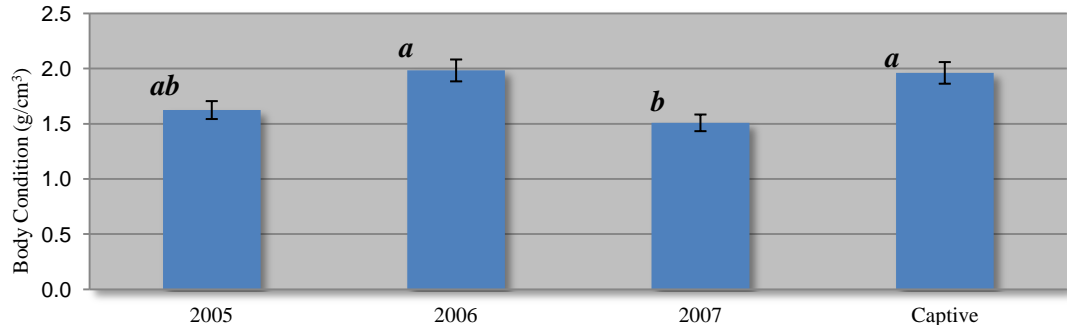


Figure 6.3.1.1 Body condition for each treatment. Treatments with differing superscripts differ significantly.

6.3.2 Sex Ratio of Spermatozoa

All samples collected had a Y sperm ratio of less than the expected 50%. There was only one outlier, as slide 4 (ID C223) had a minimal density of spermatozoa (Table 6.3.2.1).

Table 6.3.2.1 Data collected from Tammar Wallaby Sperm Collections

Slide Number	Animal ID	Total Counted	Y Sperm Counted	% Y Sperm	χ^2 Value	P value
1	C291	210	96	45.7	1.719	Nil
2	A035	204	88	43.1	3.843	<0.05
3	Y108	199	92	46.2	1.131	Nil
4	C223	85	30	35.3	7.353	<0.01
5	C229	201	94	46.7	0.975	Nil
6	C293	200	93	46.5	0.980	Nil
Total		1099	493	44.8		
Average		183.2	82.2	44.9		
Average (without outlier 4)		202.8	92.6	45.6		

The ratio of Y sperm in our sample of male wallabies differed significantly from the expected (Fisher, 1930; Hamilton, 1967; Quaas & Foote, 1982; Amann, 1989; James, 1996; Welch & Johnson, 1999; Johannisson et al., 2002) ratio of 50% ($\chi^2=16.001$, $P<0.001$, $n=493$), when all samples were analysed against the null hypothesis. If the outlier was excluded the observed was still significantly different from the expected ($\chi^2=8.648$, $P<0.01$, $n=463$). Therefore, the Null Hypothesis was rejected, and the observed value (average of all samples) was significantly different from expected.

When the captive population is tested on its own, full weight retains a normal distribution (Shapiro-Wilk=0.9568, $P=0.240$), while BC shows a slight skew (Shapiro-Wilk=0.9421, $P=0.094$), and the percentage of Y sperm counted per male sample is heavily skewed by outlier, individual 4 (SW=0.7038, $P=0.007$). Removal of this outlier weakens the power of this data to predict a relationship between body condition and % Y sperm, as the ability to pick up a 30% correlation drops from 0.132 with 6 replicates to 0.113 with 5 replicates. The best fit data appears to be (% Y Sperm)². However, there was no significant relationship with body condition (test statistic=0.19, p value=0.688, df of 1) or full weight (test statistic=0.13, p value=0.739, df of 1), even if the outlier was not removed.

6.4 Discussion

Despite the fact that an equal representation of X and Y sperm may be expected, our sampling indicates that this was not the case for this population. We found a significant deviation from the expected 50% in our sampled group, with an average of 44.9% Y sperm across all samples and there was no association between male body condition and % Y sperm in this sample. A diminished representation of Y sperm has been identified in other mammalian species, including humans (Johannisson et al., 2002; Cameron & Dalerum, 2009). A study on bovine semen samples found a ratio of 45% Y sperm across samples (Chandler et al., 2002), while other ungulate studies have shown the opposite is true of dominant males in good body condition (Gomendio et al., 2006). In humans, there is strong evidence that normal males produce a roughly 50:50 ratio of each sex sperm (Martin et al., 1995; Hilsenrath et al., 1997; Irving et al., 1999)

In the wild, the body condition amongst the males varied significantly from year to year, with annual fluctuations in resource availability the determining factor. Thus, although there was no relationship between body condition and sex ratio in this sample, due to uniformity in conditions, such a relationship cannot be ruled out. The captive individuals in this experiment correlated with good condition (high rainfall, *i.e.* 2006) wild males, but differed significantly from wild males culled during drought or poor rainfall years (*i.e.* 2007), with moderate rainfall years (2005) falling between the two groups. The captive animals were fed *ad libitum* and had constant access to water and shade. Given the good body condition of these males, if the principles of the TWM were applied, it would logically translate to a higher Y count in the semen of good condition males, as male offspring would be the desired result of a breeding episode, yet, there was an observed lower than expected Y percentage in these counts. Some studies have shown that inherited genetic anomalies can cause differential expression of the sex chromosomes (Chandler et al., 2002; Ward & Burgoyne, 2006). However, these males might still be producing 50% Y sperm, as the ability to fertilize would not become apparent until after copulation. With the current data, the influencing stimuli were acting on spermatogenesis and reducing the production of Y sperm. Therefore, there must have been other factors involved in the decrease in Y sperm production amongst the captive sample, other contributing factors might be age, diet, number of ejaculate episodes, semen or spermatozoa quality and/or stress load.

Though body condition and size are important for establishing rank in the hierarchy, and access to receptive females, other factors influence the actual quality and fertility of the

sperm of a given male. Age could play a significant role in the testes mass per individual and the resulting sperm condition. Very large males might be quite old in a tammar population, and allocate even less energy to spermatogenesis as they become more dominant in the hierarchy (Sarasa et al., 2010). The largest male, who weighed 1.7kg more than the next largest male and totalled 9.3kg, had the smallest amount of ejaculate of all the males, and the lowest %Y sperm ratio. A male of this size was likely to be older than the other males in the study, at least 4 to 5 years given his capture date (Perryman, unpublished). There was only one male from the wild collections that resembled the same size, and he was 8.2kg with an age of roughly 3.2 years. . However, it was quite hard to draw any concrete assumptions from this one male, as the reduced amount of ejaculate could have been due to some other individual defect. Such a male in the wild would most likely hold the most dominant position in the tammar hierarchy, as the largest/heaviest male in the group, or the α male (Hynes et al., 2005). Such an α male in a tammar group secures roughly 60% of the initial copulations with receptive females, but does not exhibit any increase in testosterone, which may allow an larger/older male to reduce resource expenditure on reproduction.

In 2001, a study on Soay sheep found that males at the top of the hierarchy and in the best condition, with the largest reproductive excessory organs, use their behavioural success to secure greater reproductive success, but produce inferior semen samples towards the end of the rutting period, after large numbers of copulations (Preston et al., 2001). Paris *et al.* (2005) found similar results in the tammar. During the main breeding season, the male reproductive organs were heaviest, volume and coagulation of ejaculates were at a maximum and sperm motility had increased, but there were low numbers of spermatozoa in these ejaculates. In fact, during the minor breeding season (September-November), the number and motility of spermatozoa in semen samples was highest, but coagulation and volume were not as high as during the major breeding season. Preston *et al.* (2001) assert that their findings in ungulate species suggest that the dominant males, which produced smaller ejaculates at the end of the breeding season, had greater capacity for ejaculate production at the start of the season and suffered reduced semen quality due to increased copulation activity, and that Parker's theory is an oversimplification. However, the sample size presented here was small, and this theory does not explain the reduced average %Y spermatozoa seen in this data set.

Several marsupial species, especially those that breed easily in captivity, have shown a correlation between field and laboratory reproductive data (Taggart et al., 1999), other

species have shown a pronounced difference in their sex ratios while in captivity (Merton et al., 1984; Trewick, 1997; Robertson et al., 2006; Linklater, 2007), most likely due to environmental factors (Nees et al., 2009) or nutritional changes (Robertson et al., 2006). Diet plays a significant role in reproductive physiology and the increased level of nutrition experienced in captivity can change output dramatically. However, the change in nutrition that accompanies captivity can also have deleterious effects, *e.g.* supplementing ruminant diets with fatty acids has a harmful effect on total sperm motility, viability and acrosome integrity (de Graaf et al., 2007). The males in this study were fed a diet specially formulated for wallabies, with maximum levels of protein and digestible energy, which contributed to their good body condition. Given the optimal nature of the diet provided on an *ad libitum* basis, it was reasonable to assume that semen quality should be at a maximum, unless a damaging component to the feed was undetected. However, this assumption is based on the TWM theory, which suggests that the optimum outcome for each breeding episode, when there is no restriction on resource availability, should be a male offspring. Perhaps that is not the case in this sample.

There are other stressors associated with captivity that might cause a reduced Y sperm count. For example, the current captive population was wild caught, and the stress of capture, transport and confinement could have caused stress that impacted on sex ratio, but this is supposition. In some cases of stress, including psychological, animals will produce young of the opposite sex (Schuster & Schuster, 1972). A study done on mice that were stressed in wire-screen cocoons for several hours a day for the week preceding conception had a female bias of 66.6% in their offspring (Lane & Hyde, 1973). Schuster & Schuster (1972) performed a similar stress induced test and found that when only the male was stressed the resulting sex ratio was 36% male progeny. As males under some form of stress tend to have a declining testosterone secretion response (Semple, 1986), it is plausible that the stress of the experimental parameters and increased animal handling caused some sort of endocrine response that resulted in the decrease in Y bearing sperm. In humans, war events have been associated with physiological stress in the resident population that sex ratios 6 to 9 months later are significantly impacted, and sperm motility and quality drop (Zorn et al., 2002).

Other mammals in captivity under similar conditions to this captive population, 1) recently introduced to captivity from the wild, 2) fed a balanced diet and in good physical condition and 3) kept in large, semi-natural enclosures for large parts of their captivity, showed no difference in sperm quality parameters to their wild counterparts (Wildt et al., 1987; Crosier

et al., 2007; Ganan et al., 2010). The only trait found to vary between the two groups was the % motile spermatozoa, which was higher in the captive group (Ganan et al., 2010). However, the fact that the captive males were isolated from females, but could see and smell them, might have acted as a form of stress. The males in this experiment were also exposed to further psychological stress caused by a rat infestation, though this would not have impacted on their body condition as feeding occurred daily. In addition, these captive males ejaculated solely during the sperm sampling episodes, which only occurred once or twice until a sample was obtained, this also may have influenced the results, as abstinence has been shown to marginally increase the proportion of X bearing sperm in ejaculates (Hilsenrath et al., 1997). The first ejaculate in a breeding season has been shown to be highly variable and have a skewed sex ratio in some mammalian species. A bovine study found that frequency and amount of semen sampling had a significant impact on % Y sperm of an individual (Chandler et al., 2002). The first sampling of the bulls after a 30-day sexual rest produced a highly variable % Y, with only 1 ejaculate out of 5 nearing the expected 50% ratio. After 3-4 samplings all the bulls were within 1 standard deviation of the mean, but the mean was 45% Y sperm, still significantly different from the expected.

As the parameters of this study are limited do to sample size and the lack of appropriate comparisons, an explanation for the reduced average % Y sperm ratio in this data set is not available. However, it is possible that the sex ratio bias reported in Chapter 2, where older, larger females had daughters and younger, smaller females produced sons, was merely one factor at work on the sex ratio in this population. If females were also influencing foetal sex ratio at conception. based on their own best interests, it is highly likely that both parental controls could be at work concurrently in the population, with competing objectives (Sunnucks & Taylor, 1997; Jimenez et al., 2003).

Although the accepted literature on polygynous mating systems suggests that males will mate with any available female in oestrus, there may well be a preference for females in better body condition, without disease, of certain ages or body sizes. In the tammar breeding system, females enter oestrus at the same time using a synchronised photosensitive mechanism (Inns, 1980; Tyndale-Biscoe & Renfree, 1987; Hinds et al., 1990a). Males will not be able to fertilize all females in the group, and they may well have to choose the best females available for mating. Thus, dominant males could choose larger, better conditioned females when possible, and allocate superior ejaculate qualities to these females (Poiani, 2006; Cornwallis & O'Connor, 2009). Thus, there may be some form of cryptic male choice

involved in these mating decisions (Reinhold et al., 2002; Wedell et al., 2002). Such a phenomenon has been seen in insects (Bonduriansky, 2001), crustaceans (Rubolini et al., 2006; Sato & Goshima, 2007), fish (Pilastro et al., 2002), birds (Hunter et al., 2000; Cornwallis & Birkhead, 2007) and mammals (Baker & Bellis, 1993), where the male selectively attributes higher quality semento more attractive and better conditioned females. Thus, dominant males might be more prudent in their sperm allocation choices, and in chosing which females to compete for (especially earlier in the breeding season, before deterioration of sperm quality), than their subordinate males (Hardling et al., 2008).

It appears that males may be able to affect sex ratio by producing more X or Y-bearing sperm during spermatogenesis. This may lead to a conflict between maternal and paternal interests in a given breeding season. A larger more fertile male may benefit from producing one sex, but a female in poor condition may suffer with greater costs in rearing that sex. Unless the reduced %Y sperm ratio seen in this sample can be observed across a larger population of *M. eugenii*, it is likely that some form of stress, possibly caused by captivity, acted on the male wallabies in this sample, causing the small reduction in average %Y sperm counted. However, the parameters of this experiment, since no samples were taken in the wild, mean any inferences that can be drawn with regards to the larger population of *M. Eugenii* are limited. Further investigation into the exact mechanism and cause of such a reduction is warranted to assess the application and existence in the wild population.

CHAPTER 7 *General Discussion*

Marsupials have physiological adaptations that have allowed them to radiate in Australasia more than on any other continent. These same adaptations make metatherian species integral to the advancement of mammalian life history tactics, by allowing the study of the divergent methods used by the separate therian clades to reach the same outcome. This is especially important to the understanding of mammalian reproduction, as much of modern reproductive knowledge is based solely on investigation of the eutherian methodology (Fisher et al., 2001b). Marsupial reproductive nuances provide a unique scenario that can be exploited to illuminate reproductive processes that are difficult to assess in eutherian species, broadening therian understanding as a whole, and elucidating and isolating control mechanisms that remain unclear and contested in the literature to date. A prime example of the usefulness of such comparative ecology is the case of adaptive sex ratio, as most of the available knowledge in the area concerns eutherian species, which has provided very little consensus on patterns and mechanisms, widening the sphere of knowledge to include metatherian species may clarify the literature in this area.

One of the most widely debated adaptive sex allocation hypothesis is the Trivers-Willard Model (TWM) (Trivers & Willard, 1973). The discussion in the literature on the merits of this, and other models like Local Resource Competition (LRC) (Clark, 1978), has raged for decades, with study of eutherian species unable to bring consensus amongst researchers. The literature available on offspring sex ratio skewing in metatherian species is sparse, with much of it no clearer in support of these hypotheses than the literature available on eutherian species. The conjecture is due to the *a posteriori* application of theories to observed data, suggesting that the only method of truly assessing a species' adherence to a pattern like the TWM is to test all of the assumptions of that theory on the same population, namely the three major assumptions of the TWM, which are:

- 1) Male offspring benefit more than female progeny from increased resource allocation from mothers;
- 2) There is no compensatory growth in the young, meaning offspring condition is an indicator of adult condition; and
- 3) The quality of the mother is associated with the quality of the progeny (Trivers & Willard, 1973).

Previous studies on the tammar wallaby (*Macropus eugenii eugenii*) on Kawau Island, New Zealand indicated support for the TWM, but this was an *a posteriori* application of the theory after observed data ‘appeared’ to fit the TWM pattern. Thus, to test the actual application of the TWM in *M. eugenii*, all of the assumptions were tested in this thesis. Firstly, there appeared to be no discrepancy in maternal resource allocation between male and female progeny. In the data presented in both Chapter 2 and Chapter 4, mothers of sons and daughters appeared equally capable of buffering their pouch young from external resource fluctuations. In addition, there was no difference in size or body condition between pouch young at any stage of development observed in this thesis. Secondly, the association between juvenile and adult condition is murky at best. Female juvenile tammars overcompensated for a period of reduced feed intake, while restricted juvenile male tammars recovered enough condition that, at least in weight, they were not significantly different from their non-restricted counterparts. Thirdly, there was significant evidence that maternal condition is associated with pouch young condition, yet mothers were capable of protecting their young from severe resource declines. Therefore, at least in this tammar population, there is minimal support for the TWM, based on the assumptions alone. Meaning, the pattern of sex ratio skew observed in Chapters 2 and 3, although possibly interpreted as *a posteriori* verification for the hypothesis when considered individually, must conform to another pattern. This is further evidenced by the fact that larger, older mothers had more daughters in the wild populations, in complete contravention of the essence of the TWM.

Despite the lack of evidence for the TWM in this population, there is evidence that sex ratio bias occurred and that it was an adaptive mechanism in response to external stimuli. The results from Chapter 3, where predominantly sons were produced, indicated that there must be other external factors influencing mothers in their ‘choice’ of offspring, and given the lack of correlation with rainfall, the external factor could be something other than resource availability. In actuality, it appears from this data highly likely that stress, perhaps by changing plasma cortisone levels or levels of other hormones like testosterone (Lane & Hyde, 1973; Moriya & Hiroshige, 1978; McGinley, 1984; Hattingh & Petty, 1992; Yamaguchi et al., 2005; Grant, 2007), could be the causal factor influencing maternal allocation. For example, the sex ratio bias observed in Chapters 2 and 3 might have been caused by an increase of maternal testosterone due to stress, while males in Chapter 6 might have reduced %Y sperm counts due to a decrease in testosterone levels caused by stress.

The causes of such stress events that might affect sex ratio bias were not concretely discovered by this thesis, but change to population structure was a plausible factor. For example, the lack of available males in Chapter 3 might have influenced mothers to produce more sons, while the lack of available females in Chapter 6 may have caused males to reduce their Y sperm number in favour of more female sperm. Given the increased mortality of males in the wild, especially during the juvenile phase of life, and the limited migration of individuals from their home ranges (Andrewartha & Barker, 1969; Inns, 1980), such changes in available mates could cause such shifts in sex ratio bias. However, the wild data reported in Chapter 2 did not present such a clear picture, as only larger, older females or smaller, younger females were showing bias in their 'choice' of offspring sex. This may suggest that there are many factors involved in influencing sex ratio bias strategy, including mate availability, resource availability and other population dynamics, and that all somehow collaborate to maintain a 1:1 overall population sex ratio.

This thesis produces just as many questions as answers, however there is clear evidence that sex allocation is an adaptive mechanism in *Macropus eugenii*, and that stress, possibly caused by a number of external stimuli has a plastic and reactionary impact on which way a mother will bias her offspring, *e.g.* towards sons or daughters. There is also evidence that males may attempt to bias offspring sex, possibly with a competing agenda to that of female partners, by influencing the number of X or Y sperm at a given mating event. That being said, there is clear indication that the TWM is not acting on this population, as the assumptions are not clearly met, and that the sex ratio pattern is much more complicated than such a theory hypothesises. Any past applications of the TWM to *M. eugenii* have not taken into account all the assumptions, as *a posteriori* application to fit data that has already been observed, rather than specifically testing for such a pattern, could turn up any number of results. Thus, the discrepancies between the wild sampling in Chapter 3 and that of Sunnucks & Taylor (1997) are explained by the convoluted nature of sex ratio bias in this species.

Although further work is needed on the actual control mechanism of sex ratio bias, which must be occurring pre- or at conception, and the possible external stimuli influencing the adaptive nature of sex ratio bias, the data presented in this thesis can be quite useful in many ways. In addition to the scientific benefit of research like this in illuminating patterns like the TWM, such research is also integral in the preservation of marsupial species from extinction. As 28% of all extinct mammalian species are marsupial species that have

become extinct post-European settlement, in the last 200 years (EPBCA, 1999; McKenzie & Burbidge, 2002; McKenzie et al., 2007; Burbidge et al., 2008; Woinarski et al., 2011), more than on any other continent, reproductive research is fundamental to conservation efforts. Understanding the sex ratio patterns observed in metatherian populations is a primary demographic parameter that is crucial to population viability (Stelkens & Wedekind, 2010). Specifically, the reproductive research presented in this thesis could have direct application to the breeding and re-introduction of the Mainland South Australian tammar wallaby (*Macropus eugenii eugenii*), which is underway as part of a cooperative between Monarto Zoo and Innes National Park (DEH, 2004). Understanding the stress that specific housing and handling situations may have on individual *M. eugenii* may aid in breeding husbandry. For example, early brush-tailed bettong (*Bettongia penicillata*) produced high numbers of sons (68%) rather than daughters, which was not conducive to increased propagation of the species (Smith, 1998). If future research on the reproductive patterns of species like the tammar wallaby maintain a practical application in conservation, many metatherian species, including those that have been difficult to breed in captivity, may be saved from extinction.

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